THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 46, pp. 33449–33461, November 15, 2013

Early B-cell Factor 1 Regulates the Expansion of B-cell Progenitors in a Dose-dependent Manner*

Received for publication, July 29, 2013, and in revised form, September 25, 2013 Published, JBC Papers in Press, September 27, 2013, DOI 10.1074/jbc.M113.506261

Josefine Åhsberg[‡], Jonas Ungerbäck[‡], Tobias Strid[‡], Eva Welinder[‡], Jenny Stjernberg[‡], Malin Larsson[§], Hong Qian[‡¶], and Mikael Sigvardsson[‡1]

From the [‡]Department of Clinical and Experimental Medicine, Experimental Hematopoiesis Unit, Faculty for Health Sciences, Linköping University, University Lab 1, Level 13, SE-581 85 Linköping, Sweden, [§]BILS (Bioinformatics Infrastructure for Life Sciences), IFM Bioinformatics, Linköping University, SE-581 85 Linköping, Sweden, and the [¶]Center for Hematology and Regenerative Medicine, Department of Medicine, Karolinska Institutet Huddinge, SE-171 77 Stockholm, Sweden

Background: Transcription factor doses play important roles in normal and malignant B-lymphocyte development.
Results: We show dose-dependent regulation of B-cell specification and expansion of committed progenitors.
Conclusion: Transcription factor dose impacts several aspects of B-cell development.
Significance: Knowing the effects of reduced transcription factor dose aids our understanding of the molecular events underlying leukemia and B-cell development.

Transcription factor doses are of importance for normal and malignant B-lymphocyte development; however, the understanding of underlying mechanisms and functional consequences of reduced transcription factor levels is limited. We have analyzed progenitor and B-lineage compartments in mice carrying heterozygote mutations in the *E2a*, *Ebf1*, or *Pax5* gene. Although lymphoid progenitors from *Ebf1* or *Pax5* heterozygote mice were specified and lineage-restricted in a manner comparable with *Wt* progenitors, this process was severely impaired in *E2a* heterozygote mutant mice. This defect was not significantly enhanced upon combined deletion of *E2a* with *Ebf1* or *Pax5*. Analysis of the pre-B-cell compartment in *Ebf1* heterozygote mice revealed a reduction in cell numbers. These cells expressed *Pax5* and other B-lineage-associated genes, and global gene expression analysis suggested that the reduction of the pre-B-cell compartment was a result of impaired pre-B-cell expansion. This idea was supported by a reduction in IL2Rα-expressing late pre-B-cells as well as by cell cycle analysis and by the finding that the complexity of the VDJ rearrangement patterns was comparable in *Wt* and *Ebf1*$^{+/-}$ pre-B-cells, although the number of progenitors was reduced. Heterozygote deletion of *Ebf1* resulted in impaired response to IL7 *in vitro* and reduced expression levels of pre-BCR on the cell surface, providing possible explanations for the observed stage-specific reduction in cellular expansion. Thus, transcription factor doses are critical for specification as well as expansion of B-lymphoid progenitors, providing increased insight into the molecular regulation of B-cell development.

The development of mature B-lymphocytes from multipotent progenitors in the bone marrow (BM)[2] depends on an orchestrated transcription factor network. The understanding of this process has been facilitated by recent advancements in the isolation of early progenitor populations (1) as well as by analysis of gene expression patterns and *in vivo* protein-DNA interactions (2–4). The formation of the earliest B-cell committed progenitors is dependent on the transcription factor EBF1 (5, 6) as well as *E2a* encoded proteins (Tcf3) (7, 8) and FOXO1 (9) because in the absence of these factors, the expression of B-lineage genes is dramatically reduced in the lymphoid progenitors, and normal lineage restriction is disrupted (9, 10). Commitment to B-lineage development is associated with the expression of the transcription factor PAX5, known to be of critical importance for restriction of alternative cell fates (11–13). However, in contrast to what is observed in the absence of EBF1 and E2A proteins, lack of PAX5 has a modest effect on the transcription of B-lineage genes in the earliest progenitors (14–16). This has resulted in the establishment of a concept postulating that although EBF1 and E2A are critical for B-cell specification, this process is linked to stable commitment through the activation of PAX5.

Although the role of lineage specific transcription factor networks in development has been rather well established, the direct link between these regulatory cues and human leukemia is now becoming an area of intense investigation. This is because detailed mapping of genetic changes in human B-cell malignancies has revealed that mutations in genes encoding key regulatory proteins, such as PAX5, EBF1, and *E2a* encoded proteins, can be found in a large fraction of the pediatric B-progenitor acute lymphoblastic leukemias (17). These mutations appear to be heterozygous, and thus it has been suggested that the impact of these mutations depends on a reduction of functional transcription factor dose, an idea supported by the finding that leukemia formation is enhanced in mice expressing a constitutively active STAT5 protein in combination with the loss of one functional allele of either *Pax5* or *Ebf1* (18). Furthermore, low levels of PAX5 expression in hematopoietic progenitors result in an expansion of cells expressing a combination of myeloid and lymphoid genes similar to what can be observed in biphenotypic

* This work was supported by grants from the Swedish Cancer Society, the Swedish Research Council through project grants, and Hemato-Linné and the Faculty of Medicine at Linköping University.
[1] To whom correspondence should be addressed. Tel.: 46-13-227887; Fax: 46-13-224314; E-mail: mikael.sigvardsson@liu.se.
[2] The abbreviations used are: BM, bone marrow; 7-AAD, 7-aminoactinomycin D; NK, natural killer; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitors.

leukemia (19). Functional transcription factor dose is also of crucial importance in normal B-cell development, where reduced levels of E-proteins (E2A, HEB, and E2-2) (20) or loss of one allele of *Ebf1* alone or in combination with *E2a* or *Runx1* results in disturbed B-cell development (5, 21–23). Hence, transcription factor dose is of critical importance for malignant transformation in leukemia as well as normal B-cell differentiation, creating a link between development and disease.

We here report an analysis of B-cell development in mice carrying heterozygote mutations in critical transcription factors. Although *E2a* gene dosage is important for B-cell specification, the loss of one allele of *Ebf1* instead appears to be of importance for the normal expansion of already committed progenitor cells. We believe that our findings have implications not only for normal B-cell development but also for the molecular understanding of leukemia formation.

EXPERIMENTAL PROCEDURES

Animal Models—Pax5$^{+/-}$ (14), *Ebf1*$^{+/-}$ (5), *E2a*$^{+/-}$ (24), and reporter mice carrying a *hCD25* reporter under the regulatory elements of the *Lambda5* (*λ5*, *Igll1*) promoter (25) were all on C57BL/6 background. Bone marrows and spleens were harvested from mice aged 7–13 weeks. Adoptive transfers were performed as described previously (16). Animal procedures were performed with consent from the local ethics committee at Linköping University (Linköping, Sweden).

FACS Staining and Sorting of Hematopoietic Cells—For analysis and cell sorting of progenitor bone marrow cells, CD16/CD32 (FC)-blocked (Clone 93, eBioscience) cells were stained with antibodies against lineage markers CD11b (M1/70), GR1 (RB6-8C5), TER119 (Ter119), CD3 (17A2, BD Pharmingen), CD11c (N418), NK1.1 (PK136), CD19 (1D3), and CD45R/B220 (RA3-6B2). Further staining with KIT (2B8), SCA-1 (E13-161.7), IL7R (A7R34), FLT3 (A2F10), Ly6D (49-H4, BD Pharmingen), and propidium iodide (Invitrogen). All antibodies were purchased from BioLegend unless stated otherwise. For progenitor isolation, bone marrow cells were subjected to magnet-activated cell sorting column enrichment of KIT$^+$ cells using anti-CD117 immunomagnetic beads (Miltenyi Biotec) prior to antibody staining. Analysis and cell sorting were performed on a BD FACSAria^{TM} cell sorter (BD Biosciences). Gates were set according to fluorescence minus one controls.

For population analysis, equal cell numbers of FC-blocked cells were stained with the following antibody combinations: progenitors, as stated above without column enrichment; B-cells, lineage markers CD11b (M1/70), GR1 (RB6–8C5), TER119 (Ter119), CD3 (17A2), CD11c (N418), and NK1.1 (PK136) and further with CD19 (ID3), CD45R/B220 (RA3–6B2), CD43 (S7), IgM (RMM-1), and IgD (11-26, eBioscience, San Diego, CA). Dead cells were excluded with 7-aminoactinomycin D (7-AAD; Sigma), and analysis was performed with a BD FACSCantoII analyzer (BD Biosciences).

Ex Vivo Analysis of Cell Cycle Status in B-cell Progenitors—Cells stained for lineage, CD19, CD43, IL2Rα (CD25), and IgM were fixated and permeabilized with the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Biosciences) and further stained with Ki-67 antibody (BD Biosciences) and 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Gates for Ki-67 staining were based on isotype control antibody. All gates were set according to fluorescence minus one controls. Analysis was performed on a FACS Aria Special Order System^{TM} (BD Biosciences).

In Vitro Evaluation of Natural Killer (NK)- and T-lineage Potentials—For evaluation of NK- and T-cell potential, cells were deposited (using FACSAria) directly into 96-well plates containing preplated (2000 cells/well) stroma cells. T-cultures (on OP9Delta stroma layers) were supplemented with 10 ng/ml KIT ligand, 10 ng/ml Fms-like tyrosine kinase 3 ligand (FLT3L), and 10 ng/ml interleukin-7. NK cultures (on OP9 stroma layers) were supplemented with the same cytokines as the OP9Delta cultures, with the addition of 20 ng/ml interleukin-15 and 40 ng/ml interleukin-2. All cytokines were acquired from Peprotech (Rocky Hill, NJ). Cultures were substituted with fresh cytokines after 7 days. OptiMEM supplemented with 10% heat-inactivated fetal calf serum, 25 mM HEPES, 50 μg/ml Gentamicin, and 50 μM β-mercaptoethanol was used for maintaining the OP9/OP9Delta stroma cell lines (26) as well as for the cocultures.

Cocultures were evaluated by FACS staining with CD19 (ID3), NK1.1 (PK136), CD11c (N418), and 7-AAD for NK OP9 cocultures and CD19 (ID3), CD90.2/Thy1.2 (53-2.1), CD3 (17A2), and 7-AAD for T OP9Delta cocultures. All cocultures were analyzed at day 14 with a BD FACSCantoII analyzer (BD Biosciences).

In Vitro Evaluation of Myeloid Potential—We have modified a previously described method to evaluate myeloid potential (27). In brief, cells were directly sorted to a concentration of 1 cell/20 μl of medium OptiMEM (supplemented as above) and 25 ng/ml KIT ligand, 25 ng/ml FLT3L, 25 ng/ml murine thrombopoietin/megakaryocyte colony-stimulating factor, 10 ng/ml interleukin-3, 25 ng/ml murine granulocyte colony-stimulating factor, and 25 ng/ml murine granulocyte/macrophage colony-stimulating factor, and 20 μl of cell suspension were plated into each well (Nunc Minitrays). Wells were scored after 5 days (using an inverted light microscope) to estimate clonal growth and clone size.

Quantitative and Single Cell RT-PCR—Quantitative RT-PCR analysis of sorted cells was performed as described previously (27). Assays-on-Demand^{TM} probes (Applied Biosystems, Foster City, CA) used were as follows: *Hprt*, Mm00446968_m1; *Pax5*, Mm00435501_m1. Multiplex single cell RT-PCR analysis was performed as described previously (27). Multiplex PCR primers used were as follows: *Hprt*, GGGGGCTATAAGTTCTTTGC (primer 1), GTTCTTTGCTGACCTGCTGG (primer 2), TGGGGCTGTACTGCTTAACC (primer 3), and TCCAACACTTCGAGAGGTCC (primer 4); *Pax5*, CTACAGGCTCCGTGACGCAG (primer 1), ATGGCCACTCACTTCCGGGC (primer 2), GTCATCCAGGCCTCCAGCCA (primer 3), and TCTCGGCCTGTGACAATAGG (primer 4); *λ5/IgII1*, AGTTCTCCTCCTGCTGCTGC (primer 1), GGGTCTAGTGGATGGTGTCC (primer 2), CAAAACTGGGGCTTAGATGG (primer 3), and CCCACCACCAAAGACATACC (primer 4); *Pou2af1/Ocab*, ACGCCCAGTCACATTAAAGAAG (primer 1), AGCCAGTGAAGGAGCTACTGAG (primer 2), ATGTTCCCTCCTCTGTCACTGT (primer 3), and CTGTCACTGTGGAAGCAGAAAC (primer 4); *Flt3*, TCTCAATTCAGGTGGCGGTG (primer 1), GCTGTGAAAAAGAAGCTCTCATGTC (primer 2), ACCAGGCATGCT-

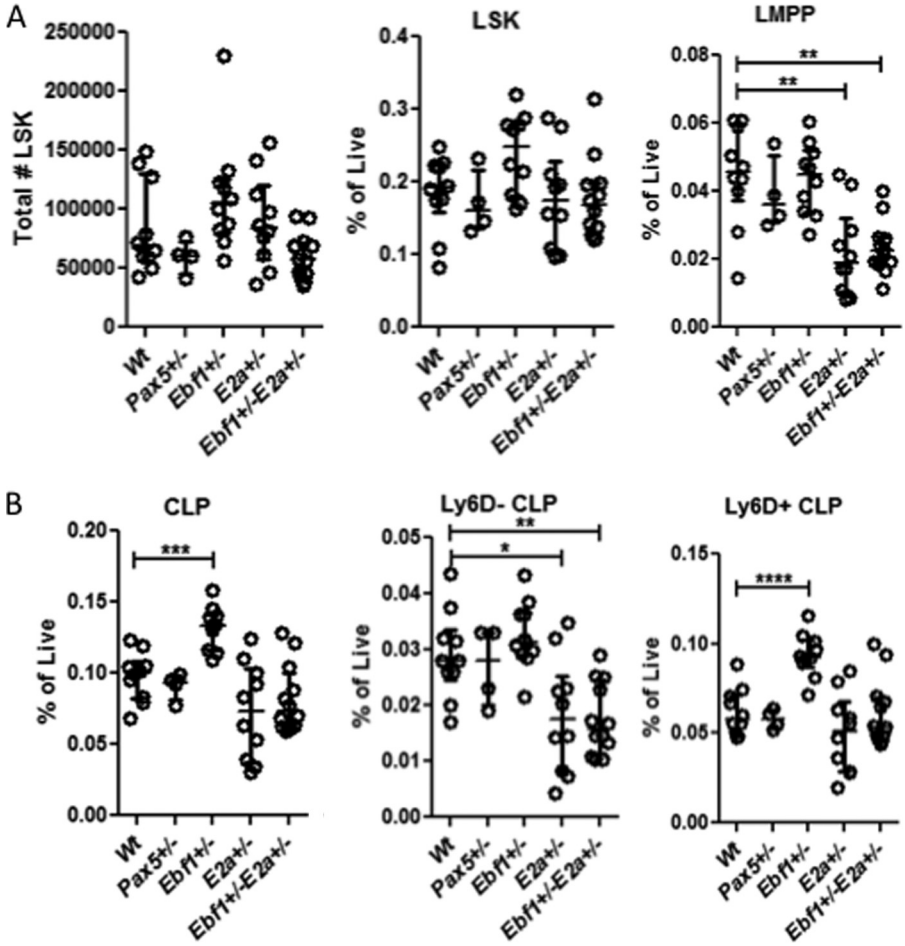

FIGURE 1. **Reduced *E2a* dose results in impaired development of early progenitors.** *A*, diagrams showing the percentages or absolute numbers of phenotypic hematopoietic progenitor cells in *Wt*, *Pax5*$^{+/-}$*Ebf1*$^{+/-}$, *E2a*$^{+/-}$, or *Ebf1*$^{+/-}$*E2a*$^{+/-}$ mice of the total number of propidium iodide negative (live) cells in mouse BM. LSK cells are defined as Lin-SCA-1^{high}KIT^{high} cells, while LMPPs are defined as FLT3^{high}LSK cells. *B*, CLPs are identified as Lin^{-}SCA-1$^{low/int}$ KIT$^{low/int}$FLT3^{+}IL7R^{+}. These cells were then further subdivided based on the expression of Ly6D on the cell surface. Each *dot* represents one mouse, and the data are presented as median (*horizontal line*) ± interquartile range. Statistical analysis was performed using the Mann-Whitney *U* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

GGAATTTG (primer 3), GAGCTGATCCAAGGGCGG (primer 4); *Cd79α/Mb1*, CCTCCTCTTCTTGTCATACG (primer 1), AAACAATGGCAGGAACCC (primer 2), TGATGATGCGGTTCTTGG (primer 3), GAACAGTCATCAAGGTTCAGG (primer 4). Primers 1 and 4 constitute the outer primer pair, whereas primers 2 and 3 constitute the inner nested primer pair.

Affymetrix Gene Expression and Data Analysis—Cells were sorted directly to RLT buffer with 2-mercaptoethanol, and RNA was extracted from purified adult BM subsets as described previously (28). RNA was labeled and amplified according to the Affymetrix^{TM} GeneChip Expression Analysis Technical Manual and hybridized against Affymetrix gene expression array chip mouse genome 430 2.0. Chips were scanned using a GeneChip^{TM} Scanner 3000 (GEO accession no. GSE51385). Probe level expression values were calculated using RMAexpress, and further analysis was done using dChip (29).

VDJ Recombination Analysis—Amplification of rearranged DNA segments was based on the use of degenerated heavy chain variable segment (VH-gene) family primers (30) and nested primers in the J-segments. The sorted cells (total number of CD43$^{low/neg}$IgM^{-} pre-B-cells from one femur) were lysed and transferred directly to the PCR, at a density of 50,000 cells/50 μl of PCR. After the primary PCR of 15 cycles using biotinylated J-primers, the products were purified on streptavidin-coated beads, and the obtained products were then subjected to a second PCR using the same heavy chain variable segment but nested J-primers at a density of 200,000 input cells/50 μl of PCR (pooled from the primary PCR). The secondary PCRs were pooled and gel-purified and used for construction of single indexed libraries for high throughput sequencing analysis, using an Illumina True Seq kit. Sequencing was performed using a MiSeq benchtop sequencer and a 500-cycle kit (Illumina, San Diego, CA).

Biotinylated Primers were as follows: J4AS1, AGACCTGGAGAGGCCATTCTTACC; J3AS1, GAATGGGAGAAGTTAGGACTCACC; J2AS1, GAATAGAAGAGAGAGGTTGTAAGG; J1AS1, CAGCTTACCTGAGGAGACGGTGACC. Inside primers were as follows: J4AS2, GAGGTTCCTTGACCCCAGTAGTCC; J3AS2, ACCTGCAGAGACAGTGACCAGAGTC; J2AS2, CACCTGAGGAGACTGTGAGAGTGG; J1AS2, AGGAGACGGTGACCGTGGTCCCTGTG. V-family primers were as follows: Vh558, TCCARCACAGCCTWCATGCARCTCARC; Vh7183, AAGAASAMCCTGTWCCTGCA-

AATGASC; VhQ52, GACTGARCATCASCAAGGACAAY-TCC. The sequences are presented 5′ to 3′. Degenerated nucleotide positions are coded as follows: R represents A or G; Y represents C or T; S represents C or G; M represents A or C; K represents G or T; W represents A or T; and B represents C, G, or T. The script used for data analysis is distributed upon request.

Generation of Tamoxifen-dependent Pro-B-cell Lines—For the generation of tamoxifen-inducible cell lines, *Ebf1*-deficient BM cells (5) were transduced with retroviruses encoding either an EBF1-estrogen receptor fusion protein (EBF1-ER) (31) or full-length EBF1. The transduced progenitors were expanded in tamoxifen (0.5 μM)-supplemented medium as CD19$^+$ cells on OP9 stroma cells.

RESULTS

E2a Dose Is Critical for Normal B-cell Specification—In order to understand the developmental disruptions observed in mice carrying heterozygote mutations in crucial B-lineage transcription factors, we analyzed the formation of early progenitors in *Pax5*$^{+/-}$, *E2a*$^{+/-}$, and *Ebf1*$^{+/-}$ as well as in *Ebf1*$^{+/-}$*E2a*$^{+/-}$ transheterozygote mice. We were unable to detect any significant impact of heterozygote loss of either *Pax5*, *E2a*, or *Ebf1* on the earliest lineage negative Lin$^-$SCA-1^{high}KIT^{high} (LSK) cells (Fig. 1*A*), whereas we confirmed an important role for *E2a* dose in the formation of the FLT3^{high}LSK, lymphoid-primed multipotent progenitor (LMPP) compartment (32–34) (Fig. 1*A*). Analysis of the Lin$^-$SCA-1$^{low/int}$KIT$^{low/int}$FLT3$^+$IL7R$^+$ lymphoid progenitor (CLP) population (35, 36), revealed that the loss of one allele of *Ebf1* resulted in a significant increase in the relative frequency of these cells (Fig. 1*B*). In order to investigate the composition of the early lymphoid restricted compartments further, we investigated the expression of Ly6D within the CLP population (8, 37). Ly6D is expressed on a subpopulation of the classical CLP that retain B-lineage potential but display reduced NK- and dendritic cell (DC)-lineage potential *in vivo* and *in vitro* as well as a reduced T-cell potential *in vivo* (8, 16). The frequency of Ly6D$^-$ cells was reduced as compared with *Wt* in mice carrying one inactivated allele of *E2a* (Fig. 1*B*). The relative frequency of Ly6D$^+$ cells was not significantly changed in *E2a*$^{+/-}$ mice; however, the loss of one allele of *Ebf1* resulted in an increase of this population (Fig. 1*B*).

In order to investigate the impact of transcription factor dose on early B-lymphoid progenitors, we analyzed the pro-B-cell (Lin$^-$B220$^+$CD19$^+$CD43^{high}IgM$^-$) compartment in mice carrying heterozygote mutations in the *Ebf1* and/or *E2a* genes (Fig. 2*A*). Although this population was comparable with *Wt* in mice lacking one allele of *Ebf1* (*Ebf1*$^{+/-}$), it was reduced in mice lacking one allele of *E2a* (*E2a*$^{+/-}$). Mice carrying combined heterozygote mutations of the *E2a* and either the *Ebf1* or *Pax5* (*Pax5*$^{+/-}$) genes did not display any reduction in cell numbers as compared with *E2a*$^{+/-}$ mice.

The reduction of pro-B-cell numbers in the *E2a*$^{+/-}$ mice suggested that reduced *E2a* dose results in a developmental disruption at an early progenitor stage, before the expression of CD19 on the cell surface. Within the Ly6D$^+$ compartment, it is possible to detect cells with an activated B-lineage transcriptional program reflected in the expression of the surrogate light chain gene *Lambda5* (*λ5*, *Igll1*) (27, 37), and in order to investigate B-lineage specification in progenitors from the mutant mice, we crossed *E2a*- and *Ebf1*-deficient mice to a reporter mouse carrying a *hCD25* reporter gene under the regulatory elements of the *λ5* promoter (25). Loss of one allele of *E2a* resulted in a 5-fold reduction of reporter-expressing CD19$^-$ progenitor cells, whereas the combined monoallelic loss of *Ebf1* and *E2a* did not enhance the phenotype as compared with *E2a*$^{+/-}$ mice (Fig. 2*B*). In order to further investigate the dependence on *E2a* dose for B-lineage specification, we performed single cell multiplex RT-PCR on CLPs from *Wt* and *E2a*$^{+/-}$ mice (Fig. 2*C*). Although we noted comparable frequencies of cells with detectable *Flt3* expression, the frequency of cells expressing *Pax5* was reduced from 24.1% in the *Wt* to 6.7% (Table 1) of the *E2a*$^{+/-}$ cells ($p < 0.0001$). Reductions were also observed in the frequency of cells expressing detectable levels of *λ5* ($p = 0.0167$), *Mb1* ($p < 0.0001$), or *Ocab* ($p < 0.0001$) in the *E2a*$^{+/-}$ as compared with *Wt* mice. These data suggest that the *E2a* dose is critical for B-lineage specification.

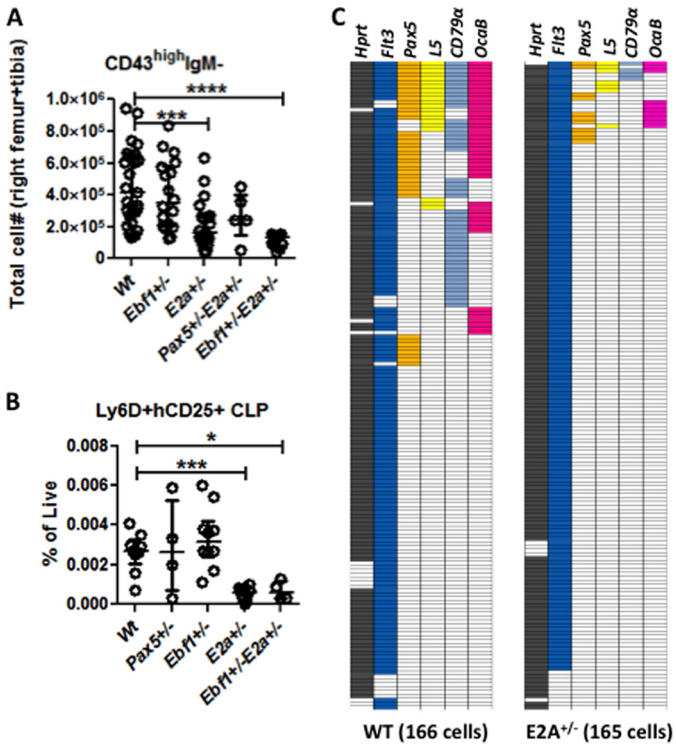

FIGURE 2. **Reduced *E2a* dose results in impaired B-lineage specification.** *A*, diagram displaying the absolute numbers of 7-AAD$^-$ pro-B-cells (Lin$^-$B220$^+$CD19$^+$CD43^{high}IgM$^-$) in BM from *Wt*, *Ebf1*$^{+/-}$, *E2a*$^{+/-}$, *Pax5*$^{+/-}$*E2a*$^{+/-}$, or *Ebf1*$^{+/-}$*E2a*$^{+/-}$ mice. *B*, diagrams showing the percentage of phenotypic Lin$^-$SCA-1$^{low/int}$KIT$^{low/int}$FLT3$^+$IL7R$^+$ CLPs expressing a λ5 promoter-regulated human CD25 (hCD25) reporter transgene in mice of the indicated genotype. Each *dot* in *A* and *B* represents one mouse. Data are presented as median (*horizontal line*) ± interquartile range. Statistical analysis was performed using the Mann-Whitney *U* test. *C*, a *color-coded panel* displaying data collected by single cell multiplex RT-PCR analysis of Lin$^-$SCA-1$^{low/int}$KIT$^{low/int}$FLT3$^+$IL7R$^+$ (CLPs) sorted from *Wt* or *E2a*$^{+/-}$ mice. Each *horizontal line* of the *boxes* represents a single investigated cell. A *colored box* indicates that an RT-PCR product from a given gene could be detected on an ethidium bromide-stained agarose gel. Statistical analysis was performed using Fisher's exact test (Table 1). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

E2a Dose Is Critical for Preserved B-lymphoid Potential in Early Lymphoid Progenitors—Because B-cell lineage restriction depends on transcription factor networks, one possible conse-

quence of reduced transcription factor dose is reduced lineage fidelity. In order to investigate the functional potential of progenitor populations, we cultured single cells under conditions driving development toward defined hematopoietic cell fates. Culturing single LMPPs (Fig. 3*A*) or Ly6D$^-$ CLPs (Fig. 3*B*) under conditions stimulating growth of myeloid cells revealed that although LMPPs from both *Wt* and transcription factor heterozygote mutant mice generated large colonies of cells, we were unable to detect any major differences in myeloid lineage restriction in Ly6D$^-$ CLPs from any of the heterozygote mutant mice (Fig. 3, *A* and *B*), suggesting that *E2a* dose is not critical for lympho/myeloid lineage restriction. The expression of Ly6D is normally associated with a reduction in the ability of lymphoid progenitors to develop into cells of NK- and DC-lineage (8, 37). In order to investigate if the Ly6D$^+$ cells generated in the presence of reduced levels of transcription factors displayed a proper lineage restriction, we incubated single Ly6D$^+$λ5(hCD25)$^-$ progenitors collected from *Wt*, *Pax5*$^{+/-}$, *Ebf1*$^{+/-}$, *E2a*$^{+/-}$, and *Ebf1*$^{+/-}$*E2a*$^{+/-}$ mice under conditions permissive for the development of B-, DC-, and NK-lineage cells from multipotent progenitors. Flow cytometric analysis of the cellular content of colonies generated from seeded *Wt*, *Pax5*$^{+/-}$, or *Ebf1*$^{+/-}$ cells revealed that 17, 12, and 23%, respectively, of the colonies contained cells expressing NK1.1 or CD11c in addition to cells expressing CD19 (Fig. 3*C*). Seeding *E2a*$^{+/-}$ or *Ebf1*$^{+/-}$*E2a*$^{+/-}$ cells resulted in 61 and 74%, respectively, of the generated colonies containing NK1.1- or CD11c-expressing cells ($p < 0.0001$ for *Wt versus E2a*$^{+/-}$ or *E2a*$^{+/-}$*Ebf1*$^{+/-}$). These cells displayed a reduced cloning frequency (Fig. 3*C*) ($p < 0.0001$ for *Wt versus E2a*$^{+/-}$ or *E2a*$^{+/-}$*Ebf1*$^{+/-}$) and impaired ability to generate colonies containing CD19$^+$ cells ($p < 0.0001$ for *Wt versus E2a*$^{+/-}$ or *E2a*$^{+/-}$*Ebf1*$^{+/-}$). These data suggest that although the *E2a* dose is critical for the generation of functional, lineage-restricted B-cell progenitors, the *Ebf1* or *Pax5* dose has a minor impact on this process.

In order to investigate functional consequences of reduced *Ebf1* or *Pax5* levels on the earliest specified B-cell progenitors, we sorted Ly6D$^+$hCD25$^+$ lymphoid progenitors and cultured

TABLE 1

Reduced *E2a* dose results in impaired B-lineage specification

The table displays data collected by single cell multiplex RT-PCR analysis of Lin$^-$SCA1$^{low/int}$KIT$^{low/int}$FLT3$^+$IL7R$^+$ (CLPs) sorted from *Wt* or *E2a*$^{+/-}$ mice. The percentage indicates the fraction of cells generating an RT-PCR product from a given gene that could be detected on an ethidium bromide-stained agarose gel. The *p* values were determined by Fisher's exact test. CI, confidence interval. NS, not significant.

mRNA	*Wt* (% of positive cells)	*E2a*$^{+/-}$ (% of positive cells)	Odds ratio	*p* value	95% CI
Hprt	91.6	96.4	0.9	NS	0.7–1.2
Flt3	92.2	91.7	1.0	NS	0.7–1.3
Ebf1	77.7	82.4	0.9	NS	0.6–1.3
Pax5	24.1	6.7	3.6	0.0001	1.7–7.2
L5	12.7	4.2	2.9	0.0167	1.2–7.2
Mb1	30.1	2.4	12.4	<0.0001	4.3–35
Ocab	27.1	6.1	4.4	<0.0001	2.1–9.1

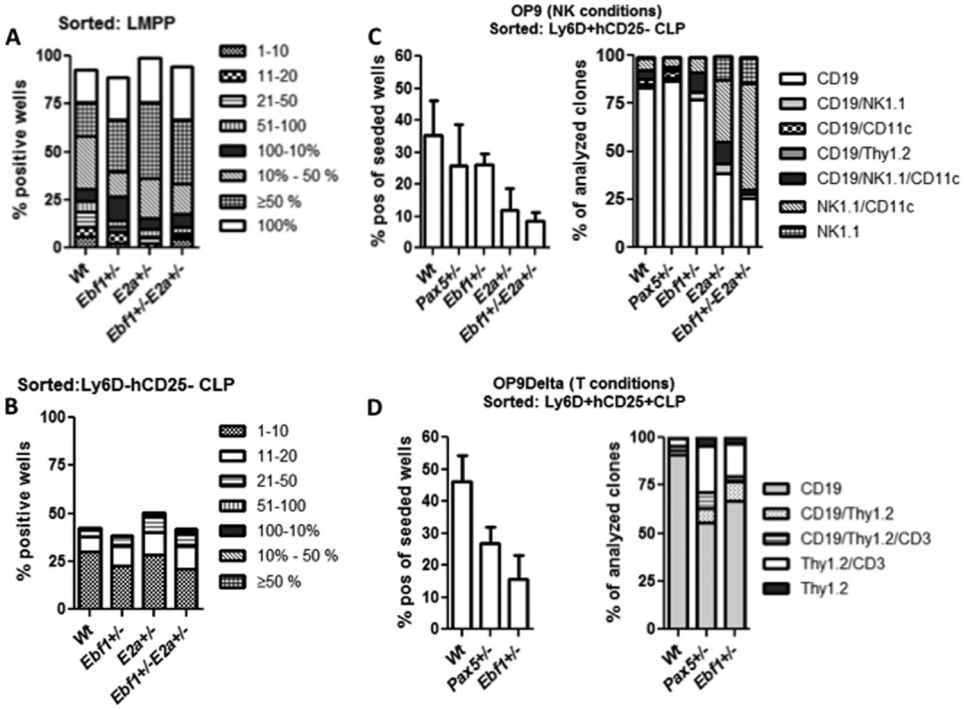

FIGURE 3. **Reduced *E2a* dose results in impaired B-lineage potential but retained lymphoid lineage restriction.** Diagrams show the cloning frequency and estimated clone size 5 days after seeding of 184 *Wt*, 187 *Ebf1*$^{+/-}$, 153 *E2a*$^{+/-}$, and 159 *Ebf1*$^{+/-}$*E2a*$^{+/-}$ single LMPPs (*A*) or 144 *Wt*, 143 *Ebf1*$^{+/-}$, 144 *E2a*$^{+/-}$, and 143 *Ebf1*$^{+/-}$*E2a*$^{+/-}$ single Ly6D$^-$ CLPs (*B*) in Terasaki plates under myeloid conditions. Cells were collected from two independent experiments. *C*, diagram of the cloning frequency and the cellular content of clones generated from single Ly6D$^+$hCD25$^-$Lin$^-$SCA-1$^{low/int}$KIT$^{low/int}$FLT3$^+$IL7R$^+$ cells after 14 days of culture under conditions permissive for B/DC/NK-cell development. The composition of the cultures was determined by flow cytometry using CD19, Cd11c, and NK1.1 as markers for B-, DC-, and NK-cells, respectively. The data are based on seeding of 576 *Wt*, 384 *Pax5*$^{+/-}$, 288 *Ebf1*$^{+/-}$, 480 *E2a*$^{+/-}$, and 480 *Ebf1*$^{+/-}$*E2a*$^{+/-}$ cells. The data were collected from four independent experiments. Statistical analysis was performed using Fisher's exact test. *D*, diagrams displaying the cloning frequency and cellular composition of single cell cultures from either 248 *Wt*, 192 *Ebf1*$^{+/-}$, or 380 *Pax5*$^{+/-}$ Lin$^-$SCA-1$^{low/int}$KIT$^{low/int}$FLT3$^+$IL7R$^+$ cells expressing a *λ5* promoter-regulated human CD25 (*hCD25*) reporter transgene incubated for 14 days on OP9Delta stroma cells to stimulate T-cell development. The data are collected from two independent experiments. Statistical analysis was performed using Fisher's exact test. *Error bars*, S.E.

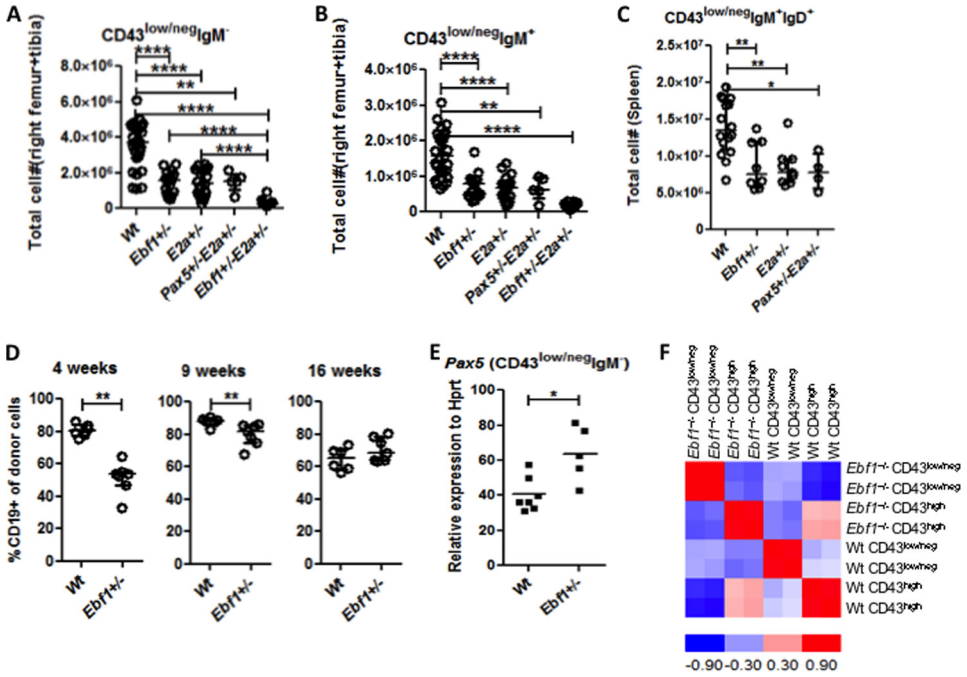

FIGURE 4. **Loss of one allele of *Ebf1* results in impaired development of B-lymphoid cells.** Diagrams display the absolute numbers of 7-AAD$^-$ pre-B-cells (Lin$^-$B220$^+$CD19$^+$CD43$^{low/neg}$IgM$^-$) (*A*) or IgM$^+$ cells (Lin$^-$B220$^+$CD19$^+$CD43$^{low/neg}$IgM$^+$) (*B*) in BM from *Wt*, *Ebf1*$^{+/-}$, *E2a*$^{+/-}$, *Pax5*$^{+/-}$*E2a*$^{+/-}$, or *Ebf1*$^{+/-}$*E2a*$^{+/-}$ mice and Lin$^-$B220$^+$CD19$^+$CD43$^{low/int}$IgM$^+$IgD$^+$ spleen cells (*C*) from *Wt*, *Pax5*$^{+/-}$, *Ebf1*$^{+/-}$, or *E2a*$^{+/-}$ mice. *D*, diagrams displaying the percentage of CD19$^+$ cells among the total number of CD45.2$^+$ cells in the peripheral blood of mice transplanted with either *Wt* or *Ebf1*$^{+/-}$ fetal liver after 4, 9, and 16 weeks, as indicated. Each *dot* in the *panel* represents one mouse, and the average values are indicated by *horizontal lines*. Statistical analysis was performed using the Mann-Whitney *U* test. *E*, diagram displaying quantitative RT-PCR data to investigate PAX5 expression in sorted pre-B (Lin$^-$B220$^+$CD19$^+$CD43$^{low/neg}$IgM$^-$) cells from *Wt* or *Ebf1*$^{+/-}$ mice. The data were normalized to the expression of *Hprt* and collected from seven individually sorted *Wt* and 5 *Ebf1*$^{+/-}$ mice analyzed in triplicate quantitative RT-PCRs. Each *dot* in the *panel* represents one mouse, and the data are presented as median (*horizontal line*) ± interquartile range. Statistical analysis was performed using a Mann-Whitney *U* test. *F*, a correlation matrix of eight samples made by dCHIP software using >1.2-fold differentially expressed genes between Wt and *Ebf1*$^{+/-}$ pre-B-cells and between Wt and *Ebf1*$^{+/-}$ pro-B-cells. The correlation is calculated and visualized by color (*red* and *blue*) in the matrix. The data were generated by hybridization of cRNA to Affymetrix™ microarrays and collected from two independently sorted populations. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

single cells on OP9Delta stroma cells under conditions permissive for T-cell development. The cloning frequency for *Wt* progenitors approached 50%, with 90% of the cells generating clones composed of only CD19$^+$ cells (Fig. 3*D*). Seeding cells from *Pax5*$^{+/-}$ or *Ebf1*$^{+/-}$ mice resulted in lower cloning frequency ($p < 0.0001$ for *Wt versus Pax5*$^{+/-}$ and *Ebf1*$^{+/-}$) and an increased frequency of colonies containing Thy1.2$^+$CD3$^+$ cells. However, considering the lower cloning efficiency of *Ebf1*$^{+/-}$ cells, the increase in frequency of colonies containing T-lineage cells in relation to the number of seeded cells only reached statistical significance using progenitors from *Pax5*$^{+/-}$ mice ($p = 0.0003$). This reveals that reduction in either the *Pax5* or *Ebf1* dose in specified B-cell progenitors results in impaired functional B-cell potential ($p < 0.0001$ for *Wt versus* either *Ebf1*$^{+/-}$ or *Pax5*$^{+/-}$), but only heterozygote loss of *Pax5* results in a significant increase in lineage plasticity *in vitro*.

Ebf1 Dose Is Critical for Normal Development of Pre-B- and Mature B-lineage Cells—Investigating the early pre-B-cell compartment (Lin$^-$B220$^+$CD19$^+$CD43$^{low/neg}$IgM$^-$), the loss of one allele of either *Ebf1* or *E2a* resulted in a 2–3-fold reduction of cell numbers as compared with *Wt* mice (Fig. 4*A*). In line with previous observations (23), the combined deletion of *Ebf1* and *E2a* resulted in a 6-fold reduction in pre-B-cell numbers, significantly lower than the reduction observed in either *Ebf1*$^{+/-}$ or *E2a*$^{+/-}$ mice. In contrast, no synergistic reduction of pre-B-cell numbers was observed in mice carrying transheterozygote mutations in the *E2a* and *Pax5* genes (Fig. 4*A*). Similar reductions in cell numbers were observed in the IgM$^+$ (Lin$^-$B220$^+$CD19$^+$CD43$^{low/neg}$IgM$^+$) (Fig. 4*B*) compartment. Heterozygote deletion of either *Ebf1* or *E2a* also resulted in a reduced fraction of CD19$^+$CD43$^{low/neg}$IgM$^+$IgD$^+$ B-cells in the spleen (Fig. 4*C*).

The impaired ability of *Ebf1*$^{+/-}$ progenitors to generate B-lineage cells was also reflected in a reduced ability to generate B-lymphoid cells after transplantation (Fig. 4*D*). During analysis of peripheral blood cell composition 4 weeks after transplantation of *Wt* or *Ebf1*$^{+/-}$ CD45.2$^+$ fetal liver cells to CD45.1$^+$ hosts, we noted a lower level of B-cell reconstitution from *Ebf1*$^{+/-}$ fetal livers than what we observed for *Wt* fetal liver cells. This effect was lost over time, and 16 weeks after transplantation, the frequency of donor (CD45.2$^+$) CD19$^+$ cells generated from *Ebf1*$^{+/-}$ fetal liver was comparable with that observed *for Wt* cells. Hence, heterozygote loss of *Ebf1* results in a reduced ability to generate mature B-lineage cells.

Loss of One Allele of Ebf1 Impairs Pre-B-cell Expansion—The enhanced phenotype upon combined reduction of *Ebf1* and *E2a* levels has been suggested to reflect a disrupted genetic program with reduced levels of PAX5 (23); however, quantitative PCR analysis of *Ebf1*$^{+/-}$ pre-B-cells did not reveal any loss of *Pax5* expression (Fig. 4*E*), arguing against a similar explanation for the reduction of B-cell progenitor populations in *Ebf1*$^{+/-}$ mice. Hence, to better understand the underlying

TABLE 2

Gene function enrichment analysis shows changes of the genes for cell division and mitosis in pre-B-cells from *Ebf1*$^{+/-}$ mice

The table displays the average relative expression values as estimated by dCHIP analysis for a set of B-lineage genes in sorted pre-B ($Lin^-B220^+CD19^+CD43^{low/neg}IgM^-$) cells from *Wt* or *Ebf1*$^{+/-}$ mice. The lower part of the table displays gene ontology analysis of microarray data from pre-B-cells from either *Wt* or *Ebf1*$^{+/-}$ mice. The data were generated by hybridization of cRNA to Affymetrix™ microarrays and collected from two independently sorted populations. The analysis was done using dCHIP based on the genes that are 1.5-fold differentially expressed between *Wt* and *Ebf1*$^{+/-}$ pre-B-cells.

mRNA	*Wt* $CD43^{low/neg}$ IgM^-	*Ebf1*$^{+/-}$ $CD43^{low/neg}IgM^-$	Change
			-fold
B-lineage genes			
Ebf1	1459	973	−1.5
Cd79b	6879	5450	−1.3
Rag2	223	100	−2.2
Vpreb3	3205	1775	−1.8
Rag1	175	106	−1.6
Pax5	131	118	−1.1
Vpreb1///Vpreb2	64	56	−1.1
CD19	2580	2397	−1.1
Blnk	2549	2301	−1.1
IgH-V J558	1407	336	−4.2
Il2ra	46	23	−2.0
Il7ra	1123	691	−1.6
Gene ontology cell division ($p = 0.000388$)			
CcnB1-rs1	56	21	−2.6
Cdc45l	60	22	−2.7
Cdc6	80	27	−3.0
Cyclin B1	149	66	−2.3
Racgap1	70	25	−2.8
Incemp	99	40	−2.5
Zc3hc1	106	55	−1.9
Kntc1	78	28	−2.8
Lig4	344	102	−3.4
Ube2c	181	68	−2.6
Rasgef1b	9	42	−4.4
Gene ontology mitosis ($p = 0.000479$)			
CcnB1-rs1	56	21	−2.6
Cdc6	80	27	−3.0
Racgap1	70	25	−2.8
Incemp	99	40	−2.5
Zc3hc1	106	55	−1.9
Kntc1	78	28	−2.8
Plk1	83	35	−2.3
Ube2c	181	68	−2.6

cause of the reduced pre-B-cell compartment in *Ebf1*$^{+/-}$ mice, we sorted pro-B- as well as pre-B-cells from *Wt* and *Ebf1* heterozygote mutant mice and performed Affymetrix-based microarray gene expression analysis. Although the overall gene expression patterns as well as *Pax5* expression in *Wt* and *Ebf1*$^{+/-}$ pro-B-cells were similar (Fig. 4*F*), gene set enrichment analysis of the microarray data suggested a reduced expression of cell division ($p < 0.001$) and mitosis ($p < 0.001$) genes in the *Ebf1*$^{+/-}$ pre-B-cells as compared with their *Wt* counterparts (Table 2). This, in combination with rather normal expression of B-lineage genes in *Ebf1*$^{+/-}$ pre-B-cells, opens up the possibility that the phenotypic loss of pre-B-cells in *Ebf1*$^{+/-}$ mice could be a result of reduced expansion of progenitors rather than a differentiation block.

The gene expression data suggested that pre-B-cells from *Ebf1*$^{+/-}$ mice displayed reduced expression of IL2 receptor α-chain (*IL2Rα*, *CD25*), a gene proposed to be expressed after functional heavy chain rearrangement on late proliferating pre-B-cells (38, 39). In line with these data, *ex vivo* analysis of the $IL2R\alpha^+CD43^{low/neg}IgM^-$ pre-B-cell population suggests that about 90% of these cells are in an active cell cycle (Fig. 5*A*). In line with the data obtained from gene expression analysis, a reduction in IL2Rα expression on cells from *Ebf1*$^{+/-}$ mice was detectable in the IgM^-pre-B-cell compartment because 46% of the *Wt* and 24% of the *Ebf1*$^{+/-}$pre-B-cells expressed this marker (Fig. 5, *B* and *C*). A significant reduction of IL2Rα expression was also observed on IgM^+ cells, where the frequency was reduced from 18% in *the Wt* to 4% in *Ebf1*$^{+/-}$ mice (Fig. 5, *B* and *C*). In order to identify the late surrogate light chain expressing pre-B-cells with an even higher accuracy, we used *Ebf1*$^{+/-}$ λ5 reporter mice to compare the presence of $IL2R\alpha^+hCD25^+IgM^-$ or IgM^+ pre-B-cell compartments in *Wt* and *Ebf1*$^{+/-}$ mice (Fig. 5*D*). The relative percentages of these cells among the progenitor B-cells were significantly reduced in both the IgM^+ and IgM^- compartments, suggesting that the *Ebf1* dose is important for normal formation of the $IL2R\alpha^+$ late pre-B-cell compartment.

It has been reported that complete loss of *Ebf1* results in increased apoptosis and a G_1 arrest in pro-B-cells (40). In order to investigate if heterozygote loss of *Ebf1* would have a similar impact on B-cell progenitors, we investigated the fraction of AnnexinV-positive cells from *Wt* or *Ebf1*$^{+/-}$ mice *ex vivo*. The percentage of $AnnexinV^+$ cells was comparable in pro-B- as well as pre-B- or IgM^+ B-lineage cells from both strains (Fig. 6*A*), suggesting that a full *Ebf1* dose is not critical for cell survival. In order to investigate if the loss of one allele of *Ebf1* was reflected in changes of cell cycle status of B-cell progenitors, we stained BM cells from *Wt* and *Ebf1*$^{+/-}$ mice for expression of surface markers as well as the intracellular proliferation marker Ki-67 and the DNA-staining dye DAPI. Approximately 50% of the *Wt* pro-B-cells resided in G_1, whereas the fraction of these cells from *Ebf1*$^{+/-}$ mice was increased (Fig. 6*B*). This was also reflected in the fact that a smaller fraction of the pro-B-cells from *Ebf1*$^{+/-}$ mice resided in G_0; however, consistent with a partial G_1 block, we were unable to detect any significant difference in the frequency of cells in $S/G_2/M$ phase. In the pre-B-cell compartment, the apparent enrichment of cells in G_1 in *Ebf1*$^{+/-}$ mice was no longer evident; rather, a trend toward a lower fraction of cells in G_1 from the *Ebf1*$^{+/-}$ mice could be detected. We also noted a significantly reduced frequency of cells in $S/G_2/M$ phase (Fig. 6*B*) in *Ebf1*$^{+/-}$ mice. Hence, although the loss of one allele of *Ebf1* does not have any major impact on cell survival, the reduced *Ebf1* dose causes alterations in cell cycle dynamics in progenitor B-cell compartments.

In order to directly investigate the impact of the functional *Ebf1* dose on cell expansion, we generated B-cell lines by transduction of *Ebf1*-deficient BM cells with retroviruses encoding either EBF1-ER (31) or full-length EBF1. The transduced progenitors were expanded in tamoxifen-supplemented medium as $CD19^+$ cells on OP9 stroma cells. Upon removal of tamoxifen, the EBF1-ER-transduced cells displayed a reduction in cell expansion, whereas this treatment had minimal impact on the growth of *Ebf1*$^{-/-}$ cells rescued by retroviral expression of conventional EBF1 (Fig. 7*A*). Hence, nuclear EBF1 is critical for normal expansion of B-cell progenitors *in vitro*. In order to investigate the functional impact of *Ebf1* dose on the expansion of these cells, we titrated the amount of tamoxifen in the cul-

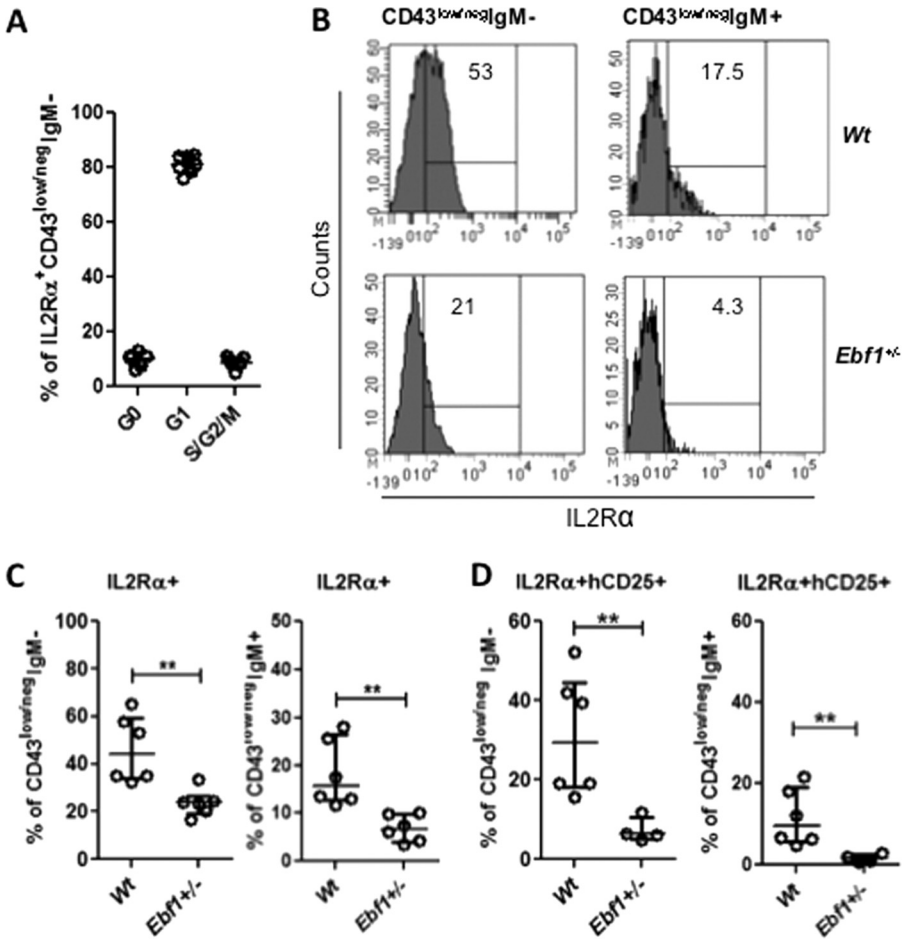

FIGURE 5. **Loss of one allele of *Ebf1* results in reductions in IL2Rα-expressing late pre-B-cells.** *A*, diagram showing the cell cycle status of IL2Rα^+ pre-B-cells ($Lin^-B220^+CD19^+CD43^{low/neg}IgM^-$) from *Wt* BM based on expression of the intracellular proliferation marker Ki-67 and the DNA-staining dye DAPI. Cells negative for Ki-67 and with DAPI staining corresponding to that of a diploid cell were classified as being in G_0. Expression of Ki-67 and a normal DNA amount identify cells in G_1, whereas high DNA content in combination with Ki-67 expression identifies cells in $S/G_2/M$ phase. *B*, representative histograms displaying expression of IL2Rα on *Wt* or $Ebf1^{+/-}$ pre-B- and IgM^+-B-cells; *C*, diagrams indicating the frequency of cells ($Lin^-B220^+CD19^+CD43^{low/neg}IgM^-$ or $Lin^-B220^+CD19^+CD43^{low/neg}IgM^+$) expressing IL2R$\alpha$ on BM cells from *Wt* or $Ebf1^{+/-}$ mice. Each *dot* in the *panels* represents one mouse, and the data are presented as median (*horizontal line*) ± interquartile range. Statistical analysis was performed using the Mann-Whitney *U* test. *D*, diagrams displaying the frequency of IL2Rα, λ5 reporter double-positive cells in the B-cell progenitor compartments in the BM of *Wt* and $Ebf1^{+/-}$. Each *dot* represents one mouse. Data are presented as median (*horizontal line*) ± interquartile range. Statistical analysis was performed using the Mann-Whitney *U* test. **, $p < 0.01$.

tures (Fig. 7*B*). This resulted in a gradual reduction of cell growth in a dose-dependent manner, providing support for the idea that the level of nuclear EBF1 directly correlates with growth of B-cell progenitors *in vitro*.

Preserved Complexity of IgH Rearrangements in Mice Lacking One Allele of Ebf1—These data suggest that the loss of one allele of *Ebf1* results in a reduced expansion of the late pre-B-cell compartment. This could be presumed to result in that the complexity of VDJ rearrangements should be comparable, although the absolute numbers of pre-B-cells are reduced in $Ebf1^{+/-}$ mice. In order to investigate the complexity of the immunoglobulin VDJ rearrangement pattern in *Wt* and $Ebf1^{+/-}$ mice, we sorted pre-B-cells from *Wt* and $Ebf1^{+/-}$ mice, amplified VDJ rearrangements by PCR, and performed deep sequencing of the generated amplicons. In order to investigate the complexity, we developed a script that allowed a comparison of the generated sequences, thereby allowing us to calculate the number of unique VDJ junctions in each sequencing run. The identification of the correctly amplified products was achieved by using only sequences containing a part of the J-region (antisense strand) not included in the primers in the mathematical analysis. The diversity was investigated in the region 30 base pairs upstream of the J-region to identify unique VDJ junctions. By dividing the number of unique sequences by the total number of VDJ sequences in a given run, we obtain a number reflecting the complexity of VDJ rearrangements in a given sample (Table 3). Analyzing six sequencing runs from three libraries of *Wt* pre-B-cells, the median number of unique sequences in relation to the total number of VDJ sequences was 0.120. The corresponding number in cells from $Ebf1^{+/-}$ mice was 0.084, a difference of no statistical significance. Hence, we were unable to detect any major difference in VDJ complexity of the pre-B-cell compartments in *Wt* and $Ebf1^{+/-}$ mice, providing an independent line of support for the theory that heterozygote loss of *Ebf1* results in impaired expansion of the pre-B-cell compartment.

Reduced Ebf1 Dose Results in Impaired IL7 Response and Reduced Surface Expression of Pre-BCR on B-cell Progenitors—It has been reported that $Ebf1^{-/-}$ pro-B-cells respond poorly to IL7 (40), and to investigate how heterozygote loss of *Ebf1*

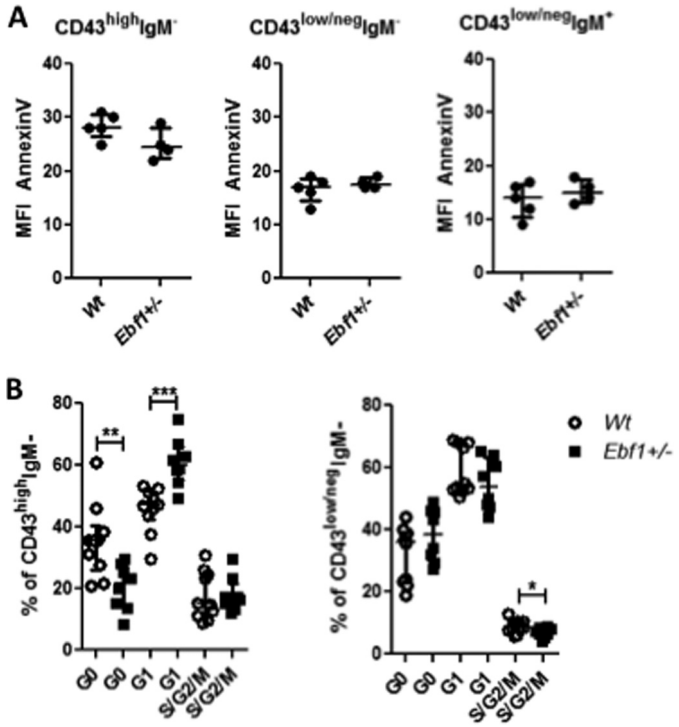

FIGURE 6. **Heterozygote loss of *Ebf1* results in disruption of cell cycle dynamics in early progenitor cells.** *A*, diagrams indicating the median fluorescence intensity of AnnexinV on Pro-B ($Lin^-B220^+CD19^+CD43^{high}IgM^-$), Pre-B ($Lin^-B220^+CD19^+CD43^{low/neg}IgM^-$), or IgM^+ ($Lin^-B220^+CD19^+CD43^{low/neg}IgM^+$) BM cells from *Wt* or $Ebf1^{+/-}$ mice. *B*, cell cycle analysis of pro-B ($Lin^-B220^+CD19^+CD43^{high}IgM^-$) and pre-B ($Lin^-B220^+CD19^+CD43^{low/neg}IgM^-$) cells from *Wt* and $Ebf1^{+/-}$ BM based on expression of the intracellular proliferation marker Ki-67 and the DNA-staining dye DAPI. Cells negative for Ki-67 and with DAPI staining corresponding to that of a diploid cell were classified as being in G_0. Expression of Ki-67 and a normal DNA amount identify cells in G_1, whereas high DNA content in combination with Ki-67 expression identifies cells in S/G_2/M phase. Each *dot* in the *panels* represents one mouse. Data are presented as median (*horizontal line*) ± interquartile range. Statistical analysis was performed using the Mann-Whitney *U* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

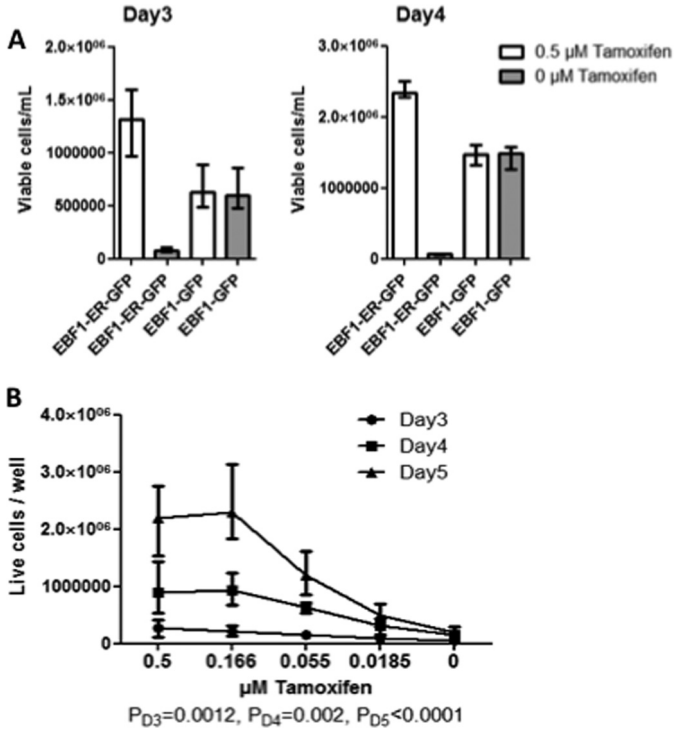

FIGURE 7. **The dose of nuclear EBF1 regulates the expansion of B-cell progenitors.** *A*, diagrams displaying the number of live cells after 3–4 days of culturing 100,000 B-cell progenitors generated from $Ebf1^{-/-}$ BM cells expressing either an EBF1-ER fusion protein or a full-length EBF1 protein in the presence or absence of 0.5 μM tamoxifen. The data are presented as medians ± interquartile range of three wells from two independent BM transductions (total of six wells). *B*, diagrams displaying the number of live cells after 3–5 days of culturing 5000 B-cell progenitors generated from $Ebf1^{-/-}$ BM cells expressing an EBF1-ER fusion protein in decreasing concentrations of tamoxifen. The data are collected from three wells from two independent BM transductions (total of six wells). Statistical analysis was performed using a Kruskal-Wallis test. *Error bars*, S.E.

impacts the response to IL7, we incubated *Wt* and $Ebf1^{+/-}$ B-cell progenitors on OP9 stroma cells in decreasing amounts of externally supplied IL7 (Fig. 8). In the presence of high levels of IL7, we noted an ~2-fold reduction in the number of viable cells after 6 days of cultivation. The effect of heterozygote *Ebf1* mutation was more pronounced when the level of IL7 was reduced, suggesting that $Ebf1^{+/-}$ B-cell progenitors display a reduced response to IL7.

Although IL7 is an important regulator of the expansion of the progenitor B-cell compartment, expansion of cells undergoing positive selection for a functional Ig-heavy chain rearrangement has been suggested to involve a combined action of pre-BCR and IL7 signaling (41). One of the critical components of the pre-BCR is λ5 (Igll1), reported to be a direct target for activation by EBF1 (42). In order to investigate if reduced levels of *Ebf1* would result in reduced transcription of the critical pre-BCR component λ5, we analyzed the expression of the λ5 promoter-controlled reporter gene (*hCD25*) in B-cell progenitors from *Wt* and $Ebf1^{+/-}$ mice (Fig. 9, *A* and *B*). Although the expression of the reporter construct was comparable on the $CD43^{high}$ progenitor cells, the expression in the $CD43^-$ pre-B and IgM^+ cell compartment was significantly reduced. This is in line with the finding that loss of one allele of *Ebf1* results in reduced expression of the surrogate light chain component λ5. The reduction in λ5 expression could potentially result in reduced pre-BCR expression levels on late pre-B-cells. To explore this possibility, we analyzed the frequency of pre-BCR^+ cells as well as the medium expression of pre-BCR on these cells using an antibody recognizing a pre-BCR-specific epitope on the IgM molecule. This suggested that both the frequency of positive cells and the pre-BCR density on the positive cells are reduced in $Ebf1^{+/-}$ mice (Fig. 9*C*). A similar result was observed when we analyzed IgM density on pre-BCR^+ cells because we noted a significant shift from IgM^{high} to IgM^{low} cells in $Ebf1^{+/-}$ mice (Fig. 9*D*).

DISCUSSION

Transcription factor dose is shown to be important for the regulation of cell differentiation in a variety of model systems, one of the most striking being sex determination in *Drosophila*, where the dose of X-encoded transcription factors establishes a regulatory network that acts to determine the sex of the embryo (43). Dose-dependent regulation of cell fate has also been reported in the hematopoietic system, where high expression of *PU.1* in multipotent progenitors results in the development of myeloid and, at lower levels, in the development of lymphoid lineage cells (44). Regulation of the functional levels of PU.1 is

TABLE 3

Heterozygote deletion of *Ebf1* does not result in significantly decreased complexity in VDJ rearrangements in pre-B-cells

The table displays data from sequencing analysis of VDJ region complexity in the bone marrow compartment of pre-B-cells ($Lin^-B220^-CD19^+CD43^{low/neg}IgM^-$) from *Wt* and *Ebf1*$^{+/-}$ BM. The data indicate the number of reads containing a genomic J segment sequence and the number of unique sequences as judged by sequence analysis of 30 bp downstream of the genomic J segment. The data were collected from four mice (2 *Wt* and 2 *Ebf1*$^{+/-}$), where the cells from each femur were sorted separately from each other. Cells from two bones from one *Wt* and one *Ebf1*$^{+/-}$ mouse and one bone from the second mouse of each genotype were used for the generation of three libraries from each phenotype. The libraries were analyzed in the indicated number of sequence runs. Statistical analysis was performed using the Mann-Whitney *U* test.

Wt $CD43^{low/neg}$	Unique Reads/ Total Reads (Ratio)	*Ebf1*$^{+/-}$ $CD43^{low/neg}$	Unique Reads/ Total Reads (Ratio)
Sample1 (Run1)	91769/465792 (0.20)	Sample4 (Run1)	76207/431128 (0.18)
Sample2 (Run1)	66471/315992 (0.21)	Sample5 (Run1)	77932/533029 (0.15)
Sample2 (Run2)	55341/632546 (0.087)	Sample5 (Run2)	66145/1354910 (0.049)
Sample2 (Run3)	86661/1060846 (0.081)	Sample5 (Run3)	109258/2127856 (0.051)
Sample3 (Run1)	105223/1003876 (0.10)	Sample6 (Run1)	42665/625899 (0.068)
Sample3 (Run2)	288112/2191349 (0.13)	Sample6 (Run2)	144534/1406333 (0.10)
	Median Ratio: 0.12		Median Ratio: 0.084

Compared cell types	Pvalue
Wt $CD43^{low/neg}$ vs *Ebf1*$^{+/-}$ $CD43^{low/neg}$	0.26

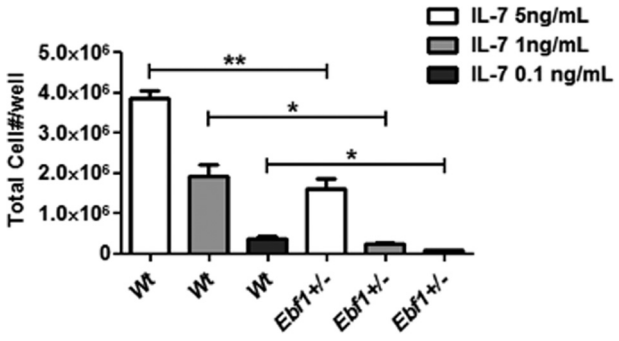

FIGURE 8. **Loss of one allele of *Ebf1* results in reduced IL7 response.** Shown is a diagram displaying the number of live cells after 6 days of culturing 40,000 *Wt* or *Ebf1*$^{+/-}$ B-cell progenitors. Statistical analysis was performed using Student's *t* test. *Error bars*, S.E. *, $p < 0.05$; **, $p < 0.01$.

mediated by the transcriptional repressor GFI-1, which replaces PU.1 on autoregulatory elements in the *PU.1* gene (45). *Gfi-1* expression is regulated by the zinc finger transcription factor IKAROS (Ikzf1) (45), known to be important for lymphoid priming in early BM progenitors (46), suggesting that a balanced network of transcription factors is of crucial importance for development of the earliest lymphocyte progenitors. In the B-lineage, the enhanced phenotype observed upon combined heterozygote loss of *E2a* and *Ebf1* (23) and the large number of shared target genes (2, 3) has led to the conclusion that they act synergistically to stimulate B-cell development. This could be a result of direct coordinated target gene activation; however, because E2A proteins appear to regulate the expression of EBF1 (2, 47, 48), the lack of lineage priming could be a result of reduced EBF1 levels in the CLP. Furthermore, E-proteins regulate FOXO1 expression in the CLP compartment (7). A lack of FOXO1 results in defective lineage priming in a manner similar to what is observed in EBF1-deficient progenitors (9). The ability of FOXO1 to bind potential regulatory elements in the *Ebf1* gene and binding of EBF1 to regions in the *FoxO1* gene suggest that these proteins act in a regulatory loop crucial for the specification of early B-cell progenitors (9). Hence, reduced levels of E2A proteins are likely to result in a disruption of this feedback loop, causing reduced expression of both EBF1 and FOXO1, leading to impaired B-lineage specification. This would be in line with the fact that *E2a*$^{+/-}$ mice display impairment in B-lineage priming already in $CD19^-$ progenitors. However, because we were unable to detect any synergistic effect of combined deletion of EBF1 and E2A on the formation of λ5-expressing progenitors or pro-B-cells (Fig. 2, *A* and *B*), we find it reasonable to propose that the synergistic effect is caused by a reduced number of primed cells in combination with reduced expansion capacity of pre-B-cells as a result of reduced EBF1 levels. Our analysis reveals that the loss of one allele of *Ebf1* results in a reduction of $CD19^+IL2R\alpha^+$ B-cell progenitors (Fig. 5), and although the link between IL2Rα and cell proliferation recently was disputed because c-*myc*-expression was not evident in $B220^+CD43^+IL2R\alpha^+$ cells (41), *ex vivo* analysis of the $CD43^{low/neg}IL2R\alpha^+$ pre-B-cell population revealed that about 90% of these cells are in the active cell cycle (Fig. 5*A*). Mutations in *λ5*, *IgM*, or *Rag-1* result in a similar phenotype with a reduction in or even loss of $IL2R\alpha^+$ B-cell progenitors (39), suggesting that reduced levels of EBF1 may result in impaired pre-BCR signaling. Although we detect modest down-regulations of mRNAs encoding pre-BCR components in *Ebf1*$^{+/-}$ pre-B-cells, slight reductions in the expression of several genes in combination may combine to reduce the number of completely assembled pre-BCRs, resulting in an impaired expansion of the pre-B-cell compartment. It is also interesting to note that the response to IL7 appears to be reduced in *Ebf1*$^{+/-}$ B-cell progenitors because IL7 signaling has been reported to act in synergy with pre-BCR signaling to regulate pre-B-cell expansion. Another possible explanation could be that EBF1 itself is directly involved in the regulation of genes controlling cell proliferation. This idea is supported by the finding that the major category of EBF1 target genes identified by chromatin immunoprecipitation was genes encoding proteins with crucial functions in the regulation of basic biological processes in pre-B-cells (2). Hence, the role of stage specific tran-

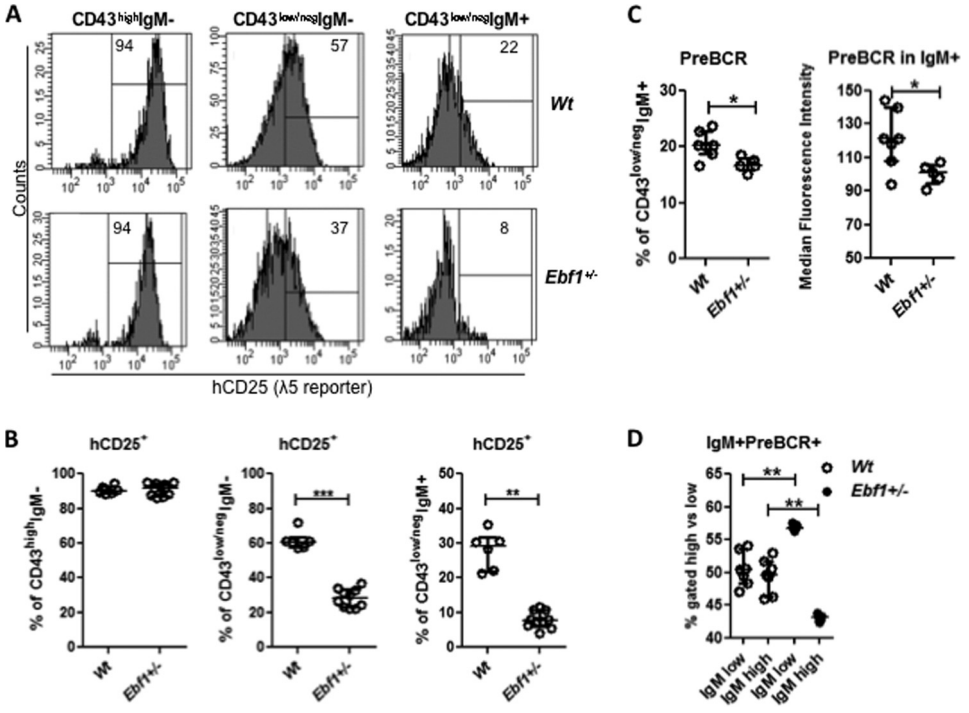

FIGURE 9. **Heterozygote deletion of *Ebf1* results in a reduction of pre-BCR expression on B-cell progenitors.** Representative histograms (*A*) and diagrams (*B*) display expression of a λ5 promoter-regulated human CD25 (*hCD25*) reporter transgene on B-lineage populations from *Wt* or *Ebf1*$^{+/-}$ mice. *C*, frequency of pre-BCR$^+$ cells and median pre-BCR expression levels on (Lin$^-$B220$^+$CD19$^+$CD43$^{low/neg}$IgM$^+$) cells; *D*, expression levels of IgM on pre-BCR$^+$ cells from *Wt* and *Ebf1*$^{+/-}$ BM. In the diagrams, each *dot* represents one mouse, and data are presented as median ± interquartile range. Statistical analysis was performed using the Mann-Whitney *U* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

scription factors may reach beyond that of regulation of a defined set of lineage-restricted target genes.

A role for *Ebf1* dose in the expansion of B-cell progenitors is also supported by the findings that *Ebf1*$^{+/-}$ mice display an accumulation of pro-B-cells in G_1, consistent with the idea that deletion of the *Ebf1* gene in B-cell progenitors results in a G_1 block (40). In the more mature CD43$^{low/neg}$ pre-B-cells, the relative fraction of cells in G_1 is rather reduced, and this, in combination with a reduced number of cells in S/G_2, supports the idea that the loss of one allele of *Ebf1* results in impaired expansion of these progenitor cells. It has been reported that ectopic expression of active STAT5 is insufficient to rescue the disturbed proliferation and cell survival in *Ebf1*$^{+/-}$ pro-B-cells; however, the finding that transgenic expression of constitutively active STAT5 collaborates with heterozygote loss of *Ebf1* in the generation of B-cell leukemia in mouse models (18) indicates that reduced *Ebf1* dose impacts cell proliferation and survival differently than a complete loss of *Ebf1* function. This is also consistent with the finding that heterozygote loss of *Ebf1* did not result in increased apoptosis, as previously reported for *Ebf1*-deficient progenitors (40). Hence, the function of reduced *Ebf1* dose in malignant transformation may be complex and possibly a result of mechanisms other than impaired differentiation. Also interesting with regard to leukemia development is the finding that reduced transcription factor dose may result in lineage plasticity in B-cell progenitors. Although our functional analysis did not reveal any dramatic increase in lineage instability of B-lineage cells, it has been reported that monoallelic loss of *Ebf1* leads to aberrant expression of NK-lineage genes in B-lymphoid progenitors (22). This indicates that alternative lineage programs cannot be properly silenced, and it should not be excluded that combined heterozygote deletions of *Pax5* and *Ebf1* may impact lineage commitment in a more dramatic manner than the heterozygote loss of either of these proteins alone. Hence, it appears as if reduced transcription factor dose may impact specification and expansion as well as lineage stability during B-cell development. Our data reveal that the impact of the transcription factor dose goes beyond a partial differentiation block, providing an extended insight into both normal and malignant blood cell development.

Acknowledgments—We thank our colleagues for help with transgenic mice and cell lines, and we also thank Liselotte Lenner and Linda Bergström for advice and assistance.

REFERENCES

1. Welinder, E., Ahsberg, J., and Sigvardsson, M. (2011) B-lymphocyte commitment. Identifying the point of no return. *Semin. Immunol.* **23,** 335–340
2. Lin, Y. C., Jhunjhunwala, S., Benner, C., Heinz, S., Welinder, E., Mansson, R., Sigvardsson, M., Hagman, J., Espinoza, C. A., Dutkowski, J., Ideker, T., Glass, C. K., and Murre, C. (2010) A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat. Immunol.* **11,** 635–643
3. Treiber, T., Mandel, E. M., Pott, S., Györy, I., Firner, S., Liu, E. T., and Grosschedl, R. (2010) Early B cell factor 1 regulates B cell gene networks by activation, repression, and transcription-independent poising of chromatin. *Immunity* **32,** 714–725
4. Revilla-I-Domingo, R., Bilic, I., Vilagos, B., Tagoh, H., Ebert, A., Tamir, I. M., Smeenk, L., Trupke, J., Sommer, A., Jaritz, M., and Busslinger, M. (2012) The B-cell identity factor Pax5 regulates distinct transcriptional programmes in early and late B lymphopoiesis. *EMBO J.* **31,** 3130–3146
5. Lin, H., and Grosschedl, R. (1995) Failure of B-cell differentiation in mice

lacking the transcription factor EBF. *Nature* **376,** 263–267
6. Zandi, S., Mansson, R., Tsapogas, P., Zetterblad, J., Bryder, D., and Sigvardsson, M. (2008) EBF1 is essential for B-lineage priming and establishment of a transcription factor network in common lymphoid progenitors. *J. Immunol.* **181,** 3364–3372
7. Welinder, E., Mansson, R., Mercer, E. M., Bryder, D., Sigvardsson, M., and Murre, C. (2011) The transcription factors E2A and HEB act in concert to induce the expression of FOXO1 in the common lymphoid progenitor. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 17402–17407
8. Inlay, M. A., Bhattacharya, D., Sahoo, D., Serwold, T., Seita, J., Karsunky, H., Plevritis, S. K., Dill, D. L., and Weissman, I. L. (2009) Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. *Genes Dev.* **23,** 2376–2381
9. Mansson, R., Welinder, E., Åhsberg, J., Lin, Y. C., Benner, C., Glass, C. K., Lucas, J. S., Sigvardsson, M., and Murre, C. (2012) Positive intergenic feedback circuitry, involving EBF1 and FOXO1, orchestrates B-cell fate. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 21028–21033
10. Tsapogas, P., Zandi, S., Åhsberg, J., Zetterblad, J., Welinder, E., Jönsson, J. I., Månsson, R., Qian, H., and Sigvardsson, M. (2011) IL-7 mediates Ebf-1-dependent lineage restriction in early lymphoid progenitors. *Blood* **118,** 1283–1290
11. Xie, H., Ye, M., Feng, R., and Graf, T. (2004) Stepwise reprogramming of B cells into macrophages. *Cell* **117,** 663–676
12. Cobaleda, C., Jochum, W., and Busslinger, M. (2007) Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* **449,** 473–477
13. Nutt, S. L., Heavey, B., Rolink, A. G., and Busslinger, M. (1999) Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* **401,** 556–562
14. Urbánek, P., Wang, Z. Q., Fetka, I., Wagner, E. F., and Busslinger, M. (1994) Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* **79,** 901–912
15. Nutt, S. L., Urbánek, P., Rolink, A., and Busslinger, M. (1997) Essential functions of Pax5 (BSAP) in pro-B cell development. Difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev.* **11,** 476–491
16. Zandi, S., Ahsberg, J., Tsapogas, P., Stjernberg, J., Qian, H., and Sigvardsson, M. (2012) Single-cell analysis of early B-lymphocyte development suggests independent regulation of lineage specification and commitment *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 15871–15876
17. Mullighan, C. G., Goorha, S., Radtke, I., Miller, C. B., Coustan-Smith, E., Dalton, J. D., Girtman, K., Mathew, S., Ma, J., Pounds, S. B., Su, X., Pui, C. H., Relling, M. V., Evans, W. E., Shurtleff, S. A., and Downing, J. R. (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* **446,** 758–764
18. Heltemes-Harris, L. M., Willette, M. J., Ramsey, L. B., Qiu, Y. H., Neeley, E. S., Zhang, N., Thomas, D. A., Koeuth, T., Baechler, E. C., Kornblau, S. M., and Farrar, M. A. (2011) Ebf1 or Pax5 haploinsufficiency synergizes with STAT5 activation to initiate acute lymphoblastic leukemia. *J. Exp. Med.* **208,** 1135–1149
19. Simmons, S., Knoll, M., Drewell, C., Wolf, I., Mollenkopf, H. J., Bouquet, C., and Melchers, F. (2012) Biphenotypic B-lymphoid/myeloid cells expressing low levels of Pax5. Potential targets of BAL development. *Blood* **120,** 3688–3698
20. Zhuang, Y., Cheng, P., and Weintraub, H. (1996) B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, *E2A*, *E2-2* and *HEB. Mol. Cell Biol.* **16,** 2898–2905
21. Lukin, K., Fields, S., Lopez, D., Cherrier, M., Ternyak, K., Ramírez, J., Feeney, A. J., and Hagman, J. (2010) Compound haploinsufficiencies of Ebf1 and Runx1 genes impede B cell lineage progression. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 7869–7874
22. Lukin, K., Fields, S., Guerrettaz, L., Straign, D., Rodriguez, V., Zandi, S., Månsson, R., Cambier, J. C., Sigvardsson, M., and Hagman, J. (2011) A dose-dependent role for EBF1 in repressing non-B cell specific genes. *Eur. J. Immunol.* **41,** 1787–1793
23. O'Riordan, M., and Grosschedl, R. (1999) Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity* **11,** 21–31
24. Zhuang, Y., Soriano, P., and Weintraub, H. (1994) The helix-loop-helix gene E2A is required for B cell formation. *Cell* **79,** 875–884
25. Mårtensson, I. L., Melchers, F., and Winkler, T. H. (1997) A transgenic marker for mouse B lymphoid precursors. *J. Exp. Med.* **185,** 653–661
26. Schmitt, T. M., and Zúñiga-Pflücker, J. C. (2002) Induction of T cell development from hematopoietic progenitor cells by δ-like-1 *in vitro*. *Immunity* **17,** 749–756
27. Mansson, R., Zandi, S., Anderson, K., Martensson, I. L., Jacobsen, S. E., Bryder, D., and Sigvardsson, M. (2008) B-lineage commitment prior to surface expression of B220 and CD19 on hematopoietic progenitor cells. *Blood* **112,** 1048–1055
28. Åhsberg, J., Tsapogas, P., Qian, H., Zetterblad, J., Zandi, S., Månsson, R., Jönsson, J. I., and Sigvardsson, M. (2010) Interleukin-7 induced Stat-5 acts in synergy with Flt-3 signaling to stimulate expansion of hematopoietic progenitor cells. *J. Biol. Chem.* **285,** 36275–36284
29. Li, C., and Wong, W. (2001) Model-based analysis of oligonucleotide arrays. Expression index computation and outlier detection. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 31–36
30. Schlissel, M. S., Corcoran, L. M., and Baltimore, D. (1991) Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. *J. Exp. Med.* **173,** 711–720
31. Gao, H., Lukin, K., Ramírez, J., Fields, S., Lopez, D., and Hagman, J. (2009) Opposing effects of SWI/SNF and Mi-2/NuRD chromatin remodeling complexes on epigenetic reprogramming by EBF and Pax5. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 11258–11263
32. Dias, S., Månsson, R., Gurbuxani, S., Sigvardsson, M., and Kee, B. L. (2008) E2A proteins promote development of lymphoid-primed multipotent progenitors. *Immunity* **29,** 217–227
33. Semerad, C. L., Mercer, E. M., Inlay, M. A., Weissman, I. L., and Murre, C. (2009) E2A proteins maintain the hematopoietic stem cell pool and promote the maturation of myelolymphoid and myeloerythroid progenitors. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 1930–1935
34. Yang, Q., Kardava, L., St Leger, A., Martincic, K., Varnum-Finney, B., Bernstein, I. D., Milcarek, C., and Borghesi, L. (2008) E47 controls the developmental integrity and cell cycle quiescence of multipotential hematopoietic progenitors. *J. Immunol.* **181,** 5885–5894
35. Kondo, M., Weissman, I. L., and Akashi, K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91,** 661–672
36. Karsunky, H., Inlay, M. A., Serwold, T., Bhattacharya, D., and Weissman, I. L. (2008) Flk2+ common lymphoid progenitors possess equivalent differentiation potential for the B and T lineages. *Blood* **111,** 5562–5570
37. Mansson, R., Zandi, S., Welinder, E., Tsapogas, P., Sakaguchi, N., Bryder, D., and Sigvardsson, M. (2010) Single-cell analysis of the common lymphoid progenitor compartment reveals functional and molecular heterogeneity. *Blood* **115,** 2601–2609
38. Chen, J., Ma, A., Young, F., and Alt, F. W. (1994) IL-2 receptor α chain expression during early B lymphocyte differentiation. *Int. Immunol.* **6,** 1265–1268
39. Rolink, A., Grawunder, U., Winkler, T. H., Karasuyama, H., and Melchers, F. (1994) IL-2 receptor α chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. *Int. Immunol.* **6,** 1257–1264
40. Györy, I., Boller, S., Nechanitzky, R., Mandel, E., Pott, S., Liu, E., and Grosschedl, R. (2012) Transcription factor Ebf1 regulates differentiation stage-specific signaling, proliferation, and survival of B cells. *Genes Dev.* **26,** 668–682
41. Sandoval, G. J., Graham, D. B., Bhattacharya, D., Sleckman, B. P., Xavier, R. J., and Swat, W. (2013) Cutting edge. Cell-autonomous control of IL-7 response revealed in a novel stage of precursor B cells. *J. Immunol.* **190,** 2485–2489
42. Sigvardsson, M., O'Riordan, M., and Grosschedl, R. (1997) EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. *Immunity* **7,** 25–36
43. Cline, T. W., and Meyer, B. J. (1996) Vive la difference. Males *vs* females in flies *vs* worms. *Annu. Rev. Genet.* **30,** 637–702
44. DeKoter, R. P., and Singh, H. (2000) Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* **288,** 1439–1441

45. Spooner, C. J., Cheng, J. X., Pujadas, E., Laslo, P., and Singh, H. (2009) A recurrent network involving the transcription factors PU.1 and Gfi1 orchestrates innate and adaptive immune cell fates. *Immunity* **31,** 576–586
46. Yoshida, T., Ng, S. Y., Zuniga-Pflucker, J. C., and Georgopoulos, K. (2006) Early hematopoietic lineage restrictions directed by Ikaros. *Nat. Immunol.* **7,** 382–391
47. Kee, B. L., and Murre, C. (1998) Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J. Exp. Med.* **188,** 699–713
48. Smith, E. M., Gisler, R., and Sigvardsson, M. (2002) Cloning and characterization of a promoter flanking the early B cell factor (EBF) gene indicates roles for E-proteins and autoregulation in the control of EBF expression. *J. Immunol.* **169,** 261–270