EMBO reports

Manuscript EMBOR-2015-40096

The thymic cortical epithelium determines the TCR repertoire of IL-17-producing γδT cells

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Review timeline:

Correspondence:	18 November 2014
Correspondence:	21 November 2014
Transfer date:	14 January 2015
Editorial Decision:	04 February 2015
Revision received:	17 February 2015
Accepted:	18 February 2015

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.)

Transfer Note:

Please note that this manuscript was originally submitted to the EMBO Journal where it was peer-reviewed. It was then transferred to EMBO reports with the original referees' comments attached. (Please see below)

Editor: Nonia Pariente

Original decision – EMBO Journal

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and I am afraid that the overall recommendation is not very positive.

The referees appreciate the characterization of a novel mouse strain that lack cTEC. However, the referees also indicate that many of the findings reported are consistent with previous work and that the novel aspects are not sufficiently worked out to consider publication in The EMBO Journal. In particular, we gain too limited insight into how cTEC control gdT and iNKT development. Given these comments from good experts in the field, I am afraid that I can't offer to consider publication

here.

However, you might want to consider EMBO Reports since you have an interesting novel mouse strain. With some further characterization of the TN mice, the paper might be suitable for publication in EMBO reports. If you are interested in considering EMBO Reports then I would suggest that you contact Nonia Pariente editor at EMBO reports at pariente@embo.org to discuss this option further.

For the EMBO Journal, I am sorry that I can't be more positive on this occasion.

REFEREE REPORTS:

Referee #1:

This study described a naturally occurring mutant mouse line with a point mutation in the gene encoding b5t, part of the thymoproteosome that is expressed by cortical thymic epithelial cells (cTEC). The authors show that tn/tn mice have severe alterations in thymus development, function and organisation. They show that the cTEC are reduced in numbers and frequency, and they equate this to several changes in thymus function, including abT-cell development, iNKT-cell development, and gdT-cell development.

The authors are correct to state that, in contrast to the availability of mouse lines with mTEC defects, mice with cTEC defects are scarce. So, this mouse line may be of use in examining the role of cTEC in thymus function. However, there are some concerns.

1. tn/tn mice do not just have alterations in cTEC. They also have a big impact on mTEC, as shown in Fig 2 and 7. So, it is not fully clear whether the changes that are seen in T-cell development specifically relate to changes in cTEC as suggested, or are caused by mTEC alterations. So, there is some over interpretation throughout this manuscript - the authors constantly refer to tn/tn mice as having cTEC defects, but the data shows that their defects are more widespread.

In some cases the authors have tried to address this problem, by using sRANKL transgenic mice to correct the mTEC defect in tn/tn mice, and then look at gdT-cell development. However, analysis of this is incomplete: for example what happens to defective a) iNKT-cell development b) SP4/SP8 thymocytes numbers c) thymus organisation in tn/tn mice crossed to sRANKL transgenic mice ?

2. If, as the authors suggest, cTEC control gdT-cell repertoire development, there is no data to indicate how cTEC control this process. While it may be acknowledged that is the basis of further study, it leaves the study somewhat incomplete.

While the area of research covered by this manuscript is interesting, given the above limitations, it may be more appropriate for a specialist Immunology journal.

Referee #2:

1. Nitta and colleagues present the phenotype of a novel spontaneous mutation, discovered within their in-house B56BL/6 colony, leading to T cell lymphopenia in mice. The mutation was mapped to the Psmb11 gene on chr 14, encoding for the proteasome subunit beta5t. There, a G220R missense mutation of cTEC-specific beta5t induced substantial loss of c-TECs. This establishes an important new experimental model for thymus biology. The authors carefully investigated the underlying mechanism inducing dominant loss of cTECs, namely impaired proteasome assembly inducing cell death.

2. Next, the MS describes the perturbations in T cell development resulting from loss of cTECs. While T cell lymphopenia possibly hints to impaired positive selection, a weaker point is the claim

of an altered TCR repertoire 5E with only n = 3-9. The results may be statistically significant with the test applied, but with only 3 n the different frequencies may as well be due to differences in age, sex, genetic background or intestinal flora. Is the repertoire also different in the thymus? Could differences in the spleen be a result of selective/ biased expansion? Thus, the conclusions should be tuned down, or further (future) studies should address the TCR repertoire shaping in depth by HT-sequencing.

3. The observed reduced numbers but no striking qualitative differences in iNKT (and Treg cell and iIEL) development further support the view that cTEC loss mainly affects the efficiency of T cell development. A few surviving cTECS may be sufficient to sustain low levels of qualitatively normal thymic T cell production.

4. Numbers (and indirectly frequencies) of IL-17 producing gd T cells may depend on self-controlling loops, see review by Klaus Ley, e.g. Immunol Res. 2006;34(3):229-42, and later work. Accordingly, homeostatic expansion of IL-17 producing gd T cells may be responsible for the observed repertoire alterations. Related observations were recently made in mice in which gd T cells develop less efficient due to restricted access to cTECS, Eur J Immunol. 2014 May;44(5):1320-9.

5. Do the authors suggest in Fig. 8 that Vg4+ IL-17 producing gd T cells are more potent pro-inflammatory effector cells than Vg6+ IL-17 producing gd T cells? Please clarify in results or discussion section.

Minor points:

Formatting Greek letters gamma and delta could be improved throughout the MS, page numbers and labels for the figures are helpful for review purposes.
Statistical significance for data shown in Fig 5 may be better analyzed by ANOVA, also, is there difference between Balbc wt and Balbc tn/tn?

Referee #3:

This study reports that the thymic cortical epithelium determines the IL-17-producing γδ T cell repertoire and influences the development of NKT cells, besides supporting and shaping conventional αβT cells during T cell development. The authors studied a spontaneous mutant mouse stain (TN) lacking naïve T cells in the periphery, and identified a point mutation (G220R) in the gene encoding β5t, a cTEC-specific proteasome subunit as the cause of the phenotype. The manuscript presents in vitro and in vivo analysis using β5tG220R transfectants and TN mice that suggests that the β5tG220R mutant impairs proteasome assembly, leading to decreased cell numbers of mature cTECs. As expected, TN mice display impaired T cell development of conventional αβT cells. However, the authors also showed altered NKT cell maturation and an altered TCR repertoire of γδT17 cells. The experiments, particularly those describing the mutant mouse and identifying the cause, are well executed and controlled. While the major phenotype is similar to that shown in other models of cTEC deficiency (appropriately referenced by the authors), the novelty of this report lies in altered development of NKT cells and gd T cells. Although the study does not identify the mechanistic cause of the NKT and gd T cell alterations (do cTEC provide key cytokines? cell surface ligands?) it is nonetheless an important report. Related to these novel findings, the manuscript could be improved by addressing the following issues.

1. The authors report NKT development is reduced by cTEC deficiency, as the proportion of iNKT/DP is reduced by about half (Figure 6C) and the authors state that NKT maturation appears normal. However, reduced tetramer/TCR level, and almost exclusive expression of NK1.1 would suggest impaired differentiation of NKT cells into NKT2 and NKT17 subsets, with normal NKT1 differentiation. The authors should use intracellular staining for key transcription factors (Tbet, RORgt, and PLZF) to define NKT subsets (NKT1, NKT2 and NKT17) as described in Nat Immunol. 2013, 14:1146. The finding that NKT2/17 differentiation depends on cTEC would be important to the field.

2. The authors interpretation that beta selection requires mature cTEC is based on reduced DN3b cells compared to DN3a. However, their DN3a gate (cKit-CD25+ CD27lo) might include other cells, as it seems illogical that there would be such high numbers of DN3a cells given the very low numbers of DN2 (and DN1) cells. This conclusion should either be removed or the authors should provide further independent evidence for a beta selection defect.

3. In Figure 4 and E4, the authors present the data to support that the β5tG220R mutant impairs normal proteasome assembly and cell survival. It should be clarified whether there are any changes on the proteasome activities of β5i-/- MEF cells expressing β5tG220R-Flag, compared to expressing β5t-Flag and mock cells.

4. In Figure 8A, the cell number of Vγ6+ cells in the skin is not included. Please add the data or explain why it cannot be shown. And how the γδT cells were prepared from mouse lung and skin should be described in Materials and Methods.

5. In the legends of Figure 2, (D) was mislabeled as (E).

6. The mutant strain showed slightly reduced mTEC numbers (Figure 2C and D), which the authors say could be due to a reduction of CD4 and CD8 SP thymocytes that support mTEC development. However reduced mTEC numbers were not seen in an inducible model of cTEC deficiency, which also resulted in reduced CD4 and CD8 SP numbers. Have the authors considered the possibility that reduced mTEC numbers in their model might be due to expression of the β5tG220R mutant in a bipotent progenitor?

Correspondence - authors 18 November 2014

Thank you for your email. As suggested, we would like to consider submitting our manuscript to EMBO reports.

Correspondence - editor 21 November 2014

I have now had time to read your study in detail, the related literature and the referee reports. EMBO reports would, in principle, be interested in considering a revised version of this study, which would be sent back to the three previous referees for final assessment. We would require that you experimentally address the following points:

- the further characterizations that referee 1 requests of the tn/tn mice crossed to sRANKL transgenic mice, which rescue the mTEC defects and would strengthen the conclusiveness of your claims regarding the roles of cTECs

- address point 2 of referee 2, but only by increasing the number of animals analyzed, as this is the less novel aspect of the work

- address points 1-4 of referee 3, which would strengthen the conclusiveness of the more novel parts of your study and provide important controls

All other issues can (and should) be addressed in the manuscript text. In addition to the referencing issues referred to by the referees, please pay particular attention to referencing throughout the study. For example, I believe that cTEC expression of Skint1 was shown by Bardee et al 2011, not Boyden et al 2008.

With respect to format, I do not want to impose a strict length limitation on the text, but please try to be as succinct as possible without losing information. You may want to combine the Results and

Discussion into a single section, which we recommend, and which will help eliminate the redundancy that is inevitable when discussing the same experiments twice. Please also place figure 3 as supplementary (I would suggest to include panel E in figure 2), as well as figure 5, given that a role on alpha beta T cell development is not so novel.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

Transfer - 1st Revision - authors' response 14 January 2015

Point-by-point response to the reviewers

Referee #1:

1. tn/tn mice do not just have alterations in cTEC. They also have a big impact on mTEC, as shown in Fig 2 and 7. So, it is not fully clear whether the changes that are seen in T-cell development specifically relate to changes in cTEC as suggested, or are caused by mTEC alterations. So, there is some over interpretation throughout this manuscript - the authors constantly refer to tn/tn mice as having cTEC defects, but the data shows that their defects are more widespread.
In some cases the authors have tried to address this problem, by using sRANKL transgenic mice to correct the mTEC defect in tn/tn mice, and then look at gdT-cell development. However, analysis of this is incomplete: for example what happens to defective a) iNKT-cell development b) SP4/SP8 thymocytes numbers c) thymus organisation in tn/tn mice crossed to sRANKL transgenic mice ?

According to the reviewer's suggestion, we included additional analysis of *tn*/*tn* sRANKL-transgenic mice in Figure E9. Histological analysis of thymus sections revealed that thymic medullary regions were markedly expanded but development of CD205+ mature cTECs was not restored by sRANKL transgene in *tn*/*tn* thymus (Figure E9B). We found no significant restoration in the frequency of CD4SP thymocytes, TCRβ^{hi}CD69^{+} post-selected DP thymocytes, and iNKT cells in *tn*/*tn* sRANKL-transgenic mice compared with non-transgenic *tn*/*tn* mice (Figure E9A). The *tn*/*tn* sRANKL-transgenic mice showed increased frequency of CD8SP thymocytes, which was also observed in wild-type sRANKL-transgenic mice (3.32 ± 0.23 %, not shown in the figure). It is likely that the sRANKL transgene causes expansion of CD8SP thymocytes irrespective of the β5t mutation, although the mechanism is unclear. These results, along with the data shown in Figure 5H, indicate that sRANKL restored development of mTECs but not of cTECs. We believe that these data strengthen our conclusion that the reduced mTEC development is not responsible for the altered repertoire of γδT cells in *tn*/*tn* thymus.

2. If, as the authors suggest, cTEC control gdT-cell repertoire development, there is no data to indicate how cTEC control this process. While it may be acknowledged that is the basis of further study, it leaves the study somewhat incomplete.

We are very much interested in investigating how cTEC controls γδT cell repertoire. Indeed, microarray screening of the candidate genes for cTEC regulation of γδT cell development is currently going on. Because additional enormous work should be required to elucidate molecular mechanism, we believe that it should be an independent study to be published in the future.

Referee #2:

1. Nitta and colleagues present the phenotype of a novel spontaneous mutation, discovered within their in-house B56BL/6 colony, leading to T cell lymphopenia in mice. The mutation was mapped to the Psmb11 gene on chr 14, encoding for the proteasome subunit beta5t. There, a G220R missense mutation of cTEC-specific beta5t induced substantial loss of c-TECs. This establishes an important new experimental model for

thymus biology. The authors carefully investigated the underlying mechanism inducing dominant loss of cTECs, namely impaired proteasome assembly inducing cell death.

We appreciate the reviewer's comment that our study established an important new experimental model for thymus biology.

2. Next, the MS describes the perturbations in T cell development resulting from loss of cTECs. While T cell lymphopenia possibly hints to impaired positive selection, a weaker point is the claim of an altered TCR repertoire 5E with only n = 3-9. The results may be statistically significant with the test applied, but with only 3 n the different frequencies may as well be due to differences in age, sex, genetic background or intestinal flora. Is the repertoire also different in the thymus? Could differences in the spleen be a result of selective/ biased expansion? Thus, the conclusions should be tuned down, or further (future) studies should address the TCR repertoire shaping in depth by HT-sequencing.

To answer the reviewer's concern, we carried out additional analysis for TCR repertoire of spleen T cells and SP thymocytes to increase the number of experiments (Figure E7E). Our new results confirmed that the usages of certain TCR Vα and Vβ were significantly altered in splenic T cells and in SP thymocytes from *tn*/*tn* mice (n = 6-12).

3. The observed reduced numbers but no striking qualitative differences in iNKT (and Treg cell and iIEL) development further support the view that cTEC loss mainly affects the efficiency of T cell development. A few surviving cTECS may be sufficient to sustain low levels of qualitatively normal thymic T cell production.

Our new results revealed that developmental defect in NKT2 population was much severer than in NKT17 in *tn*/*tn* mice, indicating that NKT cell development was qualitatively different (Fig. 4H).

4. Numbers (and indirectly frequencies) of IL-17 producing gd T cells may depend on self-controlling loops, see review by Klaus Ley, e.g. Immunol Res. 2006;34(3):229-42, and later work. Accordingly, homeostatic expansion of IL-17 producing gd T cells may be responsible for the observed repertoire alterations. Related observations were recently made in mice in which gd T cells develop less efficient due to restricted access to cTECS, Eur J Immunol. 2014 May;44(5):1320-9.

We added a discussion of this possibility in Supplementary Discussion in the revised manuscript.

5. Do the authors suggest in Fig. 8 that Vg4+ IL-17 producing gd T cells are more potent pro-inflammatory effector cells than Vg6+ IL-17 producing gd T cells? Please clarify in results or discussion section.

Although unaltered number of $V\gamma6^+$ cells exists in the skin of *tn*/*tn* mice (Fig. 6A), it has been reported that $V\gamma4^+$ γδT cells are the major source of IL-17 in IMQ-induced dermatitis model (Gray et al, *Nat Immunol* 2013). Therefore, we concluded that reduced IMQ-induced dermatitis in *tn*/*tn* mice was due to the reduction of $V\gamma4^+$ γδT cells in the skin.

Minor points:
Formatting Greek letters gamma and delta could be improved throughout the MS, page numbers and labels for the figures are helpful for review purposes.
Statistical significance for data shown in Fig 5 may be better analyzed by ANOVA, also, is there difference between Balbc wt and Balbc tn/tn?

We corrected our manuscript and figures according to the reviewer's suggestion. Statistical significance for the data in Figure E7F was now analyzed by ANOVA.

Referee #3:

1. The authors report NKT development is reduced by cTEC deficiency, as the proportion of iNKT/DP is reduced by about half (Figure 6C) and the authors state that NKT maturation appears normal. However, reduced tetramer/TCR level, and almost exclusive expression of NK1.1 would suggest impaired differentiation of NKT cells into NKT2 and NKT17 subsets, with normal NKT1 differentiation. The authors should use intracellular staining for key transcription factors (Tbet, RORgt, and PLZF) to define NKT subsets (NKT1, NKT2 and NKT17) as described in Nat Immunol. 2013, 14:1146. The finding that NKT2/17 differentiation depends on cTEC would be important to the field.

According to the reviewer's suggestion, we carried out experiments to define NKT cell subsets (NKT1, NKT2, NKT17). The new results were shown in Figure 4H. We found that *tn*/*tn* mice exhibited marked reduction of $PLZF^{+}$ NKT2 cells, majority of which are stage 2 iNKT cells. We added a sentence regarding these data in Results and Discussion. We appreciate the reviewer's helpful suggestions.

2. The authors interpretation that beta selection requires mature cTEC is based on reduced DN3b cells compared to DN3a. However, their DN3a gate (cKit-CD25+ CD27lo) might include other cells, as it seems illogical that there would be such high numbers of DN3a cells given the very low numbers of DN2 (and DN1) cells. This conclusion should either be removed or the authors should provide further independent evidence for a beta selection defect.

According to the reviewer's direction, the description about defective β-selection was removed in the revised manuscript.

3. In Figure 4 and E4, the authors present the data to support that the β5tG220R mutant impairs normal proteasome assembly and cell survival. It should be clarified whether there are any changes on the proteasome activities of β5i-/- MEF cells expressing β5tG220R-Flag, compared to expressing β5t-Flag and mock cells.

The reviewer requested experiments to clarify whether $\beta5t^{G220R}$ expression affects cellular proteasome activity. However, there is a concern that experimentally measurable proteasome activity does not accurately reflect the extent of proteasome assembly. The β5t has 60-70% weaker proteasome (Chymotrypsin-like) activity than standard subunit β5 (Murata et al, Science 2007) and the β5t-transfected cells still have expression of endogenous β5. β1i, another catalytic subunit assembled in β5t-containing thymoproteasome, also exerts Chymotrypsin-like activity, which is qualitatively indistinguishable from those of β5t and β5. These aspects imply that measuring proteasome activity is not suitable to examine the effect of $\beta5t^{G220R}$ on proteasome assembly.
Instead, we performed native PAGE followed by western blot analysis, to determine the total amount of cellular proteasomes. Cells expressing $\beta5t^{G220R}$ exhibited reduced amount of 20S proteasomes, compared with cells expressing $\beta5t^{WT}$. These results support the conclusion that the $\beta5t^{G220R}$ mutant protein impairs normal proteasome assembly and cell survival. We added these results in Figure E5C.

4. In Figure 8A, the cell number of Vγ6+ cells in the skin is not included. Please add the data or explain why it cannot be shown. And how the γδT cells were prepared from mouse lung and skin should be described in Materials and Methods.

According to the reviewer's indications, we carried out experiments and showed new data set including the cell number of $V\gamma6^{+}$ cells in the skin (Figure 6A). We also described the methods for isolation of lymphocytes from skin or lung in Supplementary Materials and Methods.

5. In the legends of Figure 2, (D) was mislabeled as (E).

We corrected the mistake in Figure 2.

6. The mutant strain showed slightly reduced mTEC numbers (Figure 2C and D), which the authors say could be due to a reduction of CD4 and CD8 SP thymocytes that support mTEC development. However reduced mTEC numbers were not seen in an inducible model of cTEC deficiency, which also resulted in reduced CD4 and CD8 SP numbers. Have the authors considered the possibility that reduced mTEC numbers in their model might be due to expression of the β5tG220R mutant in a bipotent progenitor?

We consider the possibility that defects of mTECs might be due to an effect of low level expression of $\beta5t^{G220R}$ on bipotent TEC progenitors, as described in Results and Discussion.

1st Editorial Decision 04 February 2015

Thank you for your patience while we have reviewed your revised manuscript. It was seen by previous referees 1 and 2, and -although we have not been succesful at contacting referee 3- I am making a decision now to avoid any further loss of time. As you will see from the reports below, the referees are positive about the publication of your study in EMBO reports in I think the concerns of referee 3 have also been adequately addressed.

I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed.

- As you will see, referee 1 remains concerned about the (minor) effects observed on mTECs, and I would ask you to incorporate in the discussion a mention of the fact that alterations of this compartment could also contribute to the phenotype.

- We have recently started publishing full length articles in EMBO reports, and I feel that this would be the best way to portray your findings. I apologize as this will mean that some reformatting is necessary, but I do feel that the readers of your study will be best served and the content of your manuscript clearer.

As a full Article, the complete Material and Methods should be included in the main text, and no supplementary methods presented. In addition, please separate the Result and Discussion section into two and include the supplementary discussion in the main text, as well as the relevant supplementary references. There should be no supplementary Discussion or Material & Methods.

As a longer format Article, we can accommodate more main figures. I would suggest the following figure redistribution, to make the experimental results more accessible:

- Incorporate figure E6 into figure 1
- Incorporate figure E10 into figure 6A
- Move figures E2, E3 and E5 to the main manuscript

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFREE REPORTS:

Referee #1:

This study reports the phenotype of a new mouse mutant in which thymic epithelial populations are reduced because of a point mutation in the gene encoding b5t, normally expressed by thymic epithelial cells. The authors describe altered T-cell development in the mice, and focus on gdT-cell and iNKT-cell lineages.

The experiments that are described here are well performed. However, throughout the study, the authors over-intepret their data to conclude that defects in cTEC are specifically responsible for the changes in iNKT and gdT-cell development. In addition to problems with cTEC, these mice have a clear mTEC defect (Figure 2). The authors attempt to address this in experiments using RANKL to stimulate mTEC development. While this is a reasonable attempt, it still leaves a rather unclear experimental system - it is not known, for example, whether RANKL stimulates all mTEC, and whether increasing mTEC numbers completely rules out the involvement of mTEC in the tn/tn phenotype.

I have no problem with the data presented. However, because of a significant impact on both cTEC and mTEC, I don't think their interpretation that the phenotype is down to cTEC problems, are fully justified. Even the title chooses to focus specifically on the cTEC defect, and ignores any involvement of the mTEC defect in tn/tn mice. I would recommend the authors tone down their conclusions, and describe their interesting findings in relation to a mouse that has a significant impact on both cTEC and mTEC populations.

Referee #2:

My criticisms have been properly addressed in the revised version of the manuscript.

2nd Revision - authors' response 17 February 2015

Thank you very much for your e-mail. I am glad to learn that you think our manuscript is, in principle, suitable for publication in *EMBO Reports*.

According to your suggestions, we revised the manuscript as follows:

- In order to respond the comment by the referee #1, we described the possibility that the partly reduced development of mTECs in *tn*/*tn* mice contributes to the γδT17 repertoire alteration in the Discussion section in the current manuscript (page 18).
- The complete Materials and Methods are included in the main text.
- Results and Discussion are separated into two sections.
- No supplementary methods, discussion, and references are included in the Expanded Information.

Figures were redistributed as follows:

- Figure E6 in the previous manuscript is placed as Figure 1F.
- Figure E10 in the previous manuscript is incorporated into Figure 8A in the current manuscript.
- Instead of Figure E2 that you suggested, Figure E4 in the previous manuscript is moved to the main manuscript as Figure 3, because the identification of the $\beta5t^{G220R}$ as responsible mutation is one of the important findings of this paper;
- Figure E3G in the previous manuscript is placed as Figure 2E.
- Figure E5 in the previous manuscript is placed as Figure 4B, C, and D.
- Figure E7 in the previous manuscript is moved to the main manuscript as Figure 5, because this figure indicates the defect of αβT cell development in *TN* mice, which is an important finding in this study.
- Figures 4, 5, and 6 in the previous manuscript are renumbered as Figures 6, 7, and 8,

respectively.
Finally, the current manuscript includes eight figures, five supplementary figures, and three supplementary tables.

I believe that the current revised manuscript is formally acceptable for publication as a full Article in *EMBO Reports*. Thank you very much for kind and favorable management on our manuscript.

2nd Editorial Decision 18 February 2015

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

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

