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Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch

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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 26 March 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below. As you can see the referees express an interest in the study. However, referee #2 also raise a number of important issues that have to be addressed in order to consider publication here. Among the issues raised, is that further support for a direct role of Bach2 in plasma cell differentiation class switching is needed. A better explanation of the mathematical modeling should also be included. Should you be able to address the concerns raised by referee #2 in full then we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will 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

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

Yours sincerely,

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REFEREE REPORTS

Referee #1 (Remarks to the Author):

This is a very interesting study that presents important and novel results. It will be of interest to a wide audience. The study shows that after B cell activation in culture under conditions that induce both class switching and plasma cell differentiation (LPS-treatment) that Bach2 represses Blimp1, which provides a time window for CSR to occur. When Blimp1 levels increase, cells differentiate towards plasma cells and this inhibits AID expression and class switching. Thus, Bach2 is an important component of a regulatory network that determines whether B cells will switch or differentiate to IgM-secreting cells upon activation. This study opens up many possibiltites for further research also.

They provide strong data supporting the conclusions they make, except for the points made around Fig 6D. This is a confusing figure that is very difficult to interpret. What are the structures shown? If they are germinal centers or B cell follicles, this is surprising because there is no apparent difference in unimmunized and immunized mice, except for the tone of green. The staining needs to be explained better in the legend/text, and perhaps some arrows to point to structures. The methods section does not help.

The title could be more explicit - to indicate that Bach2 inhibits CSR by stimulating differentiation to plasma cells. The effect on CSR is very strong - and the title is quite vague.

Small criticisms.
Fig 2B - need a key for the black triangles vs open circles.
Fig 3B - in legend, says they are assaying AID levels but it is AID mRNA.
Fig 6. It is unclear how long the cells were activated in these experiments.

Referee #2 (Remarks to the Author):

The manuscript by Muto et al., "Structure and Dynamics of the Bach2 gene regulatory network for antibody class switch in B cells", examines the role of the transcription factor Bach 2 in plasma cell differentiation and class switching. This is an important study that could contribute significantly to our understanding as to how class switching and plasma cell differentiation are regulated. However, some major points remain unclear and preclude publication in the current state. While Bach2 seems to be crucial for both processes it is not clear if this is a direct effect or a consequence of perturbed B cell development.

Major points

1. The authors demonstrate that differentiation of Bach2 deficient B cells into plasma cells is dramatically increased despite diminished proliferation, whereas Ig class switching is almost completely blocked. These are very interesting observations. However, the intrinsic nature of these effects is unclear. As published earlier (Muto et al., 2004), Bach2 mice have a severe B cell phenotype, which results in a very different distribution/frequency of mature B cell subsets such as follicular and marginal zone B cells. As marginal zone B cells (and B1 cells) induce Blimp1 expression and plasma cell differentiation significantly faster than follicular B cells, the phenotype of Bach2 deficient mice could be attributed simply to differential frequencies of mature B cell subsets. This different conclusion seems to be supported by Fig. 2B which shows that about 50% of the Bach2 KO cells express syndecan already after one division. Do Bach2 KO cells express this marker already at the start of the culture (in this case the interpretation of most results would be very difficult) or do they up-regulate syndecan within one division (as marginal zone B and B1 cells would do)?

The experiments presented Figures 1-3 must be repeated with sorted follicular B cells.

2. The authors conclude that Blimp1 is a direct target of Bach2. Is there any molecular evidence of

binding of Bach2 to Blimp1 regulatory sequences?

3. One of the key-experiments of the study is the rescue of Ig class switching in Bach2 KO mice by deletion of Blimp1. This is a very interesting finding that should be explored in more detail. As switching is division dependent and Bach2 KO cells appear to have a proliferation defect, one explanation could be that Blimp deficiency restores proliferation. Is that the case? CFSE profiles (including IgG staining) and cell numbers should be presented.

4. The authors leave open the question how Bach2 is shut off in activated B cells and conclude that Bach2 is silenced by an "additional, missing transcription factor that inhibits Bach2 expression". This is possible but not necessarily the case. Pax5 activity was shown to be reduced after B cell activation, which leads to the breakdown of B cell identity and the initiation of plasma cell differentiation. Thus, expression of Bach2 (a target of Pax5) may decrease due to reduced Pax5 binding to the Bach2 promoter. This should be addressed or at least adequately discussed. Similarly, the role of Bcl6 in the model as presented by Muto et al is not addressed. Bcl6 levels are reduced in Bach2 KO B cells. The authors should include Bcl6 in their analysis (such as in Fig. 4B) and include it in their model.

5. The authors attempt to show differential expression/localization of Bach2 in IgM positive and switched B cells. This experiment, as currently presented in Fig. 4A-C, is difficult to interpret and needs to be improved. In the current setting it is hard to distinguish switched antibody secreting plasma cells from switched B cells or IgM+ antibody secreting plasma cells from IgM+ B cells. This is crucial, as plasma cells do not express Bach2; which may result in a very different staining pattern. An easy way to get around this problem would be the use of cultured B cells from the Blimp1 reporter mice. These cells should be FACS sorted into Blimp1+ (plasma) cells and Blimp1 negative (B cells). Blimp1 negative B cells should be further separated into surface IgG+ and IgG- cells during the sorting process. Intracellular staining of Bach2 should be done on these sorted to be informative. In Fig. 4D the authors aim to show that Bach2 is 'high in primary follicles but low in matured GC'. This is impossible to conclude from the presented histology. The staining and/or the imaging of the histology is very poor and at the very least should be improved by adding a B cell stain such as IgD and a germinal centre stain such as PNA. Also, a negative control for the Bach2 stain should be included (Bach2 deficient mice should be used). A second, possible more convincing experiment would be to sort the cells and perform a Western Blot.

Additional points
- Please provide a quantification for the Elispot experiments in Fig. 2C.
- The analysis in Fig.4B should be done on Blimp/GFP negative B cells. Decreasing levels of Pax5 and Bach2 may simply represent increasing numbers of plasma cell in the culture.
- One of the central points of the study, the role of Bach 2 in plasma cell differentiation, is not reflected in the title.

Referee #3 (Remarks to the Author):

This manuscript from Muto et al. examines the interaction between Bach2 and Blimp-1 in regulating the choice of the induction of class switch recombination and somatic hypermutation on the one hand and differentiation into plasma cells on the other. Bach2 is already known to be induced by Pax-5, to be required for class switch recombination, and to be a repressor of Blimp-1. The authors show that the loss of Blimp-1 in Bach-2 -/- B cells restores class switch recombination, but the reduction in B cell numbers in Bach-2 -/- mice was not restored by deletion of Blimp-1. A mathematical modeling approach to examining the putative gene regulatory network is included.

The mathematical model approach used is derived from one used previously by the Dinner and Singh laboratories. The application of the three differential equations in the text to generate the data in Figure 4D is poorly explained and is not really a requirement for the fairly simple regulatory network models proposed.

Figure 6 is unnecessary and the data in Figure 6D is of poor quality.

1st Revision - authors' response 14 August 2010

Specific Responses to referees' Comments:

Referee #1

1. They provide strong data supporting the conclusions they make, except for the points made around Fig 6D. This is a confusing figure that is very difficult to interpret. What are the structures shown? If they are germinal centers or B cell follicles, this is surprising because there is no apparent difference in unimmunized and immunized mice, except for the tone of green. The staining needs to be explained better in the legend/text, and perhaps some arrows to point to structures. The methods section does not help.

REPLY: Figure 6D was aimed to show that reduction in Bach2 expression correlates with plasma cell differentiation in vivo. We now provide more strong data supporting this contention using the Blimp-1-EGFP reporter cells (revised Figure 7C and D; see our reply to Referee #2, point #5). Since Referee #3 suggested us to exclude the original Figure 6 from the manuscript and the newly added revised Figure 7C and D support our conclusions, we have decided to delete the original Figure 6D.

2. The title could be more explicit - to indicate that Bach2 inhibits CSR by stimulating differentiation to plasma cells. The effect on CSR is very strong - and the title is quite vague.

REPLY: We would like to thank the referee for suggesting improvement of the title. We change the title to read: "Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch". The concept that Bach2 directs class switch recombination by inhibiting plasma cell differentiation is reflected in the title.

3. Fig 2B - need a key for the black triangles vs open circles.

REPLY: We would like to thank the referee for pointing out the missing description. We have explained the symbols in the legend of revised Figure 2B.

4. Fig 3B - in legend, says they are assaying AID levels but it is AID mRNA.

REPLY: We have corrected the description in the legend of revised Figure 4A (original Figure 3B).

5. Fig 6. It is unclear how long the cells were activated in these experiments.

REPLY: In the revised Figure 7B and 7C (original Figure 6B and 6C), splenic B cells were stimulated by LPS plus IL-4 for 3 days in culture. We have provided this critical experimental information in the figure legend.

Referee #2

While Bach2 seems to be crucial for both processes it is not clear if this is a direct effect or a consequence of perturbed B cell development.

REPLY: To address this issue and related points raised by this reviewer, we have carried out several additional experiments. Results now allow us to conclude that the CSR defect was due to changes in the kinetics of plasma cell differentiation as originally proposed but not due to any other developmental defect in B cells including the balance between follicular (FO) and marginal zone (MZ) B cells. More detailed explanation is provided in replies to the specific comments below.

Major points

1. The authors demonstrate that differentiation of Bach2 deficient B cells into plasma cells is dramatically increased despite diminished proliferation, whereas Ig class switching is almost completely blocked. These are very interesting observations. However, the intrinsic nature of these effects is unclear. As published earlier (Muto et al., 2004), Bach2 mice have a severe B cell phenotype, which results in a very different distribution/frequency of mature B cell subsets such as follicular and marginal zone B cells. As marginal zone B cells (and B1 cells) induce Blimp1 expression and plasma cell differentiation significantly faster than follicular B cells, the phenotype of Bach2 deficient mice could be attributed simply to differential frequencies of mature B cell subsets. This different conclusion seems to be supported by Fig. 2B which shows that about 50% of

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the Bach2 KO cells express syndecan already after one division. Do Bach2 KO cells express this marker already at the start of the culture (in this case the interpretation of most results would be very difficult) or do they up-regulate syndecan within one division (as marginal zone B and B1 cells would do)?
The experiments presented Figures 1-3 must be repeated with sorted follicular B cells.
REPLY: We appreciate this reviewer for pointing out these critical issues. We totally agree with the suggestion that examination of the FO B cells in Bach2 knockout mice is necessary. Similar to the results from total splenic B cells, we found that Bach2-knockout FO B cells express the Blimp-1/EGFP reporter more frequently than WT FO B cells in vitro (revised Figure 1F; text, p. 7, line 8-). The frequency of the Blimp-1/EGFP- expressing cells from FO B cell is comparable to that of total splenic B cells in Bach2 knockout mice. Therefore, we conclude that the observed defects could not be explained by a "differential frequencies of mature B cell subsets". We described these findings in the revised text (p. 7, line 23-) and Figure 1F.
Compared to wild-type splenic B cells, we found that increased percentages of total splenic B cells expressed CD138/Syndecan-1 in the absence of Bach2 (revised Figure 1C; text, p. 6, line 18-). However, the percentage was much lower than that of LPS-activated B cells. Therefore, these observations clearly demonstrated that CD138/Syndecan-1 expressing cells which appeared after one division were newly-formed antibody secreting cells (ASCs) induced by LPS stimulation.
We think that the above additional experiments are sufficient to settle the main argument by this reviewer.

2. The authors conclude that Blimp1 is a direct target of Bach2. Is there any molecular evidence of binding of Bach2 to Blimp1 regulatory sequences?
REPLY: Many biochemical/molecular data support the contention that Bach2 represses Blimp-1 gene in B cells (Ochiai et al., JBC 2006; Ochiai et al., Int. Immunol. 2008). One experiment still lacking is chromatin immunoprecipitation (ChIP) of Bach2. We have raised several antibodies but they do not function in ChIP. As such, we cannot provide ChIP data at this time. It is our responsibility for the field to solve this issue and we are working on this very hard.

3. One of the key-experiments of the study is the rescue of Ig class switching in Bach2 KO mice by deletion of Blimp1. This is a very interesting finding that should be explored in more detail. As switching is division dependent and Bach2 KO cells appear to have a proliferation defect, one explanation could be that Blimp deficiency restores proliferation. Is that the case? CFSE profiles (including IgG staining) and cell numbers should be presented.
REPLY: According to this suggestion, we examined cell division cycle of FO B cells in the presence of both LPS and IL-4 (a condition inducing IgG1 CSR). In contrast to the LPS-only condition (Figure 2A and B), cell division proceeded similarly in wild-type and Bach2 knockout B cells. Furthermore, Blimp-1 knockout did not affect cell division cycle of Bach2 knockout B cells (revised Figure 3B). Taken together, these observations resolved two issues raised by this reviewer. First, the CSR defect is not simply due to a failure to proliferate in response to stimuli. Second, the rescue of CSR by the Blimp-1 deficiency was not due to restoration of proliferation. These data are now provided as revised Figure 3B and C and described accordingly (p. 9, line 22-).
We tried CFSE staining and IgG staining simultaneously but we found that this combination inhibited CSR in our hands. Reason for this is not clear. However, the original and newly added data strongly suggest that proliferation defect itself is not the cause of the CSR defect or the rescue by the Blimp-1 deficiency in Bach2 knockout B cells.

4. .... Pax5 activity was shown to be reduced after B cell activation, which leads to the breakdown of B cell identity and the initiation of plasma cell differentiation. Thus, expression of Bach2 (a target of Pax5) may decrease due to reduced Pax5 binding to the Bach2 promoter. This should be addressed or at least adequately discussed. Similarly, the role of Bcl6 in the model as presented by Muto et al is not addressed. Bcl6 levels are reduced in Bach2 KO B cells. The authors should include Bcl6 in their analysis (such as in Figure 4B) and include it in their model.
REPLY: Consistent with the idea suggested by this reviewer, Pax5 expression decreased in the Blimp-1 knockout B cells after stimulation (revised Figure 4A). We modified the text to explain this finding: "Alternatively, the reduction of Pax5 expression may lead to the reduction of Bach2 expression, since B cells lacking Blimp-1 showed normal decrease in Pax5 expression following activation stimuli." (p. 12, line 22-). Because reduction of Bach2 expression precedes that of Pax5 expression (revised Figure 5B, original Figure 4B), we still need to consider the possible presence of additional, missing transcription factor that inhibits Bach2 expression.

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According to the reviewer's comment, we examined Bcl6 expression in Bach2 knockout, Blimp-1 knockout, and Bach2&Blimp-1 DD B cells by quantitative RT-PCR (p. 12, line 5-; revised Figure 4A). Importantly, the expression of Bcl6 mRNA in activated Bach2 knockout B cells was comparable to that of activated wild-type B cells, indicating that we may omit Bcl6 from our modeling for simplicity. Interestingly, Bcl6 expression was dramatically increased in LPS-activated Bach2&Blimp-1 DD B cells. The overexpression of Bcl6 may be relevant to the rescue of CSR and we will address this issue in our future project.

5. The authors attempt to show differential expression/localization of Bach2 in IgM positive and switched B cells. This experiment, as currently presented in Fig. 4A-C, is difficult to interpret and needs to be improved. In the current setting it is hard to distinguish switched antibody secreting plasma cells from switched B cells or IgM+ antibody secreting plasma cells from IgM+ B cells. This is crucial, as plasma cells do not express Bach2; which may result in a very different staining pattern. An easy way to get around this problem would be the use of cultured B cells from the Blimp1 reporter mice. These cells should be FACS sorted into Blimp1+ (plasma) cells and Blimp1 negative (B cells). Blimp1 negative B cells should be further separated into surface IgG+ and IgG- cells during the sorting process. Intracellular staining of Bach2 should be done on these sorted to be informative.
In Fig. 4D the authors aim to show that Bach2 is 'high in primary follicles but low in mature dGC'. This is impossible to conclude from the presented histology. The staining and/or the imaging of the histology is very poor and at the very least should be improved by adding a B cell stain such as IgD and a germinal centre stain such as PNA. Also, a negative control for the Bach2 stain should be included (Bach2 deficient mice should be used). A second, possible more convincing experiment would be to sort the cells and perform a Western Blot.
REPLY: We appreciate the suggestion of a simple but decisive experiment. By carrying out the suggested experiment, we now found that Bach2 staining was weaker in a substantial fraction of IgM+/Blimp-1- cells than in IgG+/Blimp-1- cells. The Bach2 staining was cytoplasmic, if any, in Blimp-1+ plasma cells, whereas its staining were detected mainly nuclear in Blimp-1- cells. These data indicate that B cells undergoing CSR tended to express Bach2 at higher levels than IgM B cells and that Bach2 is rapidly shut off in newly formed plasma cells expressing Blimp-1. The newly obtained data are now provided as Figure 7C and 7D, and described in the text (p. 18, line 16-). The conclusion of this part has been changed to reflect these observations: "Diminution of Bach2 protein correlates well with Blimp-1 expression and plasma cell differentiation, supporting its role in Blimp-1 gene repression. While some of the activated IgM+ B cells showed reduction in the Bach2 protein level, they may represent cells that are to detour CSR."
According to the comments of referee #3, we decided to delete entire data of investigation for Bach2 protein levels in germinal center.

Additional points
6. Please provide a quantification for the Elispot experiments in Fig. 2C.
REPLY: According to the referee's comment, we counted spot numbers and presented the quantified data as revised Figure 2C.

7. The analysis in Fig.4B should be done on Blimp/GFP negative B cells. Decreasing levels of Pax5 and Bach2 may simply represent increasing numbers of plasma cell in the culture.
REPLY: This is a rather technically challenging issue. By using Blimp-1/EGFP-negative cells, correlation with Blimp-1 mRNA will be lost. We will address this issue using single cell time-lapse imaging technology in the near future.

8. One of the central points of the study, the role of Bach 2 in plasma cell differentiation, is not reflected in the title.
REPLY: We are grateful for understanding the importance of our study and the helpful comments to improve the title. Incorporating this suggestion, we now change the title to read: "Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch".

Referee #3

The mathematical model approach used is derived from one used previously by the Dinner and Singh laboratories. The application of the three differential equations in the text to generate the

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data in Figure 4D is poorly explained and is not really a requirement for the fairly simple regulatory network models. proposed.
REPLY: We apologize for our poor presentation. More detailed description of our mathematical model approach has been provided in the revised manuscript (p. 13, line 3-). In brief, development of equations is described according the steps and four stages around CSR are described separately (mature-B, time keeper stage for CSR, a sensor stage for initiation of plasma cell differentiation, and plasma cell stage).

Figure 6 is unnecessary and the data in Figure 6D is of poor quality.
REPLY: Since referees #1 and #3 also raised concerns about Figure 6D, we decided to Figure 6D. Other data in the original Figure 6 has been strengthened by newly added experiments using Blimp-1/EGFP reporter cells (please refer to our reply to Referee #2, comment #5).

2nd Editorial Decision 01 September 2010

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original referee #2 and the comments are provided below. As you can see this referee appreciates the introduced changes and supports publication in the EMBO Journal. However, the referee also lists a few minor points that have to be clarified before publication here. I would like to ask you to respond to the last remaining concerns in a final revision.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

The authors have significantly improved their manuscript. Most of the major points raised have been adequately addressed. There are only some minor points that I would like to comment on and would ask to have clarified:

Fig. 1C is somewhat puzzling. The authors show B220+ gated FACS plots containing large numbers of plasma cells. Mature plasma cells should have down-regulated B220; thus, it appears that the plot contains only freshly activated plasma blasts. Is that so? And could the authors provide some better data on the number of plasma cells in Bach2-deficient mice?

Fig. 3C seems to suggest a higher rate of cell death in Bach2 deficient B cells. Is that so? And is that significant?

Fig. 5A. The model in the middle of the panel is wrong. Blimp1 is not expressed in B cells undergoing CSR. This should be removed.
There is data published that suggest that down-modulation of Pax5 precedes Blimp1 expression and is responsible for the initiation of plasma cell differentiation. This would be compatible with Fig 4A and could be incorporated in the model.

2nd Revision - authors' response 20 September 2010

Specific Responses to Referee #2:

The authors have significantly improved their manuscript. Most of the major points raised have been adequately addressed. There are only some minor points that I would like to comment on and would ask to have clarified.
REPLY: We are grateful for understanding the improvement of our manuscript and helpful comments to clarify the descriptions.

Fig. 1C is somewhat puzzling. The authors show B220+ gated FACS plots containing large numbers of plasma cells. Mature plasma cells should have down-regulated B220; thus, it appears that the plot contains only freshly activated plasma blasts. Is that so? And could the authors provide some better data on the number of plasma cells in Bach2-deficient mice?
REPLY: To answer this comment, we provide additional panels of FACS data indicating expression profiles of B220 and CD138 in Figure 1C. According to the referee's comment, we added data on the population of plasma cells in the total spleen cells (revised text, page 7, line 7-). Our data indicated clearly that plasma cells are increased in Bach2-deficient mice.

Fig. 3C seems to suggest a higher rate of cell death in Bach2 deficient B cells. Is that so? And is that significant?
REPLY: In another set of experiments, we have found that Bach2-deficient B cells show higher rate of cell death upon activation. This may explain the reduced cell numbers in Figure 3C. However, precise molecular mechanism is still unclear. Therefore, we would like to report the cell death phenotype as another paper after adding further studies.

Fig. 5A. The model in the middle of the panel is wrong. Blimp1 is not expressed in B cells undergoing CSR. This should be removed.
There is data published that suggest that down-modulation of Pax5 precedes Blimp1 expression and is responsible for the initiation of plasma cell differentiation. This would be compatible with Fig 4A and could be incorporated in the model.
REPLY: We would like to thank the referee for pointing out our mistake. We change the panels in Figure 5A so that Blimp-1 expression levels are not depicted by the relative heights. We prefer to retain the modified CSR panel because this panel is essential to describe a time keeper stage for sustaining activation time of Pax5 in the Delay Driven Diversity model.
We decided to cite a paper describing down-modulation of Pax5 before Blimp1 expression according to the referee's comment. We think that there is no discrepancy between this previous finding and our model in this paper and discuss about the relationships among Pax5, Bach2 and Blimp-1 upon plasma cell differentiation (revised text, page 22, line 13-).

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