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Molecular role of the PAX5-ETV6 oncoprotein in promoting B-cell acute lymphoblastic leukemia

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   26 September 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the slightly extended duration of the review period in this case. Your study has now been seen by three referees and their comments are shown below.

As you will see from the reports, all referees express high interest in the findings reported in your manuscript and support publication in The EMBO Journal, pending adequate revision. Given the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:
-> further compare and discuss the effect of Pax5-fusion proteins in mouse and human. As mentioned by all three refs this point is essential for the translational implications of this work.
-> we had initially consulted with an external expert advisor on your manuscript and this person found that while the amount of data generated in this study is impressive, the analysis itself - especially at the genomics level - could have been taken further in order to better understand the possible functional contribution from the genes affected by fusion protein expression. This concern does not emerge as a central point in the comments from the referees (apart from ref #2 who asks

you to explain the rationale behind the focus on CXCL12) but I would nonetheless ask you to consider this point for the revised manuscript, in case you have further data that could be included.

REFEREE REPORTS

Referee #1:

Smeenk et al. have established a new mouse model that recapitulates the main characteristics of Pax5 fusions-associated B-ALL and showed mechanistic aspects of transcriptional activity of the fusion proteins with respect to Pax5 targets.
The study is of high interest and has an impact on our perception of B-ALL and Pax5 biology. The authors should further study/explain differences between the human disease and mouse model of disease. A few other points need to be clarified as outlined below:

Main points:
"RNA-sequencing (RNA-seq) comparison of ex vivo sorted Pax5+/+ and Pax5+/- pro-B cells furthermore revealed only 1 up-regulated and 16 down- regulated genes in Pax5+/- pro-B cells (Fig. 1D and Appendix Fig. S1A)."
-what are the levels of Pax5 mRNA in Pax5+/+ compared to Pax5+/- mice/cells?
-Are any of the down- or up-regulated genes direct Pax5 targets? This an important question especially as the authors show later in the manuscript that Pax+/+ and Pax5Prd have a substantially overlapping repertoire of targets.

"Moreover, the large pre-B cells expressing the pre-BCR were also decreased in Pax5Etv6/+ and Pax5Foxp1/+ mice, whereas pro-B cells were moderately increased."
-As this is the first mouse model to express the chimeric proteins in a a physiological in vivo setting, the authors should perform basic characterization of the other hematopoietic lineages (T and myeloid ones, for instance) to exclude the presence of additional abnormalities.

"The considerably higher (5-6-fold) number of differentially expressed genes identified in human PAX5-ETV6+ B-ALLs compared to the mouse Cdkn2ab+/- Pax5Etv6/+ B-ALLs could be explained by the fact that we used the quiescent small pre-B cells as a reference cell type in the human comparison in contrast to the cycling large pre-B cells in the mouse comparison."
-This is an important point as this model can be used to test novel drug treatments and combinatorial treatments for this disease subtype.
-Are a) the relative protein levels and b) the genomic binding sites of Pax5 and the Pax5-fusion proteins comparable between human and mouse B-ALL?
-Could this difference be due to additional genetic alterations occurring in the case of the human B-ALL. Analysis of WGS data in this type of human B-ALL could provide evidence for additional genetic alterations.

Other points
"we inserted human cDNA sequences, starting in exon 4 and encoding the remaining PAX5-ETV6 or PAX5-FOXP1 protein, in frame into exon 4 of the mouse Pax5 locus to generate the Pax5Etv6 and Pax5Foxp1 knock-in alleles."
-The main difference compared to the human case is the presence of the mouse Pax5 exons 1-4 in the fusion protein. The authors should add a comment on the homology between the human and mouse Pax5 proteins.

"Pax5-Etv6 primarily bound to sites of open chromatin (DHS) containing active histone modifications (H3K4me2, H3K9ac, H3K27ac; sectors a-c, g), whereas these active marks as well as the repressive H3K27me3 modification were largely absent at unique Pax5- and Prd-binding sites"
-Do sites cobound by wild type Pax5 and Px5-Etv6 have different amounts of active marks compared to sites bound by Pax5 only?

"In contrast, the Pax5-Etv6 protein activated 76 genes and repressed 70 genes in Pax5Etv6/+ pro-B cells (Fig. 3B and Table S1). Notably, Pax5-Foxp1 activated a similar number of genes (100), but repressed 3 times more genes (213) compared to Pax5-Etv6 (Fig. 3C and Table S1)"

-How many of these genes overlap with genes that change in Pax5+/- or PaxPrd compared to Pax5+/+ genotypes?

"Immunoblot analysis of nuclear extracts with a Pax5 paired domain-specific antibody indeed revealed that the Pax5Etv6/+, Pax5Foxp1/+ and Pax5Prd/+ pro-B cells expressed the Pax5-Etv6, Pax5-Foxp1 or Prd protein at the same level as wild-type Pax5"
-Fig 2B (two left panels) shows additional, albeit weak, bands between the chimeric proteins and Pax5 itself. Are those degradation products of the Pax5-Etv6 and Pax5-FoxP1 proteins or different proteins?

Referee #2:

PAX5 haploinsufficiency is frequently observed in B-ALL and recurrent chromosomal translocations resulting in the fusion of PAX5 with an array of other genes have been identified in a small percentage of these malignancies. The functions of PAX5 and PAX5 fusions have mostly been studied at the molecular level using transient overexpression assays and, biologically, in mouse B cell lines. These approaches do not recapitulate the complexity of the association between PAX5 haploinsufficiency and expression of PAX5 fusion proteins, observed in human B-ALL. The manuscript by Smeenk et al shows that PAX5 haploinsufficiency is without major effect on B cell development and gene expression and reports novel mouse models in which 2 distinct PAX5 fusions, namely PAX5-ETV6 and PAX5-FOXP1, are each expressed from the natural mouse Pax5 promoter/enhancers thus combining expression of these fusion proteins with Pax5 haploinsufficiency.
The data clearly show that (i) B cell development is impaired in vivo at the pro-B/pre-B transition by expression of either of these fusion proteins; (ii) unlike the widely held notion that PAX5 fusions become oncogenic through interference with PAX5 transcriptional activity, only a small overlap actually exists between PAX5-regulated genes and PAX5-ETV6 or PAX5-FOXP1 deregulated genes in pro-B cells; (iii) a distinct transcriptional profile is imposed by each fusion proteins in pro-B cells; (iv) in line with its structure, the binding of PAX5-ETV6 to specific chromatin sites in cultured pro-B cells is linked to the presence of both ETS and/or PAX5 DNA binding motifs in this fusion protein; (v) chromatin binding sites are shared between PAX5 and PAX5-ETV6; (vi) a rather small number of genes are directly cis-regulated either positively or negatively by PAX5-ETV6; (vii) the mere expression of these fusion proteins in mice is not leukemogenic per se and that the simultaneous inactivation of a relevant tumor suppressor locus (cdkn2a/b) can uncover PAX5-ETV6 pre-B ALL inducing activity; (viii) PAX5-ETV6 expression is associated with a specific set of target genes presumably involved in B-ALL maintenance; (ix) an overlap is observed between genes deregulated in mouse PAX5-ETV6 B-ALL (B-ALL vs large pre-B cells) and diagnostic PAX5-ETV6+ B-ALL cases (B-ALL vs hu pre-B cells), indicating the relevance of the reported mouse models.
This study is the first to describe knock-in models for PAX5 fusion genes and provides novel and important insights into the mode of action of these fusion proteins including strong effects on normal B cell development and specific requirements for in vivo leukemogenic potential. This study also clearly demonstrates that PAX5-ETV6 and PAX5-FOXP1 do not just interfere with PAX5 function but also enforce a specific gene expression program through the activity of their dual DNA binding domains. The (many) data provided are convincing and generally support the conclusions drawn by the authors (see below for few possible exceptions).

Specific comments.

1. It is unclear from figure 1C whether the difference in immature B cell proportions between wild type and Pax5+/- mice is statistically significant. Please provide numbers.

2. Fig. 4F should be explained in more details in legend.

3. It is unclear how the identification of PAX5-ETV6 genes associated with adhesion/migration control (Fig S4G) lead the authors to investigate the response of PAX5-ETV6/pro-B cells to CXCL12 as none of these genes appear connected to this chemokine or its receptor. Sphingosine-1-P would have been a logical candidate to investigate as S1pr3 is highly induced in PAX-ETV6 cells

and this receptor has been linked to cell migration of immature B cells and to B cell development (Donovan Eur J Immunol. 2010). The logic behind the choice for CXCL12 should be indicated or amended. Also, given the above, the claim (p. 12) that the few PAX5-ETV6 deregulated genes found in pro-B cells do not provide clues for a potentially oncogenic function of this fusion protein could be premature. For example abnormal relocalisation of PAX5-ETV6-expressing pro-B/pre-B cells in bone marrow niches may contribute to disease initiation.

4. Fig 5C: the authors should more precisely specify what "other tumors" exactly means. Do PAX5-ETV6 mice developing B-ALL are devoid of these "other tumors"? Please clarify.

5. B-ALL arising in PAX5-ETV6/cdkn2a/b ko mice are pre-BCR-positive and arrested at a transcriptional stage close to large pre-B cells (Fig. 5 and 6), suggesting a potential function of tonic pre-BCR signaling in leukemia maintenance. Available evidence shows that tonic pre-BCR signaling is restricted to less than 15% B-ALL diagnostic cases that belong to specific molecular subgroups (Geng et al Cancer Cell 2015). PAX5-ETV6 B-ALL were not included in that published study. It would thus be important to determine if PAX5-ETV6+ human B-ALL are also pre-BCR-positive and thus whether the knock-in model of PAX5-ETV6-induced B-ALL recapitulates the human disease in that respect as well. This might have important translational implications.

Referee #3:

In the manuscript entitled „Molecular role of the PAX5-ETV6 oncoprotein in promoting B cell acute lymphoblastic leukemia" by Dr. Busslinger and colleagues [EMBOJ-2016-95495] novel mouse strains, including Pax5-Etv6 and Pax5-Foxp1 knock-in mice, were generated to investigate the role of Pax5 expression in B-ALL.
The authors report that heterozygous loss of Pax5 does not impair B cell development in mouse, while heterozygous expression of the Pax5 fusion proteins Pax5-Etv6 and Pax5-Foxp1 leads to developmental block at the pro to pre B cell transition and this developmental block is caused by the fusion protein and not by the Pax5 heterozygosity. Moreover, the authors report that the Pax5 fusion proteins are unlikely to act as dominant negative versions of Pax5 as they mostly regulate different genes as compared with Pax5. Despite the developmental defects, heterozygous expression of Pax5-Etv6 or Pax5-Foxp1 fusion protein did not lead to B-ALL development in mice and, furthermore, detailed characterization of Pax5-Etv6 target genes failed to elucidate the molecular mechanisms underlying malignant transformation induced by this fusion protein. By generating and analyzing additional mouse strains, the authors concluded that additional mutations are required for B-ALL development in Pax5-Etv6 or Pax5-Foxp1 knockin mice. In fact, heterozygous loss of the Cdkn2a/b tumor suppressor locus together with Pax5-Etv6, but not with Pax5-Foxp1, promotes B-ALL development in mice. Further analysis revealed that Pax5-Etv6 regulated target genes might have important functions in signaling pathways, thereby contributing to B-ALL in Cdkn2ab+/- Pax5Etv6/+ mice. In summary, this is a very interesting study and the generated mouse models improve our understanding of the molecular mechanisms underlying human B-ALL.

There are only a few minor points

- In some western blots the loading controls are missing.
- Since tumor cells seem to express the pre-BCR in Cdkn2ab+/- Pax5Etv6/+ mice, the authors may discuss some reports on autonomous pre-BCR signaling and its potential role for proliferation.
- The authors may wish to mention why the sorted cells in Fig. S3-A: (2,4 &5) are so few. Are they sufficient for the analysis? Also, what is the reason for measuring CD2 for Pax5+/+ & Pax5Foxp1+/- samples and then measuring CD25 for Pax5+/-, Pax5prd+/- & Pax5ETV6+/-?
- If already available, the authors might include some in-depth measurements like RNA-seq for Cdkn2ab-/- cells as compared with Cdkn2ab+/-cells in order to characterize the differentially expressed genes that might be involved in malignant transformation?
- The authors may explain why they compared the mouse B-ALL cells with large pre- B cells, while human B-ALL cells were compared with small pre-B cells.

1st Revision - authors' response   10 December 2016

Point-by-point reply to the reviews for the manuscript EMBOJ-2016-95495

We thank all three reviewers for their constructive criticism (indicated in blue), which has significantly improved our manuscript, as explained below.

External expert advisor:

We had initially consulted with an external expert advisor on your manuscript and this person found that while the amount of data generated in this study is impressive, the analysis itself - especially at the genomics level - could have been taken further in order to better understand the possible functional contribution from the genes affected by fusion protein expression.

The comment of the external advisor is very general. We spent a lot of time to perform extensive bioinformatic analyses of our genome-wide data sets, which included Gene Ontology, KEGG and Ingenuity pathway analyses, extensive gene set enrichment assays (GSEA) as well as SNP and indel detection analyses (see below point 3b of reviewer #1). We have shown only the positive results in the manuscript and do not know what we could have done more with regard to bioinformatic analyses.

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Referee #1:

Smeenk et al. have established a new mouse model that recapitulates the main characteristics of Pax5 fusions-associated B-ALL and showed mechanistic aspects of transcriptional activity of the fusion proteins with respect to Pax5 targets. The study is of high interest and has an impact on our perception of B-ALL and Pax5 biology. The authors should further study/explain differences between the human disease and mouse model of disease. A few other points need to be clarified as outlined below:

We are pleased that the reviewer considers our study to be of high interest and worthy of publication.

Main points:
1) "RNA-sequencing (RNA-seq) comparison of ex vivo sorted Pax5+/+ and Pax5+/- pro-B cells furthermore revealed only 1 up-regulated and 16 down- regulated genes in Pax5+/- pro-B cells (Fig. 1D and Appendix Fig. S1A)."
1a) -what are the levels of Pax5 mRNA in Pax5+/+ compared to Pax5+/- mice/cells?

The expression of *Pax5* mRNA is as followed: 394.9 TPM (SEM 4.2 TPM) in $Pax5^{+/+}$ pro-B cells and 230.4 TPM (SEM 57.4) in $Pax5^{+/-}$ pro-B cells. The *Pax5* mRNA is thus expressed in $Pax5^{+/-}$ pro-B cells at 58% of the mRNA level observed in $Pax5^{+/+}$ pro-B cells. Hence, there is no increase in expression of the wild-type *Pax5* allele to compensate for the null allele in $Pax5^{+/-}$ pro-B cells. This is now mentioned in the legend of Figure 1D (page 36, middle).

We have added the requested information in Appendix Figure 1A of the revised manuscript, which indicated that 9 (53%) of 17 deregulated genes in $Pax5^{+/-}$ pro-B cells were previously identified as direct Pax5 target genes (Revilla-i-Domingo et al., 2012). This analysis therefore indicates that 9 direct Pax5 target genes are deregulated already by a 2-fold *Pax5* expression change (Fig. 1D), which is now mentioned on page 6 (top) in the result section.

We have performed the requested flow cytometric experiments with bone marrow cells and thymocytes, which are now shown in Appendix Figure S2G. As revealed by analysis of absolute cell numbers, only B cells were significantly reduced in $Pax5^{Etv6/+}$ and $Pax5^{Foxp1/+}$ mice compared to $Pax5^{+/+}$ mice. No significant differences were observed for granulocytes, macrophages and NK cells in the bone marrow and for all T cell developmental stages in the thymus. These new data are now mentioned on page 6 (bottom) in the result section.

<u>Part a</u>. Human PAX5-ETV6$^+$ B-ALLs are relatively rare tumors. For this reason, we did not have access to fresh PAX5-ETV6$^+$ B-ALL material before and during the revision period. Hence, we could not perform a Western blot analysis to demonstrate similar expression of the full-length Pax5 and Pax5-Etv6 fusion proteins in PAX5-ETV6$^+$ B-ALL samples. As an alternative approach, we have used the RNA-seq data of the 9 human and 3 mouse B-ALL samples to quantify the expression levels of the full-length *PAX5* and *PAX5-ETV6* fusion transcripts, as described in detail in the Appendix Supplementary Methods on page 11 (top). Although the ratio between the full-length *PAX5* and *PAX5-ETV6* fusion transcripts was varying in the different human B-ALL samples, the average ratio of all 9 human B-ALL samples was close to 1 (48.7%/51.3%; Appendix Figure S7B), which was close to the average ratio (46.6%/53.4%) determined for the 3 mouse $Cdkn2ab^{+/-}$ $Pax5^{Etv6/+}$ B-ALLs analyzed (Appendix Figure S7C), Hence, these data further validate the mouse tumor as a faithful model for the human disease. This new characterization of the PAX5-ETV6$^+$ B-ALL samples is now mentioned on page 19 in

the result section.

Part b. Unfortunately, we would be unable to provide an answer to this question even if we had access to fresh PAX5-ETV6^{+} B-ALL material for performing ChIP-seq analysis for the following reason. We could not discriminate wild-type Pax5 and Pax5-Etv6 with an anti-paired domain antibody that recognizes the Pax5 sequences present in the Pax5-Etv6 protein (see Western blot data shown in Figure 2B). Moreover, if we used an anti-Etv6 antibody, we could not discriminate Pax5-Etv6 from endogenous Etv6, which is also expressed in pro-B cells (data not shown). The power of the Pax5-Etv6 mouse model is precisely the fact that we could add a C-terminal tag to Pax5-Etv6, which facilitated the specific precipitation of Pax5-Etv6 in the presence of untagged wild-type Pax5.

3b) -Could this difference be due to additional genetic alterations occurring in the case of the human B-ALL.

As suggested by the reviewer, the human PAX5-ETV6^{+} B-ALLs are genetically heterogeneous compared to the mouse $Cdkn2ab^{+/-}$ $Pax5^{Etv6/+}$ tumors, which arose in the genetically identical inbred C57BL/6 mouse strain.

3b) -Analysis of WGS data in this type of human B-ALL could provide evidence for additional genetic alterations.

We only had access to RNA of PAX5-ETV6^{+} B-ALLs to perform RNA-sequencing and had no patient consent to analyze genomic DNA. Hence, we could not perform whole genome sequencing (WGS). In the absence of genomic DNA analysis, we tried to identify SNPs by mining our paired-end RNA-seq data of the PAX5-ETV6^{+} B-ALLs. After time-consuming in-depth bioinformatic analyses, we came to the conclusion that the RNA-seq data were too noisy for identifying SNPs with statistical confidence. Moreover, the RNA-seq data would not allow the identification of nonsense mutations or out-of-frame indels due to the elimination of the corresponding mRNA by the nonsense mRNA decay (NMD) pathway. Hence, the available RNA-seq data were suboptimal for these analyses and unfortunately did not yield any conclusive data that would have allowed us to address the request of the reviewer.

Other points

4) "we inserted human cDNA sequences, starting in exon 4 and encoding the remaining PAX5-ETV6 or PAX5-FOXP1 protein, in frame into exon 4 of the mouse Pax5 locus to generate the Pax5Etv6 and Pax5Foxp1 knock-in alleles."

-The main difference compared to the human case is the presence of the mouse Pax5 exons 1-4 in the fusion protein. The authors should add a comment on the homology between the human and mouse Pax5 proteins.

The human and mouse Pax5 protein sequences only differ by three amino acid substitutions. The Pax5 sequences encoded from exon 1 to exon 6 contain only one amino acid substitution (human Ser13 to mouse Ile13), which is present upstream of the start of the paired domain (first functional domain) in the very N-terminal sequence encoded by *Pax5* exon 1 (Adams et al., Genes Dev. 6: 2589-1607). This fact is now mentioned in the legend of Figure 2A in the revised manuscript.

There must be a misunderstanding here, as the answer to this question is presented in Figure S4A. The sites shown in the sectors a+b are not only co-bound by Pax5 and Pax5-Etv6, but are also present in highly accessible chromatin with increased DNase I hypersensitivity (DHS) and elevated levels of the active histone marks H3K4me2, H3K9ac, H3K27ac as well as H3K4me3 (at promoters) or H3K4me1 (at enhancers). In contrast, the sites shown in the sectors d+f are bound only by Pax5 and are characterized by less accessible chromatin with no or only low levels of active histone marks.

We have now added the requested information in Appendix Figure S1. Notably, this analysis revealed that most of the activated genes identified in $Pax5^{+/-}$ or $Pax5^{Prd/+}$ pro-B cells were repressed by Pax5-Etv6 and Pax5-Foxp1. Hence, genes, whose expression is already decreased in response to only a 2-fold reduction of full-length *Pax5* expression, appear to be 'repressed' by Pax5-Etv6 and Pax5-Foxp1, although this effect is likely caused by the loss of one wild-type *Pax5* allele rather than the expression of the fusion protein in $Pax5^{Etv6/+}$ and $Pax5^{Foxp1/+}$ pro-B cells. We mention this observation in the legend of Figure 3E as well as Appendix Figures S1 and S3C.

The weak bands are potential degradation products. We mention now this in the legend of Figure 2B (page 37).

Referee #2:

We thank the reviewer for his/her insightful comments and for highlighting the novel discoveries that we made with the knock-in mouse models.

Specific comments.

1. It is unclear from figure 1C whether the difference in immature B cell proportions between wild type and Pax5+/- mice is statistically significant. Please provide numbers.

Statistical evaluation of the data shown in Figure 1C by two-way analysis of variance (ANOVA) revealed that the relative frequency of the $Pax5^{+/+}$ and $Pax5^{+/−}$ granulocytes, macrophages and B lymphocytes at the different developmental stages was not significantly different ($P > 0.1$). This is now mentioned in the legend of Figure 1C on page 36 (top).

2. Fig. 4F should be explained in more details in legend.

We have described the corresponding data in more detail in the legend of Figure 4F (page 39, middle), as requested.

3. It is unclear how the identification of PAX5-ETV6 genes associated with adhesion/migration control (Fig S4G) lead the authors to investigate the response of PAX5-ETV6/pro-B cells to CXCL12 as none of these genes appear connected to this chemokine or its receptor. Sphingosine-1-P would have been a logical candidate to investigate as S1pr3 is highly induced in PAX-ETV6 cells and this receptor has been linked to cell migration of immature B cells and to B cell development (Donovan Eur J Immunol. 2010). The logic behind the choice for CXCL12 should be indicated or amended. Also, given the above, the claim (p. 12) that the few PAX5-ETV6 deregulated genes found in pro-B cells do not provide clues for a potentially oncogenic function of this fusion protein could be premature. For example abnormal relocalisation of PAX5-ETV6-expressing pro-B/pre-B cells in bone marrow niches may contribute to disease initiation.

As correctly mentioned by the reviewer, *Cxcl12* is not regulated by Pax5 or Pax5 fusion proteins and therefore cannot explain the observed migration phenotype. However, we used CXCL12 as a tool to analyze the migration behavior of wild-type and $Pax5^{Etv6/+}$ pro-B cells in transwell migration experiments (Appendix Figure S4H). We added the cytokine CXCL12 to the lower chamber to activate the receptor CXCR4, which induced the migration of pro-B cells from the upper to the lower chamber. Moreover, CXCL12 is a physiological cytokine expressed by reticular cells in the bone marrow, where pro-B cells are located (Tokoyoda et al., Immunity 20, 707-718). CXCR4 is equally expressed on pro-B cells of both genotypes, and therefore the logic for the transwell migration assay was to uncover the role of deregulated intracellular signal transducers involved in cell migration. The reviewer furthermore suggested that we could have

performed migration assays with sphingosine-1-phosphate (S1P), as the gene coding for the S1pr3 receptor is strongly activated by Pax5-Etv6 to a mRNA level of 10 TPM (RNA-seq data) in *Pax5*[Etv6/+] pro-B cells (Appendix Figure S4G). In this context, it is important to note that the mRNA of the related S1pr1 receptor is expressed at a 3-fold higher level (30 TPM) in both wild-type and *Pax5*[Etv6/+] pro-B cells. Hence, the migration response to S1P is likely dominated by the non-regulated and more highly expressed S1pr1 receptor in pro-B cells of both genotypes, which may mask any potential effect of the differentially expressed S1pr3 receptor. Hence, the suggested S1P experiment would likely result in non-conclusive data.

As suggested by the reviewer, we have changed the statement at the end of the paragraph on page 12 (bottom) by mentioning that the identified regulated Pax5-Etv6 target genes involved in adhesion/migration could potentially contribute to the oncogenic function of Pax5-Etv6 in leukemic cells.

4. Fig 5C: the authors should more precisely specify what "other tumors" exactly means. Do PAX5-ETV6 mice developing B-ALL are devoid of these "other tumors"? Please clarify.

It is known that heterozygous and homozygous *Cdkn2ab* mutant mice generate tumors of many different kinds (including solid tumors) with the notable exception of B cell leukemias (Krimpenfort et al., Nature 448, 943-946). As we did not consider it important to phenotype all of the *Cdkn2ab*^{+/−} and *Cdkn2ab*^{−/−} tumors, we routinely investigated by flow cytometry only whether a tumor expressed B cell markers. We now mention in the legend of Figure 5C (page 39, bottom) that "other" tumors refer to "non-B cell" tumors that were not further characterized.

5. B-ALL arising in PAX5-ETV6/cdkn2a/b ko mice are pre-BCR-positive and arrested at a transcriptional stage close to large pre-B cells (Fig. 5 and 6), suggesting a potential function of tonic pre-BCR signaling in leukemia maintenance. Available evidence shows that tonic pre-BCR signaling is restricted to less than 15% B-ALL diagnostic cases that belong to specific molecular subgroups (Geng et al Cancer Cell 2015). PAX5-ETV6 B-ALL were not included in that published study. It would thus be important to determine if PAX5-ETV6+ human B-ALL are also pre-BCR-positive and thus whether the knock-in model of PAX5-ETV6-induced B-ALL recapitulates the human disease in that respect as well. This might have important translational implications.

PAX5-ETV6^{+} B-ALLs are rare tumors and, for this reason, may not have been included in the study of Geng et al. (Cancer Cell 27, 409-425). As mentioned under point 3a (reviewer #1), we did not have access to fresh PAX5-ETV6^{+} B-ALL material during the revision period to analyze the cell surface expression of the pre-BCR on tumor cells by flow cytometry. Instead, we have interrogated the RNA-seq data of all 9 PAX5-ETV6^{+} B-ALLs. This analysis indicated that the mRNAs coding for the extracellular components (IGHM [Igµ], IGLL [λ5], VPREB1, VPREB3) and the signal-transducing chains (CD79A [Igα], CD79B [Igβ]) of the pre-BCR were on average highly expressed (> 1,000 TPM) in these B-ALLs (new Figure 7F). The high expression of all 5 pre-BCR components strongly suggests that the pre-BCR is expressed on PAX5-ETV6^{+} B-ALLs. We describe and discuss these new data in the result and discussion sections on pages 20 (middle) and 26 (middle). For further discussion of the pre-BCR issue, see

below our response to comment 2 of reviewer #3.

Referee #3:

In the manuscript entitled „Molecular role of the PAX5-ETV6 oncoprotein in promoting B cell acute lymphoblastic leukemia" by Dr. Busslinger and colleagues [EMBOJ-2016-95495] novel mouse strains, including Pax5-Etv6 and Pax5-Foxp1 knock-in mice, were generated to investigate the role of Pax5 expression in B-ALL.
The authors report that heterozygous loss of Pax5 does not impair B cell development in mouse, while heterozygous expression of the Pax5 fusion proteins Pax5-Etv6 and Pax5-Foxp1 leads to developmental block at the pro to pre B cell transition and this developmental block is caused by the fusion protein and not by the Pax5 heterozygosity. Moreover, the authors report that the Pax5 fusion proteins are unlikely to act as dominant negative versions of Pax5 as they mostly regulate different genes as compared with Pax5. Despite the developmental defects, heterozygous expression of Pax5-Etv6 or Pax5-Foxp1 fusion protein did not lead to B-ALL development in mice and, furthermore, detailed characterization of Pax5-Etv6 target genes failed to elucidate the molecular mechanisms underlying malignant transformation induced by this fusion protein. By generating and analyzing additional mouse strains, the authors concluded that additional mutations are required for B-ALL development in Pax5-Etv6 or Pax5-Foxp1 knockin mice. In fact, heterozygous loss of the Cdkn2a/b tumor suppressor locus together with Pax5-Etv6, but not with Pax5-Foxp1, promotes B-ALL development in mice. Further analysis revealed that Pax5-Etv6 regulated target genes might have important functions in signaling pathways, thereby contributing to B-ALL in Cdkn2ab+/- Pax5Etv6/+ mice. In summary, this is a very interesting study and the generated mouse models improve our understanding of the molecular mechanisms underlying human B-ALL.

We thank the reviewer for acknowledging that our manuscript reports a very interesting study that improves our understanding of the molecular mechanisms underlying human B-ALL.

There are only a few minor points

1) - In some western blots the loading controls are missing.

Although the Western blot analyses shown in Figure 2B and Figure S2E,F are without the requested control lanes, these loading controls would not have added any further information for the following reasons. The Western blot results shown in Figure 2B are internally controlled by Pax5, as we only determined the ratio between full-length Pax5 and the Pax5-Etv6, Pax5-FoxP1 and Pax5-Prd proteins, which turned out to be similar (close to 1) in all three cases. It is not possible to analyze a pulldown experiment (Figure S2E,F) with a loading control, as all control proteins are eliminated by the pulldown and washing procedure.

2) - Since tumor cells seem to express the pre-BCR in Cdkn2ab+/- Pax5Etv6/+ mice, the authors may discuss some reports on autonomous pre-BCR signaling and its potential role for proliferation.

In response to point 5 of reviewer #2, we have now shown that human PAX5-ETV6$^+$ B-ALLs are likely to express the pre-BCR. Moreover, the newly added GSEA analysis (Appendix Figure S7F) further supported this conclusion, as genes, that are up-regulated in human pre-BCR$^+$ B-ALLs relative to pre-BCR$^-$ B-ALL (Geng et al., Cancer Cell, 27, 409-425), were also significantly enriched as up-regulated genes in human PAX5-ETV6$^+$ B-ALLs compared to PAX5-JAK2$^+$ B-ALLs, while down-regulated genes in pre-BCR$^+$ B-ALLs were also down-regulated in PAX5-ETV6$^+$ B-ALLs (Appendix Figure S7F). This analysis is now mentioned in the result section on page 20 (middle). Moreover, we have expanded the discussion dealing with the role of pre-BCR signaling in leukemia formation (page 26, bottom), as suggested by the reviewer.

3a) - The authors may wish to mention why the sorted cells in Fig. S3-A: (2,4 &5) are so few. Are they sufficient for the analysis?

We normally use relatively few cells for FACS reanalysis in order not to lose too many of the precious sorted cells. Hence, the few cells in the indicated gates of the Appendix Figure S3A give a wrong impression. Regardless of this visualization issue, we have used one million or more sorted pro-B cells as starting material for cDNA library preparation and RNA-sequencing. Moreover, we have improved the quality of the Appendix Figure S3A so that more dots are now visible in the gates (bottom row) of the FACS reanalysis.

3b) - Also, what is the reason for measuring CD2 for Pax5+/+ & Pax5Foxp1+/- samples and then measuring CD25 for Pax5+/-, Pax5prd+/- & Pax5ETV6+/-?

The expression of the cell surface markers CD2 and CD25 are known to be similarly induced in the pro-B-to-pre-B cell transition in adult bone marrow and are therefore interchangeably used for the definition of pro-B and pre-B cells. Hence, pro-B cells can be defined as CD19$^+$c-Kit$^+$CD2$^-$ or CD19$^+$c-Kit$^+$CD25$^-$ cells. We now mention this in the legend of Figure S3A (on page 4, middle). This is also mentioned under "Flow cytometric definition of mouse hematopoietic cell types" in the Appendix Supplementary Methods. In conclusion, there is no particular reason for the use of the CD2 or CD25 marker for sorting of pro-B cells other than that the sorts were performed on different dates.

4) - If already available, the authors might include some in-depth measurements like RNA-seq for Cdkn2ab-/- cells as compared with Cdkn2ab+/-cells in order to characterize the differentially expressed genes that might be involved in malignant transformation?

It was not possible to address this request within the revision period for the following reason. We have shown that loss-of-heterozygosity occurred at the *Cdkn2ab* locus in 7 (78%) of 9 B-ALL tumors that arose in compound heterozygous *Pax5*$^{Etv6/+}$ *Cdkna2ab*$^{+/-}$ mice (Figure 6A-C). Moreover, two of the sequenced tumors (Tu-3 and Tu-10) underwent loss-of-heterozygosity (*Cdkna2ab*$^{-/-}$), and only one sequenced tumor (Tu-8) retained the *Cdkna2ab*$^{+/-}$ genotype (Figure 6A). As tumors retaining the *Cdkna2ab*$^{+/-}$ genotype are generated at low frequency, we did not obtain a second tumor of this subtype for RNA-sequencing. The lack of a second RNA-seq sample did not allow us to perform a statistically sound in-depth bioinformatic analysis,

which would have required two *Pax5*^{Etv6/+} *Cdkna2ab*^{+/−} and two *Pax5*^{Etv6/+} *Cdkna2ab*^{−/−} tumors for RNA-seq comparison.

5) - The authors may explain why they compared the mouse B-ALL cells with large pre- B cells, while human B-ALL cells were compared with small pre-B cells.

Large pre-B cells are relatively rare, as they constitute only a transient stage of B cell development. Moreover, their numbers decline with progressing age. In the mouse, we sort these cells from the bone marrow of several young mice. Bone marrow of healthy human individuals is difficult to get. We obtained the pre-B cell RNA-seq data from Charles Mullighan's lab that sorted pre-B cells, but not specifically pre-BCR$^+$ large pre-B cells. Hence, we had only the RNA-seq data of human small pre-B cells at our disposition for comparison with the RNA-seq data of PAX5-ETV6$^+$ B-ALLs, which we mention in the result section (page 19, bottom).

Other changes

In the meantime, we have annotated all 71 commonly activated and 19 commonly repressed Pax5-Etv6 target genes identified in human PAX5-ETV6$^+$ B-ALLs and mouse *Pax5*^{Etv6/+} *Cdkna2ab*^{+/−} B-ALLs. As a consequence, we have replaced the previous heat map of Figure 7E with a new heat map containing only the genes that code for proteins implicated in the five pathways shown. In addition, we present now the expression pattern of all commonly activated and repressed genes in a new heat map shown in Appendix Figure S7D.

2nd Editorial Decision   04 January 2017

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by two of the original referees whose comments are shown below. As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can go on to officially accept the manuscript there are a few editorial issues that I need you to address. In addition, you will see that ref #1 suggests that you extend the discussion with a paragraph on the genetic heterogeneity of human pre-BCR+ B-ALL relative to the mouse model; I will leave it up to you to decide if you want to include this.

I would therefore invite you to submit a final version of your manuscript in which you address the following:

-> For the supplemental data we can accommodate up to 5 EV figures (these will be typeset and presented in-line with the main manuscript in the html version). In your case, I would therefore suggest that you either: a) combine the current 7 EV figures to 5, b) select 5 of the 7 figures to be EV and make the rest Appendix, or c) make all 7 figures Appendix figures. For the last option, this means including all 7 figures in a single pdf with a Table of Contents on the first page. You should also include the Supplemental methods document in the Appendix file. For all figures in the Appendix file the corresponding legends need to be moved to the Appendix file as well. In addition, you will need to update the figure callouts in the main text file.
Feel free to contact us with any questions about formatting.

-> For the supplemental tables, these should be kept as individual excel files as they are now but be renamed as Table EV1, Table EV2 etc (please note that table files do not count in the maximum number of 5 EV figures). And please also update the corresponding callouts in the main text file.

-> Please make sure that the literature references fit with the journal style. We ask that the list of author names for each manuscript in the reference list is truncated with an 'et al' after 20 names. We noticed several cases in your literature list where more than 20 authors are listed and would ask you to correct it. This can be done automatically when using the 'EMBO Journal' style in most reference manager programs.

-> I noticed that the RNA seq data for human samples is not currently available in a public database. Could you clarify is this is due to patient confidentiality or other specific issues?

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

-> Papers published in The EMBO Journal include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

REFEREE REPORTS

Referee #1:

The current version of the manuscript is significantly improved compared to the original one. Overall the authors have generated and thoroughly analyzed an impressive amount of data that helped them connect their mouse model to human disease (a subset of B-ALL) and delineate the role of migration-related genes and pre-BCR signaling.

Potential genetic heterogeneity of human pre-BCR+ B-ALL in comparison to mouse model, that might account for small (molecular) differences between the model and humans with B-ALL, should be discussed at the end of the manuscript.

Referee #2:

The revised manuscript by Smeenk et al has met the vast majority of the referee's requests. It should be published without further delay as it reports a novel model and very interesting insights to PAX5-ETV6 leukemogenic properties.

2nd Revision - authors' response	09 January 2017

Enclosed I return to you the edited version of our manuscript, which we have modified according to your instructions. We decided to turn all 7 Supplementary Figures into Appendix Figures and combined them in a single pdf document with the Appendix Supplementary Methods and Appendix Supplementary References. We now provide all Supplementary Tables as Excel files (Tables EV1-EV7). As requested, we provide the Source Data for all Southern, Western and PCR analyses. With regard to the human RNA-seq data, we have to keep the statement "The human RNA-seq data are available on request" due to patient confidentiality. We have also designed a synopsis figure with 4 bullet point sentences. Finally, we do not want to inflate the discussion with a new paragraph stating the obvious that human patients represent a genetically heterogeneous population in contrast to our mouse model that was established on the defined inbred C57BL6 background.

3rd Editorial Decision	10 January 2017

Thank you again for submitting the final revision, I am pleased to inform you that your manuscript has now been officially accepted for publication in The EMBO Journal.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Meinrad Busslinger
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

→ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
→ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
→ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
→ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
→ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

→ a specification of the experimental system investigated (eg cell line, species name).
→ the assay(s) and method(s) used to carry out the reported observations and measurements
→ an explicit mention of the biological and chemical entity(ies) that are being measured.
→ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

→ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
→ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
→ a statement of how many times the experiment shown was independently replicated in the laboratory.
→ definitions of statistical methods and measures:
  • common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  • are tests one-sided or two-sided?
  • are there adjustments for multiple comparisons?
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  • definition of 'center values' as median or average;
  • definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The minimum number of mice or sample size for each specific experiment is indicated together with the statistical evaluation of the data in each figure legend (p35-43; Figures 1-7).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The number of mice analyzed per genotype is specified for each specific experiment (see figure legends, p35-43)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No mice were excluded (see page 1 of Appendix Supplementary Methods).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Both sexes were used for each genotype, and mice from different breeding pairs were included in the experiments to increase randomization.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No steps were taken.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not used in this study.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes, the variation of each group of data was estimated calculating its standard deviation.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

Question	Answer
6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	See pages 2 and 3 of the Appendix Supplementary Methods, where the name and clone number for each antibody is specified.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

Question	Answer
8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	See page 1 of Appendix Supplementary Methods and page 27 of the manuscript. Mice of different age and genetic background (as specified in Materials and Methods) were maintained on the C57BL/6 genetic background.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	See page 1 of Appendix Supplementary Methods and page 27 of the manuscript. All animal experiments were carried out according to valid project licenses, which were approved and regularly controlled by the Austrian Veterinary Authorities

10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	See page 28 of the manuscript and page 6 of the Appendix Supplementary Methods. Informed consent for all patient samples was obtained from all subjects.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	See page 29 of the manuscript. The human RNA-seq data are available upon request.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	See page 29 of the manuscript and Supplementary Table S6.
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G- Dual use research of concern

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