THE EMBO JOURNAL

Manuscript EMBO-2010-76879

The transcription factor Pax5 regulates its target genes by recruiting chromatin-modifying proteins in committed B cells

Shane McManus, Anja Ebert, Giorgia Salvagiotto, Jasna Medvedovic, Qiong Sun, Ido Tamir, Markus Jaritz, Hiromi Tagoh and Meinrad Busslinger

Corresponding author: Meinrad Busslinger, Research Institute of Molecular Pathology IMP

Review timeline:

Submission date:	25 December 2010
Editorial Decision:	27 January 2011
Revision received:	11 March 2011
Editorial Decision:	16 March 2011
Accepted:	05 April 2011

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 27 January 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below, as you will see the referees express interest in the identification of direct Pax5 target genes and modes of activation and repression, nevertheless they require further experimental analysis before the manuscript is suitable for publication in The EMBO Journal. The major points are the reassignment of enhancers based of H3K4me1 and the identification of Pax5 consensus sequences and some further insight into the mode of repression by Pax5. Given the interest in the study should you be able to satisfactorily address these issues, we would be happy to consider a revised version of the 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 you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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

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

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

This is an excellent, comprehensive analysis elucidating the mechanism of gene activation and repression of the key B lineage transcription factor Pax5. Pax5 binding sites were identified by ChIP-chip on a custom NimbleGen array containing 1306 genes, including 170 genes known to be regulated directly or indirectly by Pax5. By determining which of the genes that were previously demonstrated to be activated by Pax5 do not bind Pax5, one can conclude that the effect of Pax5 in activating those genes must be indirect. In addition, many new potential target genes were identified on the array. The approach of using a mouse into which the authors had knocked-in a biotin acceptor sequence and an IRES-BirA to the Pax5 gene (Pax5 bio/bio) was excellent. This tag did not affect Pax5 function or B cell development at all, and allowed a cleaner ChIP-Chip to be performed without additional signals present on the ChIP-chip performed with polyclonal anti-Pax5 antibody. Four histone post-translational modifications were also analyzed on the custom arrays, permitting a glimpse into the epigenetic profile of these genes before (Pax 5-/- pro-B cells) and after (Pax5+/+ pro-B cells) Pax 5 expression. This analysis revealed a varied pattern of either maintenance of the chromatin status seen in Pax5-/- pro-B cells, addition of active histone marks (H3K9ac, H3K4me2, or H3K4me3), or removal of previously existing repressive H3K27me3 mark. Interestingly, most genes which become repressed by Pax5 do not gain H3K27me3, perhaps explaining their rapid ability to become reinduced if Pax5 is lost later in B cell differentiation as was done in floxed Pax5 mice by the authors in previous publications.

The use of Pax5-ER fusion proteins allowed insight into the rapid induction of active histone marks at the regulated genes. Importantly, the streptavidin pull-down demonstrated that Pax5 interacts with several chromatin regulators including components of the BAF complex, Brg1, TBP, CBP, PTIP, and components of the MLL complex. The rapid ER induction system demonstrated rapid recruitment of those factors to the target genes within 2 hours of Pax5 expression as demonstrated by ChIP. Together, the data provided here make a comprehensive analysis of the role of Pax5 in the epigenetic regulation of a large number of genes that play important roles in cell fate decision (B vs. non-B cells) and in a variety of B cell functions.

Specific comments:

1. My only major concern is the incorrect definition of enhancers. Enhancers are now commonly defined as bearing H3K4me1 but not H3K4me3, and have recently been further subdivided into active enhancers (H3K27ac+) vs. poised enhancers that lack H3K27ac. The definition of enhancers used here (H3K4me2+ H3K4me3-, H3K9ac+) is not correct; H3K4me2 is often absent from enhancers. I would recommend that the authors do ChIP-seq for H3K4me1 to call these elements enhancers, since this is now the accepted chromatin signature of enhancers.

2. Fig. 6 nicely shows rapid induction of chromatin changes by Pax5. However, only active histone marks were analyzed. What is the kinetics of loss of H3K27me3 on repressed genes after Pax5 expression as shown in Fig. 3?

Minor comments:

1.It is not clear why the authors only included two-thirds of the genes that they previously identified as being up or down regulated by Pax5 on their NimbleGen ChIP-chip array. Was this due to space limitations on the array? How were the genes chosen to be included or excluded?

2. α-amanitin was used to block transcription in fig S4B. Did the cells remain viable during the course of the experiment?

Referee #2 (Remarks to the Author):

The manuscript by McManus et. al. reports on the identification of Pax5 binding sites in multiple genes in pro-B cells. The authors developed a mouse model for strepavidin bead affinity purification of Pax5 by creating knock-in mice carrying the biotin acceptor sequence at the C-terminus of Pax5 followed by the gene for the ligase BirA. The authors utilize ChIP-chip (and Bio-ChiP-chip) with an oligonucleotide tiling array that includes multiple known Pax5 regulated genes in B cells (although it is <2% of the mouse genome). The authors make a number of interesting findings most of which are strongly supported by their data:

1. Identification of 398 common Pax5 peaks between the bio-ChIP-chip and standard Pax5 antibody-ChIP-chip, corresponding to 241 genes.

2. Of the 102 known Pax5 regulated genes in pro-B cells, 45 were bound by Pax5.

3. Induction of active chromatin modifications "at the majority of Pax5- binding sites in both promoter (64%) and enhancer (65%) positions".

4. Loss of H3K27me3 in a subset of activated Pax5-target genes concomitant with Pax5-dependent activation.

5. Loss of active histone modifications at repressed Pax5- target genes, though only 16 of the 68 genes repressed by Pax5 in pro-B cells were bound by Pax5. However, a majority of these repressed genes did not acquire H3K27me3.

6. Identification of 178 novel genes that are bound by Pax5 but not obviously regulated by Pax5.

7. Pax5 associates with a number of chromatin regulators in committed pro-B cells.

Overall, the data and analysis increase our understanding of Pax5 target genes in B lymphocytes and suggest a major role for Pax5 in activation of genes involved in B cell signaling, adhesion and migration.

Concerns:

1. The title of the paper is "Epigenetic regulation of B cell commitment by the transcription factor Pax5". This title seems not to fit well with the data presented. First, Pax5 has been associated with B cell "commitment", which is the process of preventing access to alternative cell fates. Commitment, in contrast to specification, is thought to involve the repression of gene expression, and this role for Pax5 has been strongly promoted by this lab. The data in Figure 3B suggests that Pax5 mostly activates transcription, and Figure 5B demonstrates that only ~10% of Pax5-binding sites are associated with genes repressed by Pax5. It is possible that these few genes are critical for commitment (Tox1, Gata3, Tcf7, etc could be important) but these genes are not examined in this paper. The data largely indicate that Pax5's known function in commitment may be indirect (perhaps mediated by the transcription factors induced by Pax5). Second, "Epigenetic" indicates that a chromatin state is propagated to subsequent generations in a heritable manner. This issue is not addressed in the manuscript, and given the dedifferentiation phenotype of Pax5-deleted cells it seems that Pax5 does not induced a stable epigenetic change, rather Pax5 is necessary to maintain the state. Thus the title is inappropriate and should be something like "Pax5 activates multiple B cell genes through recruitment of chromatin modifying proteins" or "Identification of direct Pax5 target genes in pro-B cells".

2. Though the authors have done a great job identifying a subset of genes targeted by Pax5, the manuscript would benefit from inclusion of a binding site analysis (such as MEME) for enriched sequences associated with the Pax5 binding. There are many critical questions that could be resolved with this analysis. Is there a clear consensus for Pax5 binding? Is this consensus the same at activated and repressed genes (or enhancers, promoters or intergenic regions)? Is there evidence for co-association with other factors, for example, Ebf1, E2A or Foxo? Can any of these associations explain differential recruitment of co-activators versus co-repressors?

3. The cyclohexamide experiments on page 14 are very nice, but the experimental design does not rule out the possibility that Pax5 recruits other transcription factors that ultimately recruit chromatin remodelers (alone or in cooperation with Pax5). The authors need to address this point.

4. In figure 7, the Pax5 co-immunoprecipitation is great and shows interaction with Brg1, CBP, and basal transcription complexes, and such in vivo recruitment of these complexes would be expected given the changes detected in the chromatin. However, the authors fail to comment on the instances when Pax5 is recruited to repressed target genes. Would these complexes not be recruited in these instances to these repressed genes? In other words, if these complexes are "stably" formed, then how is repression achieved? Was TLE3 or other Groucho-related protein identified in the IPs and recruited to repressed genes? What about Ncor? These questions should be addressed and include experiments looking at potential recruitment of repressive complexes to Pax5 at repressed target genes, as some repressive chromatin modifiers were identified in the mass spec analysis in figure S5.

5. It is not clear how statistically meaningful the data in figure 4 are. When 3 transgenic lines are made and 1 shows B cell specific expression, one does not, and one is not expressed, it is really reasonable to conclude that the construct contains an important B cell specific cis-regulatory element? The authors conclude from this experiment that enhancer C "confers B-cell-specific activity...in conjunction with enhancer A to the distal promoter B" of this particular gene. These conclusions are weak and overall this figure does not add substantially to the paper.

6. On page 19, the authors conclude that lack of H3K27me3 at Pax5- repressed genes demonstrates that these genes do not undergo reprogramming to a repressed chromatin state. However, the authors cannot make this conclusion because H3K27me3 is not absolutely required for repressive chromatin. The authors did not look at other histone modifications such as H3K9me3 or recruitment of HP1, nor did they assess whether these genes were in heterochromatin. This statement needs to be modified or additional experiments need to be performed.

Minor points:

There are many places in the manuscript where the authors overstate their conclusions. Below we point out a number of these overstatements that should be modified.

1. On page 8, 3rd line from bottom, "Pax5 induced activate chromatin" should be "Pax5 binding correlated with active chromatin".
2. On page 9, the sentence "Pax5-binding converted the repressive state to active chromatin..." needs to be modified to reflect "correlation".
1. On page 12, line 7, "where its binding predominantly caused the loss" should be "where its binding predominantely correlated with a loss".
2. On page 13 4th line from the bottom, remove the sentencing pertaining to mono-methylation of H3K4 being a pre-requisite for di- and tri-methylation, the data do not prove this point.

Referee #3 (Remarks to the Author):

Previous observations performed have established a key and unique function for Pax5 in early B cell development. These studies demonstrated that Pax5 in conjunction with its colleagues, E2A and EBF, acts to induce a B-lineage program of gene expression and to suppress the expression of genes associated with alternate cell lineages. However, it has remained unclear as to how Pax5 acts to modulate gene expression. Although a large subset of genes have been identified in previous studies the entire spectrum of Pax5 target genes remains to be determined. Here a global screen for Pax5 binding sites has been performed. These data identify many of the previous known targets as well as others not yet known to be controlled by Pax5, including the interesting Nedd9 target. The authors also examine the patterns of histone modifications in wild-type and Pax5-deficient pre-pro-B cells. This analysis is quite revealing. Briefly, the data show that Pax5 rapidly induces chromatin and

transcriptional changes by direct recruitment of chromatin-remodeling factors as well as histone-modifying and basal transcription factors components to the appropriate promoters.

In my view this is an important study. Although it is now well established that Pax5 acts in early B cell development to promote developmental progression it has remained unclear as to how it promotes its wide-ranging activities. This study finally provides insight into this issue. The work is very well done and quite convincing. Finally the manuscript is well written and the data presented in a logical manner.

Minor comment

I recommend that the authors present the consensus Pax5 binding site in Figure 1. Also should be included associated binding sites, including those for E2A, EBF, RUNX and FOXO1. If possible statistical values should be presented in Figure 1 for associated regulatory elements.

1st Revision - authors' response 11 March 2011

This is an excellent, comprehensive analysis elucidating the mechanism of gene activation and repression of the key B lineage transcription factor Pax5. Pax5 binding sites were identified by ChIP-chip on a custom NimbleGen array containing 1306 genes, including 170 genes known to be regulated directly or indirectly by Pax5. By determining which of the genes that were previously demonstrated to be activated by Pax5 do not bind Pax5, one can conclude that the effect of Pax5 in activating those genes must be indirect. In addition, many new potential target genes were identified on the array. The approach of using a mouse into which the authors had knocked-in a biotin acceptor sequence and an IRES-BirA to the Pax5 gene ($Pax5^{Bio/Bio}$) was excellent. This tag did not affect Pax5 function or B cell development at all, and allowed a cleaner ChIP-Chip to be performed without additional signals present on the ChIP-chip performed with polyclonal anti-Pax5 antibody. Four histone post-translational modifications were also analyzed on the custom arrays, permitting a glimpse into the epigenetic profile of these genes before ($Pax5^{-/-}$ pro-B cells) and after ($Pax5^{+/+}$ pro-B cells) Pax5 expression. This analysis revealed a varied pattern of either maintenance of the chromatin status seen in $Pax5^{-/-}$ pro-B cells, addition of active histone marks (H3K9ac, H3K4me2, or H3K4me3), or removal of previously existing repressive H3K27me3 mark. Interestingly, most genes, which become repressed by Pax5 do not gain H3K27me3, perhaps explaining their rapid ability to become reinduced if Pax5 is lost later in B cell differentiation as was done in floxed Pax5 mice by the authors in previous publications.

The use of Pax5-ER fusion proteins allowed insight into the rapid induction of active histone marks at the regulated genes. Importantly, the streptavidin pull-down demonstrated that Pax5 interacts with several chromatin regulators including components of the BAF complex, Brg1, TBP, CBP, PTIP, and components of the MLL complex. The rapid ER induction system demonstrated rapid recruitment of those factors to the target genes within 2 hours of Pax5 expression as demonstrated by ChIP. Together, the data provided here make a comprehensive analysis of the role of Pax5 in the epigenetic regulation of a large number of genes that play important roles in cell fate decision (B vs. non-B cells) and in a variety of B cell functions.

We are pleased that this reviewer likes our manuscript and considers it to be of interest for publication in the EMBO Journal.

Specific comments:

1. My only major concern is the incorrect definition of enhancers. Enhancers are now commonly defined as bearing H3K4me1 but not H3K4me3, and have recently been further subdivided into active enhancers (H3K27ac+) vs. poised enhancers that lack H3K27ac. The definition of enhancers used here (H3K4me2+ H3K4me3-, H3K9ac+) is not correct; H3K4me2 is often absent from enhancers. I would recommend that the authors do ChIP-seq for H3K4me1 to call these elements enhancers, since this is now the accepted chromatin signature of enhancers.

The reviewer is correct in pointing out that enhancers in established human cell lines are often identified by the

chromatin signature H3K4me1+ H3K4me3-, as initially described by Heintzman et al. (Nat. Genet. 39, 311-318; Nature 459, 108-112). However, this correlation seems to be less stringent in mouse pro-B cells as shown by Lin et al. (Nat. Immunol. 11, 635-643). Figure 2d of this publication clearly demonstrates that H3K4me1 marks are present in many H3K4me3+ promoter regions. Figure 2c also nicely demonstrates that the K3K4me2 modification is present at all H3K4me1+ enhancer regions contrary to the opinion of this reviewer. Clearly, the most predictive histone modification is H3K4me3, as it is present at promoters (H3K4me3+) and absent at distal enhancers (H3K4me3-). As suggested by the reviewer, we have reinvestigated the chromatin signatures at the 398 high-confidence Pax5-binding sites (identified by ChIP-chip analysis in our paper) by comparing the corresponding ChIP-sequencing data of Rag-deficient pro-B cells (H3K4me1, H3K4me2, H3K4me [Lin et al.] and H3K9ac [our own unpublished data]). This analysis is now shown in suppl. Figure S3 and demonstrates that our enhancer prediction based on ChIP-chip data is confirmed by the corresponding ChIP-sequencing data. Active promoters have the chromatin signature H3K4me1$^{low/-}$ H3K4me2^{high} **H3K4me3^{high}** H3K9ac^{high}, whereas active enhancers are characterized by H3K4me1^{+} **H3K4me2^{+} H3K4me3$^{low/-}$ H3K9ac^{medium}**. We conclude therefore that we used the correct criteria (indicated in bold) for identifying active enhancers and promoters in our manuscript. We now refer to suppl. Figure 3 on page 8, where we introduce the chromatin signatures of active enhancers and promoters.

2. Fig. 6 nicely shows rapid induction of chromatin changes by Pax5. However, only active histone marks were analyzed. What is the kinetics of loss of H3K27me3 on repressed genes after Pax5 expression as shown in Fig. 3?

We presume that the reviewer meant "*... loss of H3K27me3 on **activated** genes after Pax5 expression as shown in Fig. 3?"*
We have performed the requested experiment and show now in the new suppl. Figure S7C that Pax5-ER activation leads to the loss of H3K27me3 modifications at the Pax5-binding sites of the activated target genes *Blnk*, *Ikzf3* and *Bcar3*. We mention this result on page 14 (middle; marked in blue).

Minor comments:

1. It is not clear why the authors only included two-thirds of the genes that they previously identified as being up or down regulated by Pax5 on their NimbleGen ChIP-chip array. Was this due to space limitations on the array? How were the genes chosen to be included or excluded?

We now describe on page 7 (middle) in Supplementary Material how we selected the genes for inclusion into the DNA microarray: "We designed a custom-made DNA microarray (NimbleGen Systems) by selecting 102 of the 170 previously identified Pax5-activated genes (Schebesta et al., 2007) and 68 of the 110 identified Pax5-repressed genes (Delogu et al., 2006) based on the following criteria. We included genes of known function, whose differential expression was validated in *Pax5*$^{-/-}$ and *Rag2*$^{-/-}$ pro-B cells by semiquantitative RT-PCR and which were extensively characterized in both studies. In addition, important regulatory genes of different hematopoietic lineages constituted the second part of the DNA microarray."

2. a-Amanitin was used to block transcription in fig S4B. Did the cells remain viable during the course of the experiment?

We now show in suppl. Figure S8C that the *Rag2*$^{-/-}$ pro-B cells were perfectly viable after an 8 or 6.5 hr treatment with a-amanitin or cycloheximide, respectively, as shown by their FSC/SCC profiles. We now mention this fact on page 15 (upper part).

Referee #2:

The manuscript by McManus et. al. reports on the identification of Pax5 binding sites in multiple genes in pro-B cells. The authors developed a mouse model for strepavidin bead affinity purification of Pax5 by creating knock-in mice carrying the biotin acceptor sequence at the C-terminus of Pax5 followed by the gene for the ligase BirA. The authors utilize ChIP-chip (and Bio-ChiP-chip) with an oligonucleotide tiling array that includes multiple known Pax5 regulated genes in B cells (although it is <2% of the mouse genome). The authors make a number of interesting findings most of which are strongly supported by their data:

1. Identification of 398 common Pax5 peaks between the bio-ChIP-chip and standard Pax5 antibody-ChIP-chip, corresponding to 241 genes.

2. Of the 102 known Pax5 regulated genes in pro-B cells, 45 were bound by Pax5.

3. Induction of active chromatin modifications "at the majority of Pax5- binding sites in both promoter (64%) and enhancer (65%) positions".

4. Loss of H3K27me3 in a subset of activated Pax5-target genes concomitant with Pax5-dependent activation.

5. Loss of active histone modifications at repressed Pax5- target genes, though only 16 of the 68 genes repressed by Pax5 in pro-B cells were bound by Pax5. However, a majority of these repressed genes did not acquire H3K27me3.

6. Identification of 178 novel genes that are bound by Pax5 but not obviously regulated by Pax5.

7. Pax5 associates with a number of chromatin regulators in committed pro-B cells.

Overall, the data and analysis increase our understanding of Pax5 target genes in B lymphocytes and suggest a major role for Pax5 in activation of genes involved in B cell signaling, adhesion and migration.

We are grateful that this reviewer considers our manuscript to be of interest for publication in the EMBO Journal.

Concerns:

1. The title of the paper is "Epigenetic regulation of B cell commitment by the transcription factor Pax5". This title seems not to fit well with the data presented. First, Pax5 has been associated with B cell "commitment", which is the process of preventing access to alternative cell fates. Commitment, in contrast to specification, is thought to involve the repression of gene expression, and this role for Pax5 has been strongly promoted by this lab. The data in Figure 3B suggests that Pax5 mostly activates transcription, and Figure 5B demonstrates that only ~10% of Pax5-binding sites are associated with genes repressed by Pax5. It is possible that these few genes are critical for commitment (Tox1, Gata3, Tcf7, etc could be important) but these genes are not examined in this paper. The data largely indicate that Pax5's known function in commitment may be indirect (perhaps mediated by the transcription factors induced by Pax5). Second, "Epigenetic" indicates that a chromatin state is propagated to subsequent generations in a heritable manner. This issue is not addressed in the manuscript, and given the dedifferentiation phenotype of Pax5-deleted cells it seems that Pax5 does not induced a stable epigenetic change, rather Pax5 is necessary to maintain the state. Thus the title is inappropriate and should be something like "Pax5 activates multiple B cell genes through recruitment of chromatin modifying proteins" or "Identification of direct Pax5 target genes in pro-B cells".

We agree with the reviewer and have thus changed the title to “The transcription factor Pax5 regulates its target genes by recruiting chromatin-modifying protein in committed pro-B cells”.

2. Though the authors have done a great job identifying a subset of genes targeted by Pax5, the manuscript would benefit from inclusion of a binding site analysis (such as MEME) for enriched sequences associated with the Pax5 binding. There are many critical questions that could be resolved with this analysis. Is there a clear consensus for Pax5 binding? Is this consensus the same at activated and repressed genes (or enhancers, promoters or intergenic regions)? Is there evidence for co-association with other factors, for example, Ebf1, E2A or Foxo? Can any of these associations explain differential recruitment of co-activators versus co-repressors?

We tried very hard to find a Pax5 consensus motif in the Pax5 peaks identified by ChIP-chip by using different *de novo* motif discovery programs. However, we were unsuccessful most likely for the following two reasons. First, we previously demonstrated that the Pax5 consensus recognition sequence is not only 16 nucleotides long but also highly degenerate (Czerny et al., Genes Dev. 7, 2048-2061). Second, the size of the Pax5 peaks obtained by ChIP-chip is generally quite large, which is not helpful for the *de novo* discovery of a degenerate consensus motif. However, we would like to point out that we analyzed only high-confidence Pax5-binding sites that were detected by two independent methods (antibody- and streptavidin-mediated ChIP-chip).

3. The cycloheximide experiments on page 14 are very nice, but the experimental design does not rule out the

possibility that Pax5 recruits other transcription factors that ultimately recruit chromatin remodelers (alone or in cooperation with Pax5). The authors need to address this point.

As this point of criticism cannot be experimentally addressed, we modified the text describing the cycloheximide and a-amanitin experiments by eliminating the word “directly” in the entire paragraph (pages 14 [bottom] and 15 [top]) and by replacing it by “in a transcription- and protein synthesis-independent manner” in the concluding sentence.

4. In figure 7, the Pax5 co-immunoprecipitation is great and shows interaction with Brg1, CBP, and basal transcription complexes, and such in vivo recruitment of these complexes would be expected given the changes detected in the chromatin. However, the authors fail to comment on the instances when Pax5 is recruited to repressed target genes. Would these complexes not be recruited in these instances to these repressed genes? In other words, if these complexes are "stably" formed, then how is repression achieved? Was TLE3 or other Groucho-related protein identified in the IPs and recruited to repressed genes? What about Ncor? These questions should be addressed and include experiments looking at potential recruitment of repressive complexes to Pax5 at repressed target genes, as some repressive chromatin modifiers were identified in the mass spec analysis in figure S5.

We tried to perform the requested Groucho ChIP experiments. Although we tested several available Grg3 (TLE3) and Grg4 (TLE4) antibodies, none of them were good enough for ChIP analysis. Due to the lack of suitable antibodies, we could not investigate the Pax5-dependent recruitment of Grg3 or Grg4 to repressed Pax5 target genes, as requested (now mentioned on page 19, bottom). The reviewer correctly pointed out that we identified, by mass spectrometry, the components TBLR1 (Tbl1xr1) and NCoR1 of the NCoR1 corepressor complex as Pax5-interacting proteins. We recently realized that our mass spectrometry data additionally contained TBL1 as a third component of NCoR1 corepressor complex (now added to the list in suppl. Figure 9). We now demonstrate in suppl. Figure S10 that Pax5 is specifically co-precipitated with NCoR1 antibodies from pro-B cell nuclear extracts (panel A). Moreover, Pax5 rapidly recruits NCoR1 to the Pax5-binding sites of five repressed Pax5 target genes (panel B). These experiments are now mentioned on pages 16 (middle), 17 (middle) and 19 (bottom).

5. It is not clear how statistically meaningful the data in figure 4 are. When 3 transgenic lines are made and 1 shows B cell specific expression, one does not, and one is not expressed, it is really reasonable to conclude that the construct contains an important B cell specific cis-regulatory element? The authors conclude from this experiment that enhancer C "confers B-cell-specific activity...in conjunction with enhancer A to the distal promoter B" of this particular gene. These conclusions are weak and overall this figure does not add substantially to the paper.

We disagree with the statement that the transgenic analysis of the *Nedd9* enhancers does not add substantially to this paper. In contrast, we regard it important to provide in vivo evidence that the Pax5-dependent induction of H3K4me2 and H3K9ac (in the absence H3K4me3) identifies active Pax5-regulated enhancers that confer B cell-specific activity to Pax5 target genes. In the literature, enhancer validation is usually performed by artifact-prone transient transfection experiments in established cell lines, whereas we have used transgenic analysis as a gold standard for testing enhancer activity in vivo within the entire hematopoietic system. In the field of mouse developmental biology, it is common knowledge that transgenic analysis with proper statistical presentation (as shown in Figure 4C) often produces few transgenic lines recapitulating the correct expression pattern due to position effects at the transgene integration site. As transgenic experiments are time-consuming, we could not repeat the *Nedd9* transgenic analysis to obtain more *ABC* and *DC* transgenic lines. However, we would like to emphasize that the B cell-specific *ABC* and *DC* transgenic lines faithfully recapitulate the endogenous activity of the *Nedd9* promoters B and D during B cell development as shown by RT-PCR (suppl. Figure 4B). Moreover, *Nedd9* is an interesting gene, as it codes for an essential adaptor of integrin signaling, which plays an important role in B cell trafficking, as explained on page 10 (top).

6. On page 19, the authors conclude that lack of H3K27me3 at Pax5-repressed genes demonstrates that these genes do not undergo reprogramming to a repressed chromatin state. However, the authors cannot make this conclusion because H3K27me3 is not absolutely required for repressive chromatin. The authors did not look at other histone modifications such as H3K9me3 or recruitment of HP1, nor did they assess whether these genes were in heterochromatin. This statement needs to be modified or additional experiments need to be performed.

We have changed the text on page 20 (bottom) accordingly to eliminate the above statements. We now specifically refer to PRC2-mediated silencing.

Minor points:

There are many places in the manuscript where the authors overstate their conclusions. Below we point out a number of these overstatements that should be modified.

1. On page 8, 3rd line from bottom, "Pax5 induced activate chromatin" should be "Pax5 binding correlated with active chromatin".

We have made the corresponding change on page 8 (bottom).

2. On page 9, the sentence "Pax5-binding converted the repressive state to active chromatin..." needs to be modified to reflect "correlation".

We have made the corresponding change on page 9 (middle).

3. *On page 12, line 7, "where its binding predominantly caused the loss" should be "where its binding predominantly correlated with a loss".*

We have made the corresponding change on page 12 (middle).

4. On page 13, 4th line from the bottom, remove the sentencing pertaining to mono-methylation of H3K4 being a pre-requisite for di- and tri-methylation, the data do not prove this point.

We did not eliminate this sentence, as it describes an interesting novel finding. However, we weakened the statement to: "... which suggests that mono-methylation may be a prerequisite for di- and tri-methylation by H3K4 methyltransferases" (page 14, top)/

Referee #3:

Previous observations performed have established a key and unique function for Pax5 in early B cell development. These studies demonstrated that Pax5 in conjunction with its colleagues, E2A and EBF, acts to induce a B-lineage program of gene expression and to suppress the expression of genes associated with alternate cell lineages. However, it has remained unclear as to how Pax5 acts to modulate gene expression. Although a large subset of genes have been identified in previous studies the entire spectrum of Pax5 target genes remains to be determined. Here a global screen for Pax5 binding sites has been performed. These data identify many of the previous known targets as well as others not yet known to be controlled by Pax5, including the interesting Nedd9 target. The authors also examine the patterns of histone modifications in wild-type and Pax5-deficient pre-pro-B cells. This analysis is quite revealing. Briefly, the data show that Pax5 rapidly induces chromatin and transcriptional changes by direct recruitment of chromatin-remodeling factors as well as histone-modifying and basal transcription factors components to the appropriate promoters.

In my view this is an important study. Although it is now well established that Pax5 acts in early B cell development to promote developmental progression it has remained unclear as to how it promotes its wide-ranging activities. This study finally provides insight into this issue. The work is very well done and quite convincing. Finally the manuscript is well written and the data presented in a logical manner.

We thank this reviewer for acknowledging the importance of our study.

Minor comment:

I recommend that the authors present the consensus Pax5 binding site in Figure 1. Also should be included associated binding sites, including those for E2A, EBF, RUNX and FOXO1. If possible statistical values should be presented in Figure 1 for associated regulatory elements.

Unfortunately, the Pax5-binding sites were defined as relatively large regions in our ChIP-chip analysis, which prevented the identification of a Pax5 consensus recognition motif by *de novo* motif discovery programs, as discussed in detail under point 2 of reviewer #2.

Additional new and important control experiments to demonstrate the specificity of the Pax5-ER induction system.

Although the reviewers did not ask for this control experiment, we felt it important to demonstrate that the hormone-binding domain of the estrogen receptor does not contribute to the recruitment of Pax5-binding proteins or the induction of active chromatin at Pax5-binding sites of target genes in response to 4-hydroxytamoxifen (OHT) treatment. For this, we generated *Pax5*$^{-/-}$ pro-B cells expressing the Prd-ER protein consisting of the N-terminal DNA-binding Paired (Prd) domain of Pax5 fused to the hormone-binding domain of the estrogen receptor (referred to as KO-Prd-ER pro-B cells; suppl. Figure S7A). Although the Prd-ER protein interacted with the Pax5-binding sites in the *Blnk* and *Ikzf3* promoters upon OHT addition (as shown by ER ChIP), it failed to induce active chromatin (H3K9ac) and to recruit the Pax5-interacting Brg1 protein of the BAF complex in marked contrast to the Pax5-ER protein (suppl. Figure 7B). This experiment therefore demonstrates that the central and C-terminal sequences of Pax5 are essential for inducing active chromatin and for recruiting Pax5-interacting complexes. We discuss these data on pages 13 (middle) and 16 (bottom).

Final conclusion:

We thank the reviewers for their insightful comments. We have addressed almost all comments of the reviewers by performing the requested experiments, which has considerably strengthened the message of our manuscript. We therefore hope that our substantially improved and revised manuscript is now acceptable for publication in the EMBO Journal.

2nd Editorial Decision 16 March 2011

Thank you for submitting a revised version of your manuscript to The EMBO Journal.
I have read through your response to the referees and find that you have satisfactorily addressed all their concerns and I am happy to accept the manuscript for publication in The EMBO Journal. While reading the response I also felt that it would be better to have the NCoR1 data in the main manuscript so it will be fine to have an eighth figure. Rather than going through the entire resubmission process could you send us the following by email:
(1) a revised manuscript file, with the supplementary material removed, and also the author contribution statement added after the acknowledgments.
(2) a figure 8
(3) a separate supplementary material file.

Yours sincerely,

Editor
The EMBO Journal

