Ebf1 heterozygosity results in increased DNA damage in pro-B cells and their synergistic transformation by Pax5 haploinsufficiency.

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# Indicate equal contributions

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**Running title:** Ebf1 dose modulates DNA repair

**Funding:** This work was supported by grants from the Swedish Cancer Society, the Swedish Research Council, including a Center grant to Hematolinne, Barncancerfonden and by Linköping University. JH is supported generously by NIH grant AI081878.
Key points:

- Ebf1 regulates DNA repair in a dose dependent manner.
- Combined heterozygote loss of Ebf1 and Pax5 predispose for leukemia development.

Abstract:

Ebf1 is a transcription factor with documented dose dependent functions in normal and malignant B-lymphocyte development. To understand more about the roles of Ebf1 in malignant transformation, we investigated the impact of reduced functional Ebf1 dosage on mouse B-cell progenitors. Gene expression analysis suggested that Ebf1 was involved in the regulation of genes important for DNA repair as well as cell survival. Investigation of the DNA damage in steady state as well as after induction of DNA damage by UV light, confirmed that pro-B cells lacking one functional allele of Ebf1 display signs of increased DNA damage. This correlated to reduced expression of DNA repair genes including Rad51 and chromatin immunoprecipitation data suggested that Rad51 is a direct target for Ebf1. Although reduced dosage of Ebf1 did not significantly increase tumor formation in mice, a dramatic increase in the frequency of pro-B cell leukemia was observed in mice with combined heterozygous mutations in the Ebf1 and Pax5 genes revealing a synergistic effect of combined dose reduction of these proteins. Our data suggest that Ebf1 controls DNA repair in a dose dependent manner providing a possible explanation to the frequent involvement of EBF1 gene loss in human leukemia.
Introduction:

One of the central proteins in B-lymphocyte development is the transcription factor Early B-cell factor 1 (Ebf1). Ebf1 is critical for the activation of B-lineage restricted genes in the earliest B lineage progenitors and for restriction of lineage fate options. The activity is highly dependent on functional Ebf1 dose because mice carrying a heterozygous deletion of the Ebf1 gene display reduced numbers of CD19^+CD43^- B-cell progenitors while the CD19^+CD43^+ pro-B cell compartment remains intact. Ebf1 levels are also of relevance in leukemia because mutations resulting in reduced functional EBF1 dose and increased expression of post-transcriptional inhibitors of EBF1, ZNF521 or ZNF423 is found in B-cell acute lymphoblastic leukemia (B-ALL). A direct role for Ebf1 dose in malignant transformation was supported by the findings that combined expression of constitutively active Stat5 (caStat5) and heterozygous loss of either Ebf1 or Pax5 results in B-cell leukemia in mice. Heterozygote deletion of PAX5 is a rather common genetic alteration in human B-ALL and a genetic polymorphism causing reduced functional PAX5 activity has been found in families with a high incidence of leukemia. The finding that the developmental block observed in Pax5-deficient leukemia cells can be reversed upon restoration of Pax5 expression suggests that the reduction in Pax5 function results in a reversible disruption of differentiation. A similar mechanism of action has been proposed for Ebf1, however, reduced amounts of Ebf1 in normal cells appear to result in reduced proliferation and expansion of B-cell progenitors, indicating that the involvement of EBF1 in malignant transformation is more complex.

In order to increase our understanding of the functions of Ebf1 in malignant transformation, we identified dose dependent processes regulated by Ebf1 in early B-
cell development revealing changes in DNA repair as well as cell survival. Because these data suggested that Ebf1 functions differently than what has been reported for Pax5 in the transformation process \(^{16}\), we investigated the functional collaboration between Ebf1 and Pax5 in leukemogenesis revealing a strong functional synergy on the development of leukemia. Together, our data suggest that reduced levels of Ebf1 may contribute to malignant transformation by a combination of impaired DNA repair and increased cell survival rather than simply by a differentiation block.

**Results.**

*Reduced amounts of Ebf1 increase DNA damage in pro-B cells.*

Ebf1 is a crucial regulator of cell differentiation, however, conditional deletion of the *Ebf1* gene in B-cell progenitors reveals additional roles for this transcription factor in proliferation and cell survival \(^4,18,19\). To understand more about how Ebf1 dose impact cellular functions in B-cell development, we analyzed gene expression data from primary sorted CD19\(^+\)IgM\(^-\) B-cell progenitors from *wt* and *Ebf1*\(^{+/−}\) mice \(^6\). Gene expression data from CD43\(^{low/neg}\) progenitors revealed reduced expression of *Rad51*, *Rad51ap1* and *Smc2*, all coding for proteins with functions in DNA repair while the mRNA levels for the anti-apoptotic *Bcl2* protein was increased.

To investigate if Ebf1 directly regulates DNA repair genes in progenitor B-lymphocytes, we transduced 230-238 pre-B cells with a dominant negative Ebf1 protein. Here, the carboxy-terminal trans-activation domain of Ebf1 was replaced by the repressor domain of the *Drosophila* protein engrailed \(^20\). Expression of Ebf1-engrailed resulted in down regulation of a large number of genes involved in DNA repair processes in 230-238 cells (Table SI).
In order to investigate if the reduction in DNA repair genes result in increased DNA damage in *Ebf1*+/− pro-B cells, we analyzed the levels of phosphorylated H2AX (γH2AX) in cultured *wt* and *Ebf1*+/− cells. Western blot (Figure 1a), Flow Cytometric Analysis (FACS) (Figure 1b) and immunohistochemical (IHC) analysis (Figure 1c) determined that levels of phosphorylated H2AX (γH2AX) are higher in *Ebf1*+/− pro-B cells than in *wt* cells, even though the overall levels H2AX protein were reduced (Figure 1c). Similar differences were detected upon FACS analysis of γH2AX levels in B-cell progenitors *ex vivo* (Figure 1d). Median fluorescence intensities (MFI) revealed significant differences between γH2AX in *wt* and *Ebf1*+/− CD19⁺CD43⁺IgM⁻ pro-B cells, supporting the conclusion that *Ebf1* heterozygosity increases the level of DNA damage in early B-cell progenitors.

Increased DNA damage is often associated with an increased apoptosis, however, analysis of mRNA levels in the ProB cells by Q-RT-PCR suggested that while the levels of Bad mRNA were comparable between *Ebf1* heterozygous and *wt* cells, expression of Bcl2 message was increased (Figure 1e). In support of this, analysis of AnnexinV expression on the cultured cells suggested that frequencies of apoptotic cells were lower in the *Ebf1*+/− than in the *wt* cells (Figure 1f). Analysis of the cell cycle status of the cultured pro-B cells suggested a slight reduction of cells in the G₁ stage (Figure 1g). Hence, even though the *Ebf1*+/− cells evidenced more DNA damage as estimated by H2AX phosphorylation, this did not result in increased apoptosis.

In order to investigate how *Ebf1*+/− cells respond to induced DNA damage we exposed primary cultured *wt* and *Ebf1*+/− pro-B cells to UV light and quantified the amounts of
γH2AX 16 hours after UV-exposure by FACS and IHC (Figure 2a-b). Both assays suggested that γH2AX was more abundant in Ebf1+/– cells than in wt cells. Increased DNA damage in Ebf1+/– pro-B cells as compared to wt cells were also detected using comet assays (Figure 2c), supporting our hypothesis that reducing Ebf1 dose results in increased DNA damage. UV exposure resulted in increased Bad mRNA, as estimated by Q-RT-PCR analysis in both wt and Ebf1+/– cells (Figure 2d). Although expression of Bcl2 was down regulated in both wt and Ebf1+/– cells after exposure, Bcl2 levels remained higher in the Ebf1+/– cells (Figure 2d). In line with this finding, FACS analysis estimating the fraction of apoptotic cells based on AnnexinV staining revealed significantly less apoptosis in Ebf1+/– pro-B cells relative to wt cells (Figure 2e). These data suggest that Ebf1+/– cells possess an imbalance in DNA repair and cell survival that may drastically increase both the frequency of mutation and the continued survival of cells carrying damaged DNA.

Reduced Ebf1 dose cause impaired assembly of Rad51 complexes after induction of DNA damage.

Although Ebf1 is involved in the regulation of expression of several genes in the DNA repair machinery (Table S1), a limited number of genes were significantly down-regulated in freshly isolated Ebf1+/– progenitor B cells. These genes included Rad51 and Rad51ap and Q-PCR analysis of both primary cultured and ex vivo isolated pro-B cells confirmed the lower expression levels in the Ebf1+/– cells (Figure 3a, S1a). In order to investigate whether the reduced RNA levels translated to reduced steady state expression of Rad51 protein, we stained cultured wt and Ebf1+/– pro-B cells with antibodies for γH2AX and Rad51. Rad51 is a largely cytoplasmic protein, which upon DNA damage is recruited to the nucleus and the single stranded ends at
double strand breaks \(^{21}\). IHC staining of \(wt\) cells resulted in what appeared to be a perinuclear staining likely as a result of a rather small cytoplasm of pro-B cells (Figure 3b and c). The Rad51 staining in \(Ebf1^{+/−}\) cells was reduced supporting the idea that the levels of Rad51 protein is reduced in \(Ebf1^{+/−}\) cells relative to \(wt\) cells, while the level of γH2AX displayed an opposite pattern. These results suggest that loss of one allele of \(Ebf1\) proportionally decrease levels of Rad51 protein (Figure 3b).

In order to investigate whether the reduced levels of Rad51 impact the formation of Rad51 foci after DNA damage, we exposed \(wt\) and \(Ebf1^{+/−}\) cells to UV radiation prior to staining of Rad51. While Rad51 foci were easily detected in \(wt\) cells 16 hours after UV exposure, few were formed in \(Ebf1^{+/−}\) cells as estimated by IHC (Figure 3c). Hence, reduced levels of Ebf1 impair the assembly of Rad51 foci, with potential consequences for the efficiency of homologous DNA repair in \(Ebf1^{+/−}\) pro-B cells.

In order to investigate whether \(Rad51\) is a direct target of Ebf1, we took advantage of published Chromatin-immunoprecipitation-sequencing (ChIP-Seq) \(^{22}\) and Chromosome capture data from pro-B cells \(^{23}\) to identify putative regulatory elements for the \(Rad51\) gene interacting with Ebf1. This suggested that at least one of the \(Rad51\) regulatory elements contains an Ebf1 binding site (Figure S1b). ChIP analysis suggested that the transcription factor Tcfe2a (E2A) binds in the proximity of several putative enhancer regions. This is of interest because the gene expression analysis of primary \(Ebf1^{+/−}\) progenitors suggested that \(Tcfe2a\) transcription was down regulated (Figure S1a) \(^{6}\). Hence, reduced Ebf1 dose may impact \(Rad51\) transcription both directly and indirectly by reduction of Tcfe2a levels that also may impact expression.
of Rad51ap1 because the Rad51ap1 promoter contains a binding site for Tcfe2a (Figure S2).

**Combined heterozygous deletion of Pax5 and Ebf1 predispose for development of pro-B cell leukemia.**

Our data suggest that normal Ebf1 dosage is important for the regulation of DNA repair. This would suggest that reduced amounts of Ebf1 contribute to the transformation process in a manner that differs from reductions of Pax5, which results in impaired differentiation of progenitor cells 17. Therefore, we hypothesized that combined reduction of Pax5 and Ebf1 dose synergistically could drive malignant transformation. In order to investigate this possibility, we generated mice carrying combined heterozygous deletion of Ebf1 and Pax5 genes. Although we did not detect malignant disease in wt or Pax5+/– mice, one of 16 Ebf1+/– mice and nine of 16 Pax5+/–Ebf1+/– mice presented with severely swollen lymph nodes (LN) around week 30 (Figure 4a). Examination of lymphoid organs confirmed dramatically enlarged LNs reaching a diameter of several millimeters (Figure 4b). FACS analysis of LN cells from affected mice (Figure 4c, Table I) detected a high frequency of B220+CD19+ cells (89%±s.d.) the majority of which were CD43^High pro-B cells. Frequencies of CD43− pre-B cells varied from 3 to 36% and the frequencies of IgM+ cells ranged from 0.04 to 15%. This indicates that the LN enlargement is associated with a dramatic peripheral expansion of B-cell progenitors. We further analyzed the cellular composition of the BM and spleens of four sick Pax5+/–Ebf1+/– mice (Table SII) revealing that the LN enlargement was associated with a massive increase of B-cell progenitors in peripheral hematopoietic organs. Q-RT-PCR analysis using total live cells from four mice with enlarged LNs revealed expression of Cd79a, Cd79b and
Igll1 at levels comparable to, or higher than, sorted *ex vivo* analyzed wt CD19^+^CD43^{high} pro-cells (Figure 4d) verifying the identity of the cells and revealing that the transcriptional program of a pro-B cells is intact in the transformed cells.

In order to investigate if the expansion of B-cell progenitors in *Pax5^{+/-}Ebf1^{+/-}* mice was due to a hyperproliferative syndrome with a polyclonal expansion or a result of malignant transformation of a few or single cells, we analyzed VDJ recombination products of Immunoglobulin heavy chain gene (*Igh*) loci in LN cell preparations from mice with indications of disease. While we were able to detect VDJ rearrangements to all the three examined J segments in wt BM pro-B cells (Figure 4e), we detected rearrangements of one or two of the J-segments in cells derived from LN tumors obtained from *Pax5^{+/-}Ebf1^{+/-}* mice. This indicates that the accumulation of pro-B cells in the LNs was the result of an expansion of a single or a few B-cell progenitors.

To investigate the malignant potential of the accumulated pro-B cells *in vivo*, we transplanted 2 million CD19^+^CD43^{high} cells from LNs of 3 different leukemic *Pax5^{+/-}Ebf1^{+/-}* mice into the tail veins of sub-lethally irradiated congenic (CD45.1) mice. Approximately three weeks after transplantation, the recipients injected with each of the cell preparations presented with swollen LNs. The median frequency of CD45.2^+^ cells varied from 10-42% of total live cells from lymphnodes with the absolute majority of the cells representing B-cell progenitors (Figure S3, Table II). We also detected CD45.2^+^ pro-B cells in the BM (74-98% of the CD45.2^+^ population) peripheral blood (51-89%) and spleens (37-92%) (Figure S3, Table SIII). Similar results were obtained 3 weeks after transplantation of lymphnode cells to non-irradiated mice (Table II, Table SIII). These data support the idea that the pro-B cell
expansion observed in several of the \textit{Pax5}^{+/-}\textit{Ebf1}^{+/-} mice is caused by malignant transformation resulting in progenitor B cell leukemia.

\textit{Ectopic expression of Bcl2 in combination with heterozygous deletion of Pax5 results in pro-B cell expansion but does not cause leukemia.}

Knowing that combined reductions of Pax5 and Ebf1 result in malignant disease, we wanted to assess the potential contribution of increased Bcl2 expression \textit{per se} in the transformation process. This could be of potential interest because it has been reported that homozygous deletion of \textit{Pax5} genes in combination with transgenic expression of Bcl2 result in B-cell leukemia\textsuperscript{24}. To this end we crossed \textit{Pax5}^{+/-} mice to animals expressing human BCL2 under the regulatory elements of the \textit{vav-1} gene\textsuperscript{25}. Analysis of the B-cell compartments suggested a stage specific increase in pro-B cell numbers in \textit{Pax5}^{+/-}\textit{BCL2}^{Vav} as compared to \textit{wtBCL2}^{Vav} mice (Figure S4). However, even though we followed 16 of the \textit{Pax5}^{+/-}\textit{BCL2}^{Vav} mice for 40 weeks, we noted only a single case of leukemia. Therefore, while increased Bcl2 expression cooperates with reduction in Pax5 dose to expand the pro-B cell compartment this is insufficient to cause malignant disease.

\textit{Tumors in Pax5}^{+/-}\textit{Ebf1}^{+/-} mice display a high molecular heterogeneity.\textnewline

The expansion of a limited number of leukemic clones in each of the mice carrying tumors and late onset of decease is consistent with a molecular mechanism involving secondary genetic events, well in line with what could be anticipated to be a result of impaired DNA repair. Increased DNA damage could also be presumed to drive molecular heterogeneity, because damage-induced mutations would increase the number of possible pathways to a malignant stage. In order to investigate the
molecular heterogeneity of the generated tumors, we performed gene expression microarray and Q-RT-PCR analysis from sorted live lymph node cells from eight primary $\text{Pax5}^{+/+}\text{Ebf1}^{+/+}$ tumors, as well as sorted Lin-CD19$^\text{-}$$\text{CD43}^{\text{high}}\text{IgM}^\text{-}$$\text{BM}$ cells from healthy 10-12 week old mice. All the tumors expressed the basic gene expression patterns of B-lineage progenitors including expression of $\text{Pax5}$, $\text{Ebf1}$ and their target genes. Even though gene expression analysis identified a set of down-regulated genes, including the TGFβ-responsive $\text{Tgfbi}$ gene and genes encoding the transcription factors $\text{Runx2}$, $\text{Lef1}$ and $\text{Id3}$ (Figure 5a) in all of the tumors, the populations were heterogenous with regard to changes in gene expression patterns. Q-RT-PCR analysis of genes indicated as differentially expressed from the microarray analysis verified a high degree of heterogeneity (Figure 5b). In order to investigate this further, we performed exom-sequencing and used Mutech based analysis $^{26}$ with normal ex vivo sorted $\text{Pax5}^{+/+}\text{Ebf1}^{+/+}$ pro-B cells as reference to identify unique mutations in the individually generated tumors. This indicated that most of the tumors contained in the range of 29-53 unique mutations with an average allele depth of 0.16-0.29 (Table III). Two of the tumors presented with a higher degree of unique changes and while tumor LN327 carried a major part of the alterations on chromosome 11, the alterations in LN317 were more interspersed in the genome. In conclusion, the tumor populations arising after combined heterozygous loss of $\text{Ebf1}$ and $\text{Pax5}$ similarly represent early B-cell progenitors, but display a substantial molecular heterogeneity consistent with the idea that tumor formation involves secondary events as could be anticipated by a defect in DNA repair.
Discussion.

Several types of proto-oncogenes and oncogenic events have been defined as contributors to malignant transformation. In leukemogenesis, these events prominently include the loss or reduction of transcription factors that control differentiation. In this regard, heterozygous loss of transcription factors, including *Ebf1* and *Pax5*, has been suggested to cause a disruption in B-cell differentiation, predisposing cells for transformation. Because developmental arrest is a hallmark of pro-B and pre-B ALL cells, this represents a logical explanation and a reasonable hypothesis; however, it is not obvious how a partial developmental arrest, as imposed by a reduction in transcription factor dosage, would directly contribute to malignant transformation. Developmental changes observed due to heterozygous loss of *Pax5* in mouse models are modest and even though the loss of one allele of *Ebf1* results in a more pronounced disturbance in the generation of B-cells, this may be explained by a reduced expansion of the pre-B cell compartment due to the role of Ebf1 in cell growth and survival. Furthermore, a developmental block imposed by Rag deficiency did not result in the same degree of synergistic generation of leukemia when combined with ectopic expression of activated Stat5 as did either *Pax5* or *Ebf1* heterozygosity. Hence, it is reasonable to presume that reduced transcription factor dose impact the transformation process by multiple mechanisms.

Even though the most direct approach to identify molecular explanations to malignant transformation is a comparison of normal and leukemic cells, this provides limited information regarding the transformation process *per se*. Our analysis is based on how Ebf1 dose impact gene expression in non-transformed cells, representing an early pre-leukemic stage, allowing us to investigate events upstream of the terminal
transformation process. Our work reveals that one consequence of reduced Ebf1 levels in these non-transformed cells is impaired DNA repair. In contrast, gene expression analysis from leukemia cells generated from Ebf1+/− mice did not reveal a reduction in expression of DNA repair genes. This may reflect a post-transformation selection process because while impaired DNA repair may be crucial for tumour formation, this could represent a growth disadvantage once the malignancy is established. Even though the reduced level of Ebf1 appears to modulate the expression of several genes involved in DNA repair (Supplementary table I), the most obviously effected genes in normal Ebf1+/− cells ex vivo was Smc2, Rad51ap1 and Rad51. Rad51 expression is differentially regulated during the cell cycle being expressed from late G1 to M-phase but even though we noted some changes in cell cycle dynamics, we could not detect any accumulation of cells in G0 that could not explain the observed changes in Rad51 expression. This in combination with the CHIP analysis suggests that the Rad51 gene is a direct target for Ebf1. Rad51 has crucial functions for homologous DNA repair and preservation of DNA integrity and in myeloid leukemia, reduced Rad51 expression results in increased sensitivity to DNA damaging drugs. Hence, even though we have not established any direct link between the reduction of Rad51 levels and increase in γH2AX foci formation, we believe that the effect on expression of Rad51 and Rad51ap1 may contribute to the observed increase in DNA damage. Negative selection against a molecular mechanism active in a pre-leukemic state generates a challenge in establishing a direct link between the early event in the pre-leukemic cell and the observed characteristics of the fully developed leukemia cells. However, low clonal complexity and late onset of disease in Pax5+/− Ebf1+/− mice argues for a need of secondary genetic alterations in order to generate malignant cells, a process that should be facilitated by impaired
DNA repair. Furthermore, molecular heterogeneity among the tumours reflected in both unique gene expression patterns and mutations is well in line with what could be expected from defects in DNA repair. Additionally, the mutation range of 30-50 unique alterations in the exomes (Table III) is high as compared to what has been reported from matched human B-ALL samples. Hence, there are several lines of support for the idea that reduced Ebf1 dose could impact malignant transformation via impairments in DNA repair. Although Ebf1 may contribute to malignant transformation by other mechanisms of action, (i.e., impaired differentiation and deregulated expression of Bcl2), increased Bcl2 levels was not sufficient for the development of leukemia when combined with a heterozygote mutation in Pax5 (Figure S4). However, this does not preclude that the high expression of Bcl2 is of importance in the transformation process, since Bcl2 may promote the survival of cells carrying DNA damage in a pre-malignant stage.

Our data demonstrate that combined heterozygous deletions of Ebf1 and Pax5 precondition for the development of a lymphoid leukemia with phenotypic features resembling B-ALL in humans. In order to investigate whether combined heterozgous loss of Pax5 and Ebf1 is a feature of human B-ALL, we analyzed a SNP data set collected from 242 human childhood B- and T-ALLs. Deletion of Pax5 was detected in 57 of the 192 (30%) B-ALLs, and deletion of Ebf1 was detected in 8 of these (4%) (Figure S5A-C). Of the 8 leukemia’s carrying Ebf1 mutations, three was detected in combination with deletions of Pax5. Although the sample size is too small for reliable statistical analysis, these data reveal that combined heterozygote deletions of Pax5 and Ebf1 are a feature of human B-ALL.
Taken together, our data suggest that Ebf1 contribute to malignant transformation via its crucial functions in the regulation of genes involved in DNA repair. A role for a lineage and stage specific transcription factor in such basic cellular functions appears counterintuitive. However, epigenetic dynamics in cell differentiation may result in that stage specific transcription factors come to regulate genes involved in basic cellular functions and requirements for Ebf1 in VDJ recombination and other processes required for B cell identity and commitment may include the need for increased surveillance of genomic integrity.

**Experimental procedures**

*Animal models.*

*Pax5*+/− 31, *Ebf1*+/− 3 or vav-*BCL2* 25 were all on C57BL/6 (CD45.2) background. Adoptive transfers were performed by tail vein injection of 2 million live lymphnode cells into sublethally irradiated (4.5 Gy) or non conditioned CD45.1 mice. Animal procedures were performed with consent from the local ethics committee at Linköping University (Linköping, Sweden).

*Western blot.*

Western blot were made using extracts from cultured primary pro B cells from bone marrow of *wt* and *Ebf1*+/− mice. The membranes were incubated with the relevant primary and secondary antibodies (For details please see supplementary methods (SM)) and signals were measured with Odyssey Infrared Imaging System (LI-COR, Inc., Lincon, NE). The band intensities was quantified using Image J software.
**Immuno-fluorescence analysis**

Cultured Wt and Ebf1+/− pro-B cells from bone marrow mice or UV induced pro-B cells were fixed and incubated with the relevant antibodies and mounted using VECTA SHEILD containing DAPI (For details see SM). The fluorescence expression of stained proteins was viewed using LM Zeiss upright confocal microscope (ZEISS, Germany). The integrated density of protein expression was monitored using Image J software.

**In vitro Phospho flow.**

Cultured Wt and Ebf1+/− pro-B cells or ex vivo sorted pro-B cells were fixed and stained with the relevant antibodies (For details see SM). The stained cells were analyzed using BD FACS Canto II analyzer (BD Biosciences). Gates were set based on the antibody isotype control. All gates were set according to FMO (Fluoresce minus one control). All analysis was performed using Flow Jo software.

**Annexin V staining.**

Cultured Pro-B Wt and Ebf1+/− cells were washed and incubated with Annexin V-APC (BD Pharmingen) along with 5 μl 7AAD and the cells were analyzed using BD FACS Canto II (BD Biosciences). All the gates were set according to the unstained control, Annexin V- APC alone and 7-AAD alone.

**Cell cycle analysis.**

Cultured Pro-B Wt and Ebf1+/− cells were fixed and incubated with antibodies for Ki67 (BD Pharmingen) and DAPI G0 cells were scored as Ki67− DAPIlow (equal to that of a diploid cell), G1 Ki67+ DAPIlow and SG2M as Ki67+ DAPIHigh as in 6.
Comet assay.

The comet assay was performed using the reagents from OxiSelect Comet assay kit (Cell Biolabs, CA, USA) according to manufactures instructions (See SM for details). Comets were viewed using Zeiss upright confocal microscope (ZEISS, Germany) in FITC channel using 20X objective.

FACS staining and sorting of hematopoietic cells.

Cell sorting of and analysis of primary cells was performed as in 6 (For details see SM). Cellular composition in the primary tumors were analyzed using frozen single cell suspensions from lymph node, BM and spleen while the composition in the organs of transplanted mice was performed on freshly isolated tissues and blood.

Gene expression analysis with Quantitative RT-PCR and Affymetrix.

Q-RT-PCR and Affymetrix based global gene expression analysis of sorted cells was performed as previously described 33 (See SM for details). Data are deposited at GEO, GSE67415.

VDJ-recombination and Exome sequencing analysis.

Live cells were sorted and DNA was extracted and subjected to PCR based VDJ analysis 34. For exome sequencing the DNA was fragmented and enriched for coding regions followed by sequencing to gain an average cover of 100X and the obtained data was analysed using the Mutech software 26 (See SM for details).
Statistical analysis.

The statistical analysis is described in the corresponding figure legends.

Acknowledgements:

We wish to thank our colleges for help with transgenic mice and cell lines and we also wish to thank Liselotte Lenner, Linda Bergström and Anne Halden Waldermarsson for advice and assistance.

Author contributions:

JÅ, TS, JU, RS, MP and MS conducted, designed and interpreted the analysis of the mouse model and TF and HL, analyzed the human SNP data, RM, AD analysed High-C data and JH contributed essential reagents and experimental design. All authors participated in the writing of the manuscript.

Conflict of interest:

The authors declare that they have no conflict of interest.

References:


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### Table 1: Cellular content in lymph nodes from leukemic mice.

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<tr>
<th>Lymph Node</th>
<th>% of Live cells</th>
<th>% of CD19+CD220+</th>
<th>CD43highIgM</th>
<th>CD43lowIgM</th>
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<td>277 (Pax5+/Ebf1−)</td>
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**Table I:** Cellular composition of frozen lymph node samples from mice diagnosed with lymph node enlargement. The number in the far left column indicate the identity of the mice. The FACS analysis was based on surface expression of the myeloid markers Gr1/Mac1, the T-lineage marker CD3 and the B-lineage markers CD19 and B220. The sub-composition of the CD19+CD220+ population was further investigated based on the expression of CD43 and IgM. The first three columns are percentage of live cells while the last three columns display percentage of CD19+CD220+ cells.
Table II: Cellular composition of fresh lymph node samples from mice transplanted with leukemic cells from Pax5^+/-Ebf1^-/- mice two to three weeks after transplantation.

The numbers in the far left column indicate the identity of the original mouse with developed malignancy. The FACS analysis was based on surface expression of CD45.2 to identify transplanted cells and the NK cell marker NK1.1, the T-lineage marker CD3 and the B-lineage markers CD19 and B220. The sub-composition of the CD19^+B220^ population was further investigated based on the expression of CD43 and IgM. The data in the first column indicate the frequency of live CD45.2^+ cells in each of the samples while the remaining columns indicate percentage of the CD45.2^+ cells.

<table>
<thead>
<tr>
<th>Donor Lymph Node</th>
<th>% of Live cells</th>
<th>CD45.2</th>
<th>NK1.1</th>
<th>CD3</th>
<th>CD19^+B220^</th>
<th>CD43^+IgM</th>
<th>CD43^-IgM</th>
<th>CD43^+IgM</th>
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</thead>
<tbody>
<tr>
<td>277 (n=4^a)</td>
<td>23.8 (16.1-31.4)</td>
<td>0.0</td>
<td>0.1 (0.1-0.2)</td>
<td>86.8 (85.8-91.5)</td>
<td>0.0 (0.1-0.2)</td>
<td>3.0 (2.5-6.3)</td>
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<tr>
<td>278 (n=7^a)</td>
<td>9.7 (6.7-19.5)</td>
<td>0.1 (0.0-0.2)</td>
<td>0.0</td>
<td>96.4 (93.9-97.5)</td>
<td>83.0 (78.8-86.9)</td>
<td>4.0 (2.5-6.3)</td>
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<tr>
<td>327 (n=4^a)</td>
<td>42.2 (32.2-62.6)</td>
<td>0.0</td>
<td>0.5 (0.1-1.8)</td>
<td>98.0 (97.0-98.2)</td>
<td>97.2 (96.4-98.0)</td>
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<tr>
<td>278 (n=4^a)</td>
<td>39.5 (25.1-67.7)</td>
<td>0.0</td>
<td>0.2 (0.1-0.3)</td>
<td>98.3 (98.1-98.5)</td>
<td>91.3 (90.0-92.9)</td>
<td>2.1 (2.1-2.9)</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>292 (n=3^a)</td>
<td>55.8 (47.2-57.1)</td>
<td>0.0</td>
<td>0.2 (0.0-0.3)</td>
<td>99.2 (98.9-99.3)</td>
<td>60.5 (56.7-65.3)</td>
<td>31.7 (27.2-35.4)</td>
<td>0.1 (0.0-0.1)</td>
<td></td>
</tr>
</tbody>
</table>

* = Median ± IQR

a = Sublethally irradiated (4.5 Gy) CD45.1 recipient mice
b = non-irradiated CD45.1 recipient mice

**Table II:** Cellular composition of fresh lymph node samples from mice transplanted with leukemic cells from Pax5^+/-Ebf1^-/- mice two to three weeks after transplantation. The numbers in the far left column indicate the identity of the original mouse with developed malignancy. The FACS analysis was based on surface expression of CD45.2 to identify transplanted cells and the NK cell marker NK1.1, the T-lineage marker CD3 and the B-lineage markers CD19 and B220. The sub-composition of the CD19^+B220^ population was further investigated based on the expression of CD43 and IgM. The data in the first column indicate the frequency of live CD45.2^+ cells in each of the samples while the remaining columns indicate percentage of the CD45.2^+ cells.
Table III: Number of unique genetic differences and the average allelic fraction of unique alterations detected in tumor samples, when using sequence data from sorted pro-B cells from healthy 12 weeks old $Pax5^{+/+}/Ebf1^{+/+}$ mice as reference DNA in MuTect analyses. Unique differences were identified using a python script to eliminate identical changes in two samples.
Figure legends:

Figure 1: Heterozygous loss of Ebf1 results in increased levels of phosphorylated H2AX in B-cell progenitors. Panel (a) display a representative western blot out of three detecting phosphorylated H2AX (γH2AX) and GADPH from in vitro cultured Wt or Ebf1+/− pro-B cells. The lower panel display a quantification of the signal intensity normalized to the Wt control and represents three experiments. The data shown in band intensity Mean ± SD and statistical analysis was performed using unpaired T test, ***, P<0.001 Panel (b) display graphs over the relative median fluorescence intensity (MFI) obtained by flow cytometric analysis to detect γH2AX and indirect flow analysis of phosphorylated Chk1 (pChk1) and phosphorylated Chk2 (pCHK2) in in vitro cultured Wt or Ebf1+/− pro-B cells. The data are normalized towards the Wt control and expressed in fold MFI represents three independent experiments. The statistical analysis was done using unpaired T test Mean ± SD, **, P<0.01 and *, P<0.05 (c) Immunofluorescence analysis of phosphorylated and non phosphorylated H2AX (Total H2AX) in in vitro cultured Wt or Ebf1+/− pro-B cells detected by using rabbit γH2AX monoclonal antibody or rabbit monoclonal total H2AX antibody followed by a specific anti Alexa Fluor 594 secondary antibody. The nuclei were subsequently stained with DAPI and the images were captured using LM Zeiss upright confocal microscope. The quantification panel next to the image displaying the signal intensity collected from three experiments. The statistical analysis was performed using unpaired T test and results are plotted as change in fold integrated density compared to Wt, **, P<0.01 and ***, P<0.001 (d) display a diagram with relative median fluorescence intensity (MFI) obtained by flow cytometric
analysis to detect γH2AX, in *ex vivo* isolated *Wt* or *Ebf1*+/− pro-B cells. The data are normalized towards the *Wt* control and represents three experiments and unpaired T test was performed, Mean ± SD, ***, P<0.01 (e) Diagrams displaying Q-RT-PCR data from *in vitro* cultured *Wt* or *Ebf1*+/− pro-B cells. The data are normalized to the expression of *HPRT* in triplicate PCR reactions. Unpaired T test was performed for statistical analysis ***, P<0.001, data represent three independent experiments in Mean ± SD. The diagram in panel (f) displays the frequency of AnnexinV+ cells as estimated by flow cytometric analysis from *in vitro* cultured *Wt* or *Ebf1*+/− pro-B cells. The cells were gated on a lymphoid gate for live cells. The data represent five experiments. The error bar in the panels indicate Mean standard deviation and Statistical analysis was performed using Students t-test ***, P<0.001. Panel (g) display a diagram over cell cycle status of *Wt* or *Ebf1*+/− pro-B cells using Ki67 and DAPIi staining. G0 cells were scored as Ki67 DAPIlow, G1 Ki67+ DAPIlow and SG2M as Ki67+ DAPITH. Mean standard deviation and statistical analysis was performed using Students t-test *, P<0.05.

**Figure 2: Heterozygous loss of Ebf1 results in increased DNA damage after UV exposure of B-cell progenitors.** Panel (a) display a diagram with relative median fluorescence intensity (MFI) obtained by flow cytmetric analysis to detect γH2AX, in *in vitro* cultivated primary *Wt* or *Ebf1*+/− pro-B cells. 16 hours after UV exposure. The data are normalized towards the *Wt* control and represents three experiments, The statistics was performed using unpaired T test, the error bar represents the Mean±SD, ***, P<0.001 (b) Immunohistochemical staining of phosphorylated H2AX (γH2AX) in *in vitro* cultured *Wt* or *Ebf1*+/− pro-B cells 16 hours after UV exposure. DAPI was used to stain the nucleus. The lower panel display a quantification of the signal
intensity collected from three experiments. The representation of fold integrated density is based on unpaired T test with error bars representing Mean standard deviation ***, P<0.001. Panel (c) display representative pictures of comet assays performed 16 ours after UV exposure of in vitro cultivated primary Wt or Ebf1+/− pro-B cells. (d) Diagrams displaying Q-RT-PCR data from in vitro cultured Wt or Ebf1+/− pro-B cells before and 16 hours after UV exposure. The data are normalized to the expression of HPRT in triplicate PCR reactions and represent three independent experiments. Student U test represents the statistical analysis, ****, p<0.0001. The diagrams in panel (e) display the frequency of AnnexinV+ cells as estimated by flow cytometric analysis from in vitro cultured Wt or Ebf1+/− pro-B cells. The cells were gated on a lymphoid gate for live cells. The error bars in the diagrams indicate mean standard deviation and Statistical analysis was performed using unpaired students t-test, **, P<0.01.

**Figure 3: Heterozygous loss of Ebf1 results in lower levels of Rad51 in B-cell progenitors.** (a) Diagrams displaying Q-RT-PCR data from in vitro cultured Wt or Ebf1+/− pro-B cells. The data are normalized to the expression of HPRT in triplicate PCR reactions and represent three independent experiments. Student U test was performed to check the statistical significance, **, P<0.01 and *, P<0.05. Panel (b) display Immunofluorescence staining of γH2AX (Rabbit) and mouse Rad51 in in vitro cultured Wt or Ebf1+/− pro-B cells followed by the specific anti Alexa Fluor 594 (γH2AX) and anti Alex Fluor 488 secondary antibody (Rad51). DAPI was used to stain the nucleus. The panel adjacent to each IF images display a quantification of the signal intensity collected from three experiments and quantification is represented as fold integrated density with statistical significant value *, P<0.05 for γH2AX and ***,
P<0.001 for Rad51. Panel (c) display Immunohistochemical staining of Rad51 in in vitro cultured Wt or Ebf1+/− pro-B cells 16 hours after UV exposure. DAPI was used to stain the nucleus. The data was collected from three experiments and foci formation was counted from 3 cells from three different experiments. The error bars in the diagrams indicate standard deviation and Statistical analysis was performed using Students t-test with p value P<0.001.

Figure 4: Combined heterozygous loss of Ebf1 and Pax5 results in development of disease and increased mortality. Panel (a) Kaplan-Meier curves describing the 40-week survival of Wt, Ebf1+/−, Pax5+/− and Pax5+/−Ebf1+/− mice. A drop in the curve describes unknown death cause or development of swollen lymph nodes and subsequent sacrificing due to animal protection regulation. Mice sacrificed of known reasons other than swollen lymph nodes are censored in the curves. Overall log-rank (Mantel-Cox test) p-value is displayed. Panel (b) displays two enlarged, one inguinal and one brachial, lymph nodes from a Pax5+/−Ebf1+/− mouse. Panel (c) display representative flow cytometric analysis of the cellular content of a representative lymph node (Left panel) and the fraction of CD43high or IgM+ CD19+ cells in 4 analyzed nodes (Right panel). The diagram in panel (d) displays Q-RT-PCR data over gene expression analysis of sorted live cells in four analyzed lymphnodes. Sorted Pax5+/−Ebf1+/− BM pro-B cells and LMPPs we used as positive and negative controls. Expression levels are presented in relation to that of HPRT. The data represent one representative out of two Q-PCR experiments using triplicate PCR reactions for estimation of expression values. (e) Immunoglobulin heavy chain VDJ analysis from three of the analyzed live cell populations from nodes of mice displaying peripheral
pro-B cell expansion. Cultured Wt pro-B cells were included to show the rearrangement signature of a polyclonal population.

**Figure 5: Pax5<sup>+</sup>/Ebf<sup>+</sup> tumor cells display molecular heterogeneity.**

(a) Heat map over gene expression patterns in primary sorted CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>−</sup> <em>ex vivo</em> analyzed BM pro-B cells from 14 weeks old mice and 8 pro-B cells tumors from Pax5<sup>+</sup>/Ebf1<sup>+</sup> mice. The expression pattern is based on genes differentially expressed more than 10-fold in any of the tumor samples as compared to the normal pro-B cells. Red indicate high and blue low expression of mRNA. The data are collected from two array experiments from each cell population (b) The diagrams display Q-PCR data generated from primary sorted live lymph node cells from a set of independently generated tumors as well as primary sorted BM pro-B cells. The data were normalized to the expression of <em>Hprt</em> and the diagrams presents one representative out of two independent Q-RT-PCR experiments based on triplicate Q-RT-PCR reactions.
Figure 2

A

\[ \gamma^\text{-H2AX} \]

Fold MFI

\[ \text{Wt} \quad \text{Ebf}^{1/\text{-}} \]

B

MERGE

IF: \( \gamma^\text{-H2AX} \)

Fold Integrated Density

\[ \text{Wt} \quad \text{Ebf}^{1/\text{-}} \]

C

Comet assay

Alkaline condition

\[ \text{Wt} \quad \text{Ebf}^{1/\text{-}} \]

D

Relative expression to Hprt1

\[ \text{Bcl2} \quad \text{Bad} \]

E

\% of total apoptosis

\[ \text{Wt} \quad \text{Ebf}^{1/\text{-}} \]
Figure 3

A

<table>
<thead>
<tr>
<th>Relative expression to Hprt1</th>
<th>Rad51</th>
<th>Rad51AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ebf1+/-</td>
<td></td>
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</tbody>
</table>

B

γ-H2AX  Rad51  MERGE

Wt

Ebf1+/-

Fold Integrated Density

γ-H2AX  Rad51

Wt

Ebf1+/-

C

Rad51  DAPI  MERGE

Wt

Ebf1+/-

Fold Integrated Density

Rad51

Wt

Ebf1+/-

Average no. of Rad51 foci

Wt

Ebf1+/-
Eb1 heterozygosity results in increased DNA damage in pro-B cells and their synergistic transformation by Pax5 haploinsufficiency

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