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NFIL3/E4BP4 controls Type 2 T helper cell cytokine expression

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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 15 September 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three express interest in your work and are broadly in favour of publication, although all three do raise a number of concerns that would first need to be addressed by additional experiments. Their reports are explicit and therefore I do not need to go into detail here, but the major points would be to determine the importance of JunB for the regulation of IL4 by NFIL3 (ref 2 point 1 and ref 3 point 1), to provide some additional analysis as to the role of NFIL3 in establishing heterogeneity (ref 1 point 1 and ref 2 point 2) and to address further the question as to whether the effects of NFIL3 on interleukin expression are all cell intrinsic (ref 1 point 3).

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

NFIL3/E4BP4 controls Type 2 T helper cell cytokine expression Kashiwada et al.
In this manuscript the authors present evidence for a role for the transcription factor NFIL3/E4BP4 in the regulation of Th2 cytokines both in vitro and in vivo.
Interestingly, whilst NFIL3 appears to be a direct negative regulator of IL13 expression it acts in an indirect manner to negatively regulate expression of IL5. Surprisingly, NFIL3 acts also acts as an indirect enhancer of IL4 expression, potentially through the regulation of JunB protein levels.

The paper is well written and the experiments are well controlled and convincing. This manuscript is definitely of sufficient interest and quality to warrant publication in EMBO. However, there are a few additional experiments that could potentially increase the impact of the data.

Major points
1. Have the authors tried the NFIL3 antibody for intracellular staining? If only a subset of 'TH2' cells express NFIL3 this could provide a potential explanation for the observed heterogeneity of TH2 cytokine expression.

2. It would be interesting to repeat the retroviral transduction experiments, but instead infect during the late stages of Th2 differentiation. In addition, it would be potentially revealing to knockdown NFIL3 in polarized Th2 cells.
These experiments may reveal whether NFIL3 is important in differentiating versus polarized Th2 cells.

3. Although the authors have shown that the altered IL13/IL5 expression is not due to perturbed IL4 expression/signaling, they have not formally proven that the effects are cell intrinsic. It is possible that some of the phenotype results from altered expression of other cytokines. One simple way to address this would be co-culture experiments followed by intracellular staining.

Minor points.

1. It is obvious that NFIL3 has pleiotropic functions as both IL10 and il3 are also decreased in Th2 cells, whereas IL9 is increased, however, these observations are not mentioned in the discussion. Have the authors followed up on these observations? Do these differences result from direct transcriptional regulation (as in IL13 regulation), indirect regulation through other factors (such as for IL4) or from secondary effects resulting from altered expression of other cytokines? Although it is too much to ask for experimental evidence for each of these genes some discussion would be useful.

2. On page 8 it is written

'Moreover, while IL-10 and IL-3 production was significantly decreased in
Nfil3-/- TH2 cells, no difference in IL-10 production was observed between WT and Nfil3-/- TH1 cells (Figure 2C to H).'

However this data is presented in supplemental figure 3.

Referee #2 (Remarks to the Author):

Kashiwada et al describe the phenotype of NFIL3-deficient Th2 cells. The authors describe an interesting phenotype that NFIL3 has distinct effects on individual Th2 cytokines; promoting IL-4 while inhibiting IL-13 and IL-5. They provide a direct mechanism for regulation of Il13 by binding of NFIL3 to the gene. There is an indirect mechanism proposed for regulation of Il4 by altering of AP-1 activity.

Overall, this is a thorough examination of NFIL3-deficient mice that elucidates a novel phenotype potentially important for understanding Th2-dependent immunity. The report provides mechanism for the observations and considerable insight into the function of NFIL3. There are some additional analyses that would enhance the report.

1. The concluding figure posits that AP-1 activity is decreased in the absence of NFIL3 and that the lack of JunB expression and binding to the Il4 gene is responsible for decreased Il4 expression. Can the defect be corrected by RV expression of JunB? While it is possible that complementation of JunB alone is insufficient to rescue Il4 expression, an attempt could be discussed in showing that it is likely several factors that are required. Thus, either result would be informative.

2. The authors note the potential role of NFIL3 in establishing heterogeneity in Th2 populations but don't examine the link between NFIL3 and other factors that contribute to this including PU.1, IRF4 (which positively regulates IL-4 and IL-10, similar to NFIL3) or Pias1 that positively regulates IL-13 but not other Th2 cytokines. An examination of the expression of these factors should be added and they should be discussed in the context of NFIL3 function.

Minor issues:

When the authors mention examining the expression of Bhlhe41 they should state this encodes Dec2.

In Figure 5D, I think the term di-trimethycetylation, should be di-/tri-methylation?

There are some grammatical errors throughout the report and the text should be carefully edited.

Referee #3 (Remarks to the Author):

The authors studied the role of NFIL3 in the Th2 cell function using NFIL3 deficient mice. The authors demonstrate the production of IL-13 and IL-5 to be upregulated, while that of IL-4 decreased in the NFIL3 deficient mice. NF-IL-3 binds to the CGRE region and represses IL-13 transcription. The authors also showed a decreased protein expression of JunB, and a decreased binding of JunB to the IL-4 promoter. This study addresses an interesting issue, however, there are a few critical points that still need to be addressed as noted below;

Major points:

1) It is not clear whether the binding of JunB to the IL-4 promoter induces the transactivation of IL-4 or the chromatin remodeling of the IL-4 gene. It would therefore be helpful to perform both Luciferase assay and ChIP assay with antibodies specific for H3-K4me and H3-K9ac to clarify this point. Most importantly, the authors should investigate and elucidate whether or not the ectopic expression of JunB in NFIL-3 deficient Th2 cells is able to rescue the IL-4 expression.
2) In Figure 5, it is very likely that NFIL3 binding interferes with GATA3 binding to the consensus sequence. Therefore, whether or not the levels of GATA3 binding are affected in NFIL3-deficient Th2 cells, as well as wild type Th1 and Th2 cells, should thus be clarified. This may also provide further possible mechanistic insight into the repression of IL-13 expression by NFIL-3.

Minor points:

1) In Figure 3B, a Mock infection group is required.

2) In Figure 6C, a Th1 control is required because the IL-4 expression was substantially preserved in NFIL3-deficient Th2 cells (Figures 1-3), however the binding of JunB was negligible.

1st Revision - authors' response 24 February 2011

Referee #1

1. Have the authors tried the NFIL3 antibody for intracellular staining? If only a subset of 'TH2' cells express NFIL3 this could provide a potential explanation for the observed heterogeneity of TH2 cytokine expression.
We tried intracellular cellular staining for NFIL3 protein with several anti-NFIL3 antibodies available. However, none of these antibodies worked for intracellular staining (although the antibody used in this manuscript worked very well for Western blot and ChIP experiments).

2. It would be interesting to repeat the retroviral transduction experiments, but instead infect during the late stages of Th2 differentiation. In addition, it would be potentially revealing to knockdown NFIL3 in polarized Th2 cells.
These experiments may reveal whether NFIL3 is important in differentiating versus polarized Th2 cells.
Following the reviewer's suggestion we performed NFIL3 transduction during the late stages of T_H2 differentiation (**Supplementary Figure 8A**). NFIL3 transduction during the late stage suppressed IL-13 production as similar to early stage (**Figure 3B**). Surprisingly, however, NFIL3 transduction suppressed also IL-4 although NFIL3 transduction during early stage enhanced IL-4 production. We also knocked down *Nfil3* expression in polarized T_H2 cells (**Supplementary Figure 8B**). We used shRNA that depleted *Nfil3* mRNA expression over 70 % (data not shown). As observed in *Nfil3*$^{-/-}$ T_H2 cells, NFIL3 knockdown in polarized T_H2 cells enhanced IL-13. However, IL-4 production was also enhanced although IL-4 production was reduced in *Nfil3*$^{-/-}$ T_H2 cells (**Figure 1**). These observations suggest that NFIL3 may differently act on IL-4 production between at early and late stages of T_H2 differentiation. It has been well documented that the mechanisms for *Il4* expression during the early and late stages are different. These data suggest that NFIL3 is important for both differentiating T_H2 cells and polarized T_H2 cells to produce adequate amount of cytokines.

3. Although the authors have shown that the altered IL13/IL5 expression is not due to perturbed IL4 expression/signaling, they have not formally proven that the effects are cell intrinsic. It is possible that some of the phenotype results from altered expression of other cytokines. One simple way to address this would be co-culture experiments followed by intracellular staining.
Following the reviewer's suggestion we have performed co-culture experiments (**Figure 2D**). Co-cultured CD45.2$^+$ CD4$^+$ T cells (*Nfil3*$^{-/-}$ cells) produced significantly higher levels of IL-13 and IL-5 and less amount of IL-4 compared to CD45.1$^+$ CD4$^+$ T cells (*Nfil3*$^{+/+}$ cells). These results suggest that the altered T_H2 cytokine production in *Nfil3*$^{-/-}$ T_H2 cells is cell intrinsic.

Minor points.

1. It is obvious that NFIL3 has pleiotropic functions as both IL10 and il3 are also decreased in Th2 cells, whereas IL9 is increased, however, these observations are not mentioned in the discussion. Have the authors followed up on these observations? Do these differences result from direct transcriptional regulation (as in IL13 regulation), indirect regulation through other factors (such as for IL4) or from secondary effects resulting from altered expression of other cytokines? Although it is too much to ask for experimental evidence for each of these genes some discussion would be useful.
We are also interested in the mechanisms by which NFIL3 regulates IL-10, IL-3 and IL-9 production in T_H2 cells. As suggested, we included some discussion of this issue in the text.

2. On page8 it is written
'Moreover, while IL-10 and IL-3 production was significantly decreased in Nfil3-/- TH2 cells, no difference in IL-10 production was observed between WT and Nfil3-/- TH1 cells (Figure 2C to H).' However this data is presented in supplemental figure 3.
We would like to thank the reviewer for pointing out this error. We have fixed this labeling.

Referee #2

1. The concluding figure posits that AP-1 activity is decreased in the absence of NFIL3 and that the lack of JunB expression and binding to the Il4 gene is responsible for decreased Il4 expression. Can the defect be corrected by RV expression of JunB? While it is possible that complementation of JunB alone is insufficient to rescue Il4 expression, an attempt could be discussed in showing that it is likely several factors that are required. Thus, either result would be informative.
This is a great point. We performed JunB transduction into *Nfil3*$^{-/-}$ T_H2 cells following the suggestion by two reviewers. As shown in **Supplementary Figure 12**, transduction of JunB into *Nfil3*$^{-/-}$ T_H2 cells could not rescued IL-4 production. As the reviewer mentioned, it is possible that complementation of JunB alone is not sufficient to rescue *Il4* expression. It is also possible that JunB is unstable in T_H2 cells in the absence of NFIL3. It has been reported that JunB is degraded by Ndfip1-Itch-mediated ubiquitination, resulting in the downregulation of IL-4 production (Nat. Immunol. 3, 281, 2002 , *Immunity* 25, 929 (2006)). Therefore, it is also possible that NFIL3 regulates this pathway. We have included this observation in the Discussion.

2. The authors note the potential role of NFIL3 in establishing heterogeneity in Th2 populations but don't examine the link between NFIL3 and other factors that contribute to this including PU.1, IRF4 (which positively regulates IL-4 and IL-10, similar to NFIL3) or Pias1 that positively regulates IL-13 but not other Th2 cytokines. An examination of the expression of these factors should be added and they should be discussed in the context of NFIL3 function.
Following the reviewer's suggestion we have compared the expression of PU.1, IRF4 and Pias1 between *Nfil3*$^{+/+}$ T_H2 cells and *Nfil3*$^{-/-}$ T_H2 cells (**Figure 6**). Real-time RT-PCR showed that *Sfpi1* (for PU.1) and *Pias1* expression was similar between *Nfil3*$^{+/+}$ and *Nfil3*$^{-/-}$ T_H2 cells. *Irf4* expression was slightly reduced in *Nfil3*$^{-/-}$ T_H2 cells. However, IRF4 protein level was similar between *Nfil3*$^{+/+}$ and *Nfil3*$^{-/-}$ T_H2 cells. Thus, NFIL3 may not be involved in the regulation of PU.1, IRF4 and Pias1, which establish heterogeneity in the T_H2 cell population.

Minor issues:

When the authors mention examining the expression of Bhlhe41 they should state this encodes Dec2.
Following the reviewer's suggestion we revised the text (p.10).

In Figure 5D, I think the term di-trimethycetylation, should be di-/tri-methylation?
We would like to thank for the reviewer's pointing out. We fixed this labeling.

There are some grammatical errors throughout the report and the text should be carefully edited.
We have carefully edited the manuscript.

Referee #3

1) It is not clear whether the binding of JunB to the IL-4 promoter induces the transactivation of IL-4 or the chromatin remodeling of the IL-4 gene. It would therefore be helpful to perform both Luciferase assay and ChIP assay with antibodies specific for H3-K4me and H3-K9ac to clarify this point. Most importantly, the authors should investigate and elucidate whether or not the ectopic expression of JunB in NFIL-3 deficient Th2 cells is able to rescue the IL-4 expression.
The reviewer has highlighted an important issue. It has been demonstrated that JunB transactivates *Il4* promoter by two independent groups (*EMBO J.,* 18, 420 (1999), *J. Immunol.,*180, 5983-(2008). We performed ChIP experiments to examine whether the reduced JunB level in *Nfil3*$^{-/-}$ T_H2 cells affects chromatin modification of the *Il4* promoter. As shown in the **Supplemental Figure 11**, we could not detect any differences in both levels of H3-K4me and H3-K9ac between *Nfil3*$^{+/+}$ T_H2 cells and *Nfil3*$^{-/-}$ T_H2 cells. These results suggest that the altered JunB level in the absence of NFIL3 expression may not affect chromatin modification on *Il4* promoter. We performed JunB transduction into *Nfil3*$^{-/-}$ T_H2 cells following the suggestion by two reviewers. As shown in **Supplementary Figure 12**, transduction of JunB into *Nfil3*$^{-/-}$ T_H2 cells could not rescue IL-4 production. It is possible that complementation of JunB alone is not sufficient to rescue *Il4* expression. It is also possible that JunB is unstable in T_H2 cells in the absence of NFIL3. It has been reported that JunB is degraded by Ndfip1-Itch-mediated ubiquitination, resulting in the downregulation of IL-4 production (Nat. Immunol. 3, 281, 2002, *Immunity* 25, 929 (2006)). Therefore, it is also possible that NFIL3 regulates this pathway. We included this observation in the Discussion.

2) In Figure 5, it is very likely that NFIL3 binding interferes with GATA3 binding to the consensus sequence. Therefore, whether or not the levels of GATA3 binding are affected in NFIL3-deficient Th2 cells, as well as wild type Th1 and Th2 cells, should thus be clarified. This may also provide further possible mechanistic insight into the repression of IL-13 expression by NFIL-3.
We examined whether NFIL3 binding affected GATA-3 binding to CGRE region by ChIP (**Supplemental Figure 9**). GATA-3 binding to CGRE was not affected by NFIL3 in both T_H1 and T_H2 cells, suggesting that NFIL3 regulates *Il13* expression without inhibiting GATA-3 binding activity to CGRE region.

Minor points:

1) In Figure 3B, a Mock infection group is required.
We repeated this experiment with a mock infection control (**Figure 3B**).

2) In Figure 6C, a Th1 control is required because the IL-4 expression was substantially preserved in NFIL3-deficient Th2 cells (Figures 1-3), however the binding of JunB was negligible.
Following the reviewer's suggestion, we repeated the ChIP experiment for JunB binding to *Il4* promoter using T_H1 and T_H2 cells (**Supplementary Figure 10**). JunB binding to *Il4* promoter was very faint in T_H1 cells.

2nd Editorial Decision 15 March 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-201075657R to the EMBO Journal. It has now been seen again by all three referees, whose comments are enclosed below. As you will see, all three reviewers find that you have substantially improved the manuscript during the revision, and are now fully supportive of publication in EMBOJ. I am therefore pleased to tell you that we will be able to publish your work. However, there are just a couple of issues from the editorial side first:

1. In Figure 6D, I notice that lanes have been spliced together to generate the panel. This is acceptable, assuming that the lanes do come from the same blot, and you have simply removed intervening lanes for clarity. However, this needs to be clearly marked in the figure and mentioned in the legend. I also need to ask you to send me the original scans of these blots for our records.

2. We now require an Author Contributions statement for all accepted manuscripts. Please can you add this beneath the Acknowledgments section?

Please could you make these changes and resubmit this final revision, and send me the scans for the blots in Figure 6D by email? Once we have these things, I hope we should be able to accept the manuscript without further delay.

Many thanks and best wishes,

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript the authors present evidence for a role for the transcription factor NFIL3/E4BP4 in the regulation of Th2 cytokines both in vitro and in vivo. Interestingly, whilst NFIL3 appears to be a direct negative regulator of IL13 expression it acts in an indirect manner to negatively regulate expression of IL5. Surprisingly, NFIL3 acts also acts as an indirect enhancer of IL4 expression, potentially through the regulation of JunB protein levels.

In the revised version of the manuscript the authors have addressed all of my concerns and have made a significant attempt to address the concerns raised by the other reviewers. I now recommend the manuscript for publication in EMBO.

Referee #2 (Remarks to the Author):

The authors have addressed all of my previous comments and concerns with additional data and text. The report is highly novel and documents NFIL3 as a new transcription factor involved in Th2 cytokine production, with experiments that document function at multiple levels. Overall, this revised report is suitable for publication.

Referee #3 (Remarks to the Author):

The revision is satisfactory for this reviewer.

2nd Revision - authors' response 15 March 2011

Enclosed is a revised version of our manuscript entitled "NFIL3/E4BP4 controls type 2 T helper cell cytokine expression", which we would like you to consider publishing in the *EMBO Journal*. We would like to thank the reviewers for their time and effort. We have added a note about the Figure 6D in the figure legend and an Author Contributions in the main text following to the Editor's request. We have also sent the original data for Figure 6D by email. We believe that this version of the manuscript is now acceptable for the EMBO Journal