Runx1–Cbfβ facilitates early B lymphocyte development by regulating expression of Ebf1

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Although Runx and Cbfβ transcription factor complexes are involved in the development of multiple hematopoietic lineages, their precise roles in early mouse B lymphocyte differentiation remain elusive. In this study, we examined mouse strains in which *Runx1*, *Runx3*, or *Cbfβ* were deleted in early B lineage progenitors by an *mb1-cre* transgene. Loss of Runx1, but not Runx3, caused a developmental block during early B lymphopoiesis, resulting in the lack of IgM$^+$ B cells and reduced V_H to DJ_H recombination. Expression of core transcription factors regulating early B cell development, such as E2A, Ebf1, and Pax5, was reduced in B cell precursors lacking Runx1. We detected binding of Runx1–Cbfβ complexes to the *Ebf1* proximal promoter, and these Runx-binding motifs were essential to drive reporter gene expression. Runx1-deficient pro-B cells harbored excessive amounts of the repressive histone mark H3K27 trimethylation in the *Ebf1* proximal promoter. Interestingly, retroviral transduction of Ebf1, but not Pax5, into Runx1-deficient progenitors restored not only development of B220$^+$ cells that underwent V_H to DJ_H rearrangement but also expression of B lineage signature genes. Collectively, these results demonstrate that Runx1–Cbfβ complexes are essential to facilitate B lineage specification, in part via epigenetic activation of the *Ebf1* gene.

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Abbreviations used: ChIP, chromatin immunoprecipitation; CLP, common lymphoid progenitor; RRS, Runx recognition site.

During hematopoiesis, proper specification and lineage commitment are controlled by a complex and dynamic network of transcriptional regulators. For example, the lymphoid lineage is derived from lymphoid-primed multipotent progenitors, which give rise to common lymphoid progenitors (CLPs) in part through the activity of PU.1, Ikaros, and Bcl11a transcription factors (Georgopoulos et al., 1994; Scott et al., 1994; Liu et al., 2003). After lymphoid priming, three transcription factors, E2A, Ebf1, and Pax5, cooperatively program B lymphocyte development in bone marrow (Busslinger, 2004; Mandel and Grosschedl, 2010). Progress in bioinformatics has recently led to the discovery that many B cell–specific genes contain overlapped binding sites for E2A, Ebf1, and Pax5 (Lin et al., 2010; Treiber et al., 2010), further supporting the idea that these three factors work in concert to guide B cell development. Detailed genetic analyses in mice have shown that loss of either E2A or Ebf1 causes an arrest in B cell development at the pre-pro-B cell stage without D_H to J_H rearrangement at the *Igh* (*Ig heavy chain*) locus (Bain et al., 1994; Lin and Grosschedl, 1995), whereas Pax5 deficiency inhibits B cell development at the pro-B cell stage with impaired V_H to DJ_H rearrangement (Nutt et al., 1997). Thus, Pax5 is thought to act late as a commitment factor to seal B cell identity after B lymphoid lineage specification is orchestrated by E2A and Ebf1.

There are several lines of evidence indicating that E2A and Ebf1 are each likely to contribute to B lineage specification in a different manner. For instance, E2A expression is not limited to the B lymphocyte lineage, and its known target genes such as *Rag* and *Il7r* are required for both B and T lymphopoiesis, indicating that E2A functions upstream of Ebf1 at the CLP stage. Indeed, it has recently been shown that E2A activates Foxo1 to support B cell programming (Welinder et al., 2011). In contrast, Ebf1, whose expression is mostly restricted to B lineage cells,

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regulates expressions of many genes only required for B cell development, including *mb1*, *VpreB*, *λ5*, and *Pax5* (Mandel and Grosschedl, 2010). Furthermore, overexpression of Ebf1 can rescue various degrees of the block in B cell development caused by loss of several transcription factors such as Ikaros and E2A (Seet et al., 2004; Reynaud et al., 2008). Therefore, it is conceivable that E2A and Ebf1 serve as competence and specification factors, respectively. Thus, Ebf1 plays a central role as a B lineage–specific transcription factor in initiating the developmental program leading toward B lymphopoiesis; therefore, it is important to understand how expression of the *Ebf1* gene is initiated. Although the mouse *Ebf1* gene was shown to be transcribed from two promoters, a distal α promoter and a proximal β promoter, the dominant activity of the proximal β promoter at most stages of B cell differentiation highlights its importance in specification to the B lineage (Roessler et al., 2007).

The mammalian Runx transcription factor family contains three proteins, Runx1, Runx2, and Runx3, each of which forms a heterodimeric complex with a common non-DNA–binding partner Cbfβ. Runx1–Cbfβ complexes are essential for hematopoiesis (Speck, 2001), and recent studies have revealed that Runx–Cbfβ complexes play pivotal roles in regulating differentiation of several T lymphocyte subsets (Collins et al., 2009). Using Runx1-deficient bone marrow progenitors, Runx1 was shown to be indispensable in generating CLPs (Growney et al., 2005). In addition, expression of CD79a (also known as mb1), which is an essential signaling subunit of the pre-B cell receptor, was shown to be regulated by Ebf1 with the help of Runx1 (Maier et al., 2004). Although these results indicate that Runx1–Cbfβ complexes are important for early B lineage development, the mechanisms by which Runx1 regulates early B cell development have not been fully addressed.

In this study, we conditionally inactivated the *Runx1*, *Runx3*, or *Cbfβ* gene in mice from the early pro-B cell progenitor stage by using an *mb1-cre* transgene (Hobeika et al., 2006). We report that Runx1 deficiency in early pro-B cells results in a severe reduction of both B cell progenitors and V_H-DJ_H recombination events. Furthermore, a partial rescue of B cell development in vitro by Ebf1 transduction together with our demonstration of the direct activation of the *Ebf1* proximal β promoter by Runx1 indicate that Runx1 serves as an upstream factor together with E2A for *Ebf1* activation.

RESULTS AND DISCUSSION

Runx1–Cbfβ complexes are essential for efficient B cell development

To examine the roles of Runx transcription factors during early B cell development, we generated three mouse strains in which the *Runx1*, *Runx3*, or *Cbfβ* gene was conditionally inactivated by the *mb1-cre* transgene (Fig. 1 G). In *Runx1*$^{F/F}$*;mb1-cre* mice, there were almost no detectable CD19$^+$ IgM$^+$ splenic B cells or B220$^+$ IgM$^+$ bone marrow immature B cells. In contrast, these populations in the spleen and bone marrow of *Runx3*$^{F/F}$*;mb1-cre* mice were comparable with those of control mice (Fig. 1, A, B, and D). These results clearly indicate that Runx1, but not Runx3, is essential for B cell development. To determine the precise developmental stages during which B cell differentiation is blocked in *Runx1*$^{F/F}$*;mb1-cre* mice, we analyzed early B cell development in bone marrow by using the criteria of Hardy et al. (2000), which defines B cell precursors by their expression of CD43, B220, CD24 (HSA), and BP-1. Bone marrow of *Runx1*$^{F/F}$*;mb1-cre* mice contained extremely low numbers of overall B220$^+$ cells (Fig. 1 D). More specifically, there was a dramatic 20-fold reduction in immature B cells (CD43$^-$ B220^{hi}) and a fivefold reduction in pre-B cells (CD43$^-$ B220^{int}). The pro-B cells (CD43$^+$ B220^{int}) were only slightly decreased in percentages (Fig. 1 B). This shows that Runx1 plays a role during the transition from pro-B to pre-B cell stages. However, when the pro-B cell population was further divided by HSA and BP-1 expression, the frequency of pro-B cells (HSA$^+$ BP-1$^-$, HSA$^+$ BP-1$^+$, and HSA^{hi} BP-1$^+$ in the order of development) was significantly lower but the frequency of pre-pro-B cells (HSA$^-$ BP-1$^-$) was higher in the *Runx1*$^{F/F}$*;mb1-cre* mice compared with control mice (Fig. 1, C and D). This demonstrates that Runx1 is necessary for efficient transition from pre-pro-B to pro-B cell stages as well.

In addition to surface marker expression, the status of the *Igh* gene serves as a good molecular marker to define stages of early B cell development. Therefore, we examined *VDJ* rearrangement of the *Igh* locus in sorted pro-B cells (HSA$^+$ BP-1$^-$, HSA$^+$ BP-1$^+$, and HSA^{hi} BP-1$^+$) using a DNA-PCR assay to further examine the perturbed B cell development. As shown in Fig. 1 E, rearrangement of both V_H558 and V_H7183 gene segments to a rearranged DJ_H was easily detected in the pro-B cell population from control mice, whereas such rearrangements were absent in pro-B cells from *Runx1*$^{F/F}$*;mb1-cre* mice. D_H to J_H rearrangement was also impaired in Runx1-deficient cells, albeit to a lesser extent. Given that D_H to J_H rearrangement initiates in pre-pro-B cells and V_H to DJ_H rearrangement starts from pro-B cells, this genetic analysis indicates that inactivation of *Runx1* impairs early B cell development from the pre-pro-B to pro-B transition, consistent with the analysis using surface marker expression.

In *Cbfβ*$^{F/F}$*;mb1-cre* mice, although there were very few CD43$^-$ B220^{hi} immature B cells in the bone marrow, the frequency of the pre-B cell (CD43$^-$ B220^{int}) population was reduced to only one third of that in control mice (Fig. 1 B), indicating that the B cell developmental arrest in *Cbfβ*$^{F/F}$*;mb1-cre* mice was at a later stage than that in *Runx1*$^{F/F}$*;mb1-cre* mice. Given the putative obligatory function of Cbfβ in the function of all Runx proteins, this result suggests that there is a delayed loss of Cbfβ protein after *Cbfβ* gene inactivation. If this was the case, one might expect to see a significant effect of gene dosage and, indeed, early B cell development was impaired at earlier stages in *Cbfβ*$^{F/-}$*;mb1-cre* than in *Cbfβ*$^{F/F}$*;mb1-cre* mice (Fig. 1 F). Thus, it is likely that a gradual loss of Cbfβ

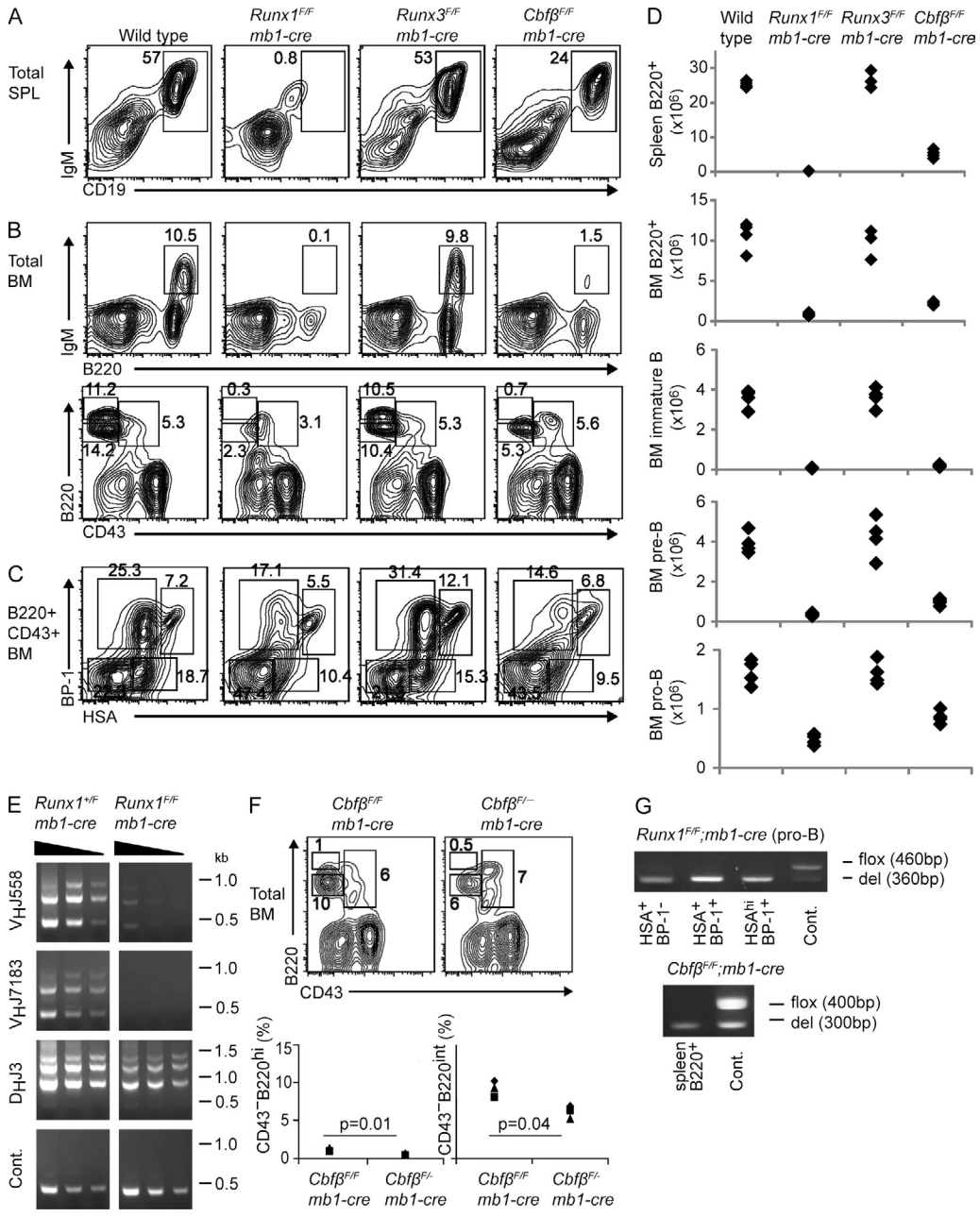

Figure 1. Defective early B lymphocyte development caused by conditional inactivation of *Runx1* or *Cbfβ* genes. (A–C) Dot plots showing IgM and CD19 expression profiles in splenocytes (SPL; A) and IgM and B220 or CD43 and B220 expression profiles in total bone marrow (B) from mice of the indicated genotypes. In C, BP-1 and HSA expression profiles in CD43$^+$ B220$^+$ bone marrow cells are shown. (D) Absolute numbers of spleen B220$^+$, bone marrow B220$^+$, CD43$^-$ B220^{hi} immature B, CD43$^-$ B220^{int} pre-B, and CD43$^+$ B220^{int} pro-B cells from each mouse strain are shown. Each symbol represents one mouse. (E) PCR analysis of V_H-DJ_H (V_H558 or V_H7183 families) and D_H-J_H rearrangement in sorted pro-B cell populations from *Runx1$^{+/F}$;mb1-cre* and *Runx1$^{F/F}$;mb1-cre* mice. The ThPOK silencer region was used as the loading control (Cont.). (F) Dot plots showing CD43 and B220 expression profiles in bone marrow from *Cbfβ$^{F/F}$;mb1-cre* and *Cbfβ$^{F/-}$;mb1-cre* mice (top). The bottom graph shows the percentages of CD43$^-$ B220^{hi} immature B and CD43$^-$ B220^{int} pre-B cells in bone marrow of *Cbfβ$^{F/F}$;mb1-cre* and *Cbfβ$^{F/-}$;mb1-cre* mice. (G) Deletion efficiencies of floxed alleles in the indicated cells are shown. Controls are *Runx1$^{F/-}$* or *Cbfβ$^{F/-}$* genotypes. (A–F) Representative data of four (A–E) or three (F) independent experiments are shown.

protein after its gene inactivation results in arrest of B cell development at a relatively later stage in *Cbfβ$^{F/F}$;mb1-cre* mice. Nonetheless, some immature B cells successfully developed into mature B cells, composing a significant population of mature B cells in the spleen of *Cbfβ$^{F/F}$;mb1-cre* mice (Fig. 1, A and D). This suggests that Runx1–Cbfβ complexes might not be essential for maintaining the mature B lymphocyte pool in the periphery even though our results do not formally exclude the possibility that Runx1 might have Cbfβ-independent function during B cell development.

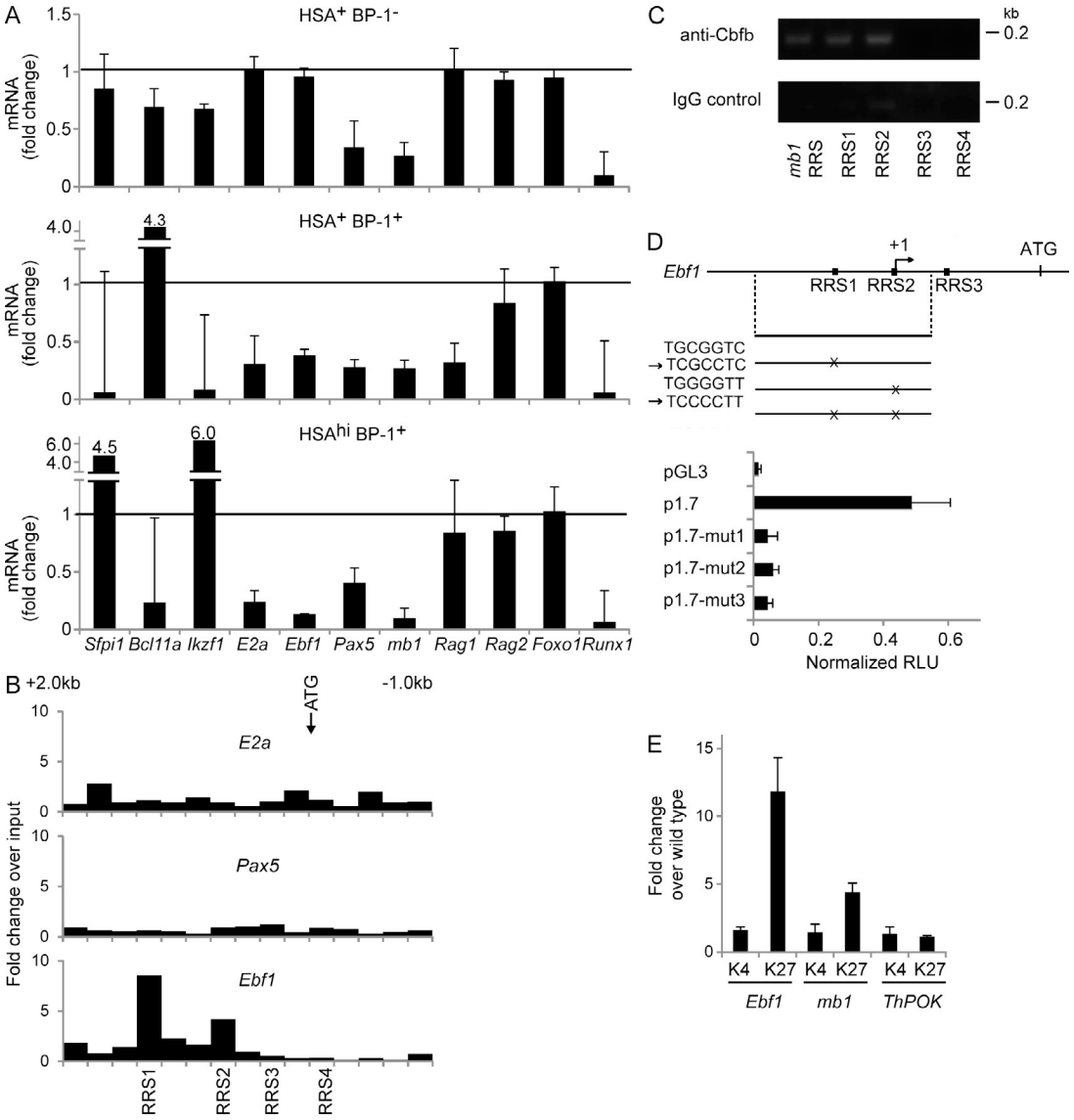

Figure 2. Direct activation of the *Ebf1* proximal β promoter by Runx1. (A) Real-time PCR analysis showing messenger RNA (mRNA) expression levels of several B cell signature genes in subsets of pro-B cells (HSA$^+$ BP-1$^-$, HSA$^+$ BP-1$^+$, and HSA^{hi} BP-1$^+$ in the order of development) from *Runx1$^{f/f}$; mb-1-cre* mice. Data were normalized to HPRT and are shown as fold changes to wild-type control. (B) A ChIP-on-chip assay was performed with anti-Cbfβ antibody to evaluate Runx–Cbfβ binding to the promoter regions of *E2a*, *Pax5*, and *Ebf1* genes in B220$^+$ bone marrow cells. Positions of putative RRSs within the *Ebf1* proximal β promoter are indicated. One representative of two independent experiments is shown. (C) Analytical ChIP assay using B220$^+$ bone marrow cells. The *mb1* promoter was used as a positive control for Cbfβ binding. One representative result from three independent experiments is shown. (D) Schematic overview of the *Ebf1* proximal β promoter is shown with three predicted RRSs. +1 indicates the transcription start site. The top panel shows the structure of each reporter construct, and the bottom panel indicates relative luciferase activity from each construct in a transfection assay in the Ba/F3 cell line. Values are shown in relative light units (RLU). (E) Relative H3K4Me3 (K4) and H3K27Me3 (K27) histone modification levels at the *Ebf1* proximal β promoter, the *mb1* promoter, and *ThPOK* silencer regions in Runx1-deficient pro-B cells relative to wild-type pro-B cells. Data are represented as relative fold changes as in A. (A, D, and E) Error bars represent mean ± SD of three independent experiments.

Ebf1 promoter activity is directly regulated by Runx1–Cbfβ complexes

To understand the molecular mechanisms underlying the inhibition of B cell development by *Runx1* inactivation, it is important to identify Runx1–Cbfβ target genes. To this end, we examined the expression of genes known to be essential for early B cell development from pro-B cell fractions, which were further fractionated into three subpopulations (HSA$^+$ BP-1$^-$, HSA$^+$ BP-1$^+$, and HSA^{hi} BP-1$^+$ of CD43$^+$ B220^{int} bone marrow cells). Consistent with a previous study showing that Runx1 activates the *mb1* gene cooperatively with Ebf1 (Maier et al., 2004), the amount of *mb1* transcripts was considerably reduced in all three fractions of pro-B cells. In addition, expression of some transcription factor genes known to

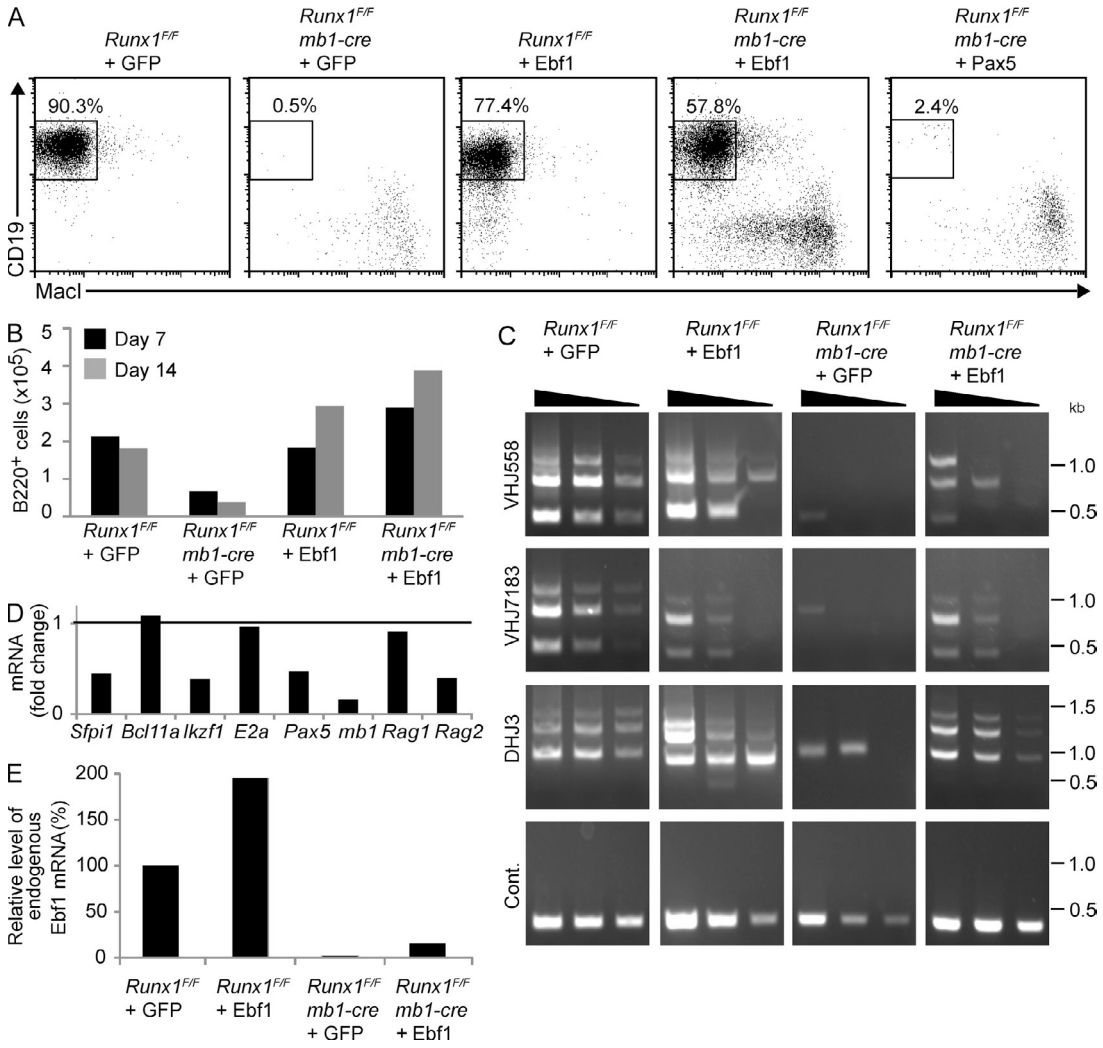

Figure 3. Rescue of B cell development from Runx1–defcient progenitors by EBF1 transduction. Purified hematopoietic progenitors, which were enriched by negative selection of lineage markers from the indicated mouse strains, were co-cultured with TSt-4 stromal cells for 3 d after transduction with retrovirus encoding GFP alone, Ebf1-IRES-GFP, or Pax5-IRES-GFP. GFP$^+$ cells were sorted and further co-cultured with TSt-4 stromal cells to facilitate B cell differentiation. (A and B) At 7 or 14 d after cell sorting, cells were analyzed for surface expression of CD19 and MacI by flow cytometry (A) and the absolute numbers of B220$^+$ cells (B). Dot blots in A are data at 14 d. (C–E) At day 17, V_H558 to J_{H3}, V_H7183 to J_{H3}, and D_H to J_{H3} joining (the *Thpok* silencer region was used as the loading control [Cont.]; C), expressions of genes known to be important for B cell development (D), and endogenous *Ebf1* expression relative to *Runx1*$^{F/F}$ cells transduced with GFP vector (E) were measured in purified B220$^+$ cells. One representative result from two independent transduction experiments with duplicate is shown.

be important for B cell development was also decreased (Fig. 2 A). Among them, expression of *Sfpi1*, *Bcl11a*, and *Ikaros* was not consistently reduced in fractions HSA$^+$ BP-1$^+$ and HSA^{hi} BP-1$^+$, whereas expression of *E2a*, *Ebf1*, and *Pax5* was consistently decreased. These results suggest that decreased expression of these three transcription factors could be involved in the B cell developmental arrest in *Runx1*$^{F/F}$*;mb1-cre* mice.

We thus examined whether Runx1–Cbfβ complexes bind to the promoter regions of *E2a*, *Ebf1*, and *Pax5* genes in vivo by a chromatin immunoprecipitation (ChIP)-on-chip assay. Within 2.0 kb upstream and 1.0 kb downstream from the transcription start site of these genes, Cbfβ protein bound only to the *Ebf1* proximal β promoter at two regions that contain putative Runx recognition sites (RRSs; Fig. 2 B). This result was further confirmed using an analytical ChIP assay (Fig. 2 C). Thus, although there are four putative RRSs within the 2.0-kb *Ebf1* proximal β promoter (Fig. 2 D), only RRS1 and RRS2 were bound by Runx1–Cbfβ complexes in vivo.

To examine the significance of RRS1 and RRS2 in activating the *Ebf1* proximal β promoter, we performed reporter transfection assays. Because a 1.7-kb fragment of the β promoter was shown to be sufficient to drive reporter *luciferase* expression in the Ba/F3 cell line (Roessler et al., 2007), we modified the parental p1.7 construct by introducing specific mutations onto RRS1, RRS2, and both to generate mut1, mut2, and mut3 constructs, respectively (Fig. 2 D). Although the p1.7 construct yielded ∼50-fold higher activation relative to the promoterless pGL3 control construct, none of the

mutant constructs were able to enhance luciferase expression much above that seen with the pGL3 control construct (Fig. 2 D). This result clearly shows that RRS1 and RRS2 sites are essential for the *Ebf1* proximal β promoter activity in transfection assays. Furthermore, increased accumulation of H3K27 trimethylation (H3K27Me3), a representative epigenetic mark for gene loci in a repressed state, was observed at the *Ebf1* proximal β promoter in Runx1-deficient pro-B cells, whereas the level of an active mark, H3K4Me3, was comparable with that in control cells (Fig. 2 E). Collectively, these results suggest that Runx1 is essential for initiating *Ebf1* expression in part via direct activation of the proximal β promoter by altering its epigenetic state, such as by removing repressive H3K27Me3 modifications, during specification to the B lineage.

Ebf1 transduction restores B cell development in the absence of Runx1

To address whether reduced Ebf1 expression is the major factor in the B cell developmental arrest in *Runx1*$^{F/F}$*;mb1-cre* mice, we performed an Ebf1 complementation experiment. Bone marrow progenitors transduced with a control retrovirus vector or a vector encoding Ebf1 were sorted and then cultured on TSt-4 stromal cells under conditions that support the generation of both B and myeloid lineage cells. Although control progenitors efficiently differentiated into CD19$^+$ cells even without exogenous Ebf1 expression, progenitors from *Runx1*$^{F/F}$*;mb1-cre* mice generated almost only MacI$^+$ cells and failed to proliferate (Fig. 3, A and B). However, Ebf1 transduction greatly restored generation of CD19$^+$ cells in both frequency (~70% compared with wild-type cells) and in absolute cell numbers. In sharp contrast, Pax5 transduction failed to rescue development of CD19$^+$ cells.

To further characterize the extent of rescue by Ebf1 transduction, we examined *VDJ* rearrangement and gene expression profiles from purified B220$^+$ cells. Not only D_H to J_H rearrangement but also V_H to DJ_H recombination were restored in Runx1-deficient cells by Ebf1 transduction to levels almost equivalent to that of wild-type cells (Fig. 3 C). Quantitative measurement of B cell signature gene expression also showed very little difference between Runx1-sufficient control and Runx1-deficient rescued B220$^+$ cells in many genes, with the exception of *mb1* (Fig. 3 D). These results indicate that exogenous expression of Ebf1 corrected, at least to some extent, the perturbed B cell differentiation program caused by Runx1 deficiency, supporting a model in which defects of early B cell development in *Runx1*$^{F/F}$*;mb1-cre* mice in part reflect secondary effects caused by *Ebf1* down-regulation. Interestingly, Ebf1 transduction induced a twofold increase in endogenous *Ebf1* messenger RNA in control cells and restored it to 15% of control cell levels even in Runx1-deficient cells (Fig. 3 E).

In this study, we showed that Runx1 is essential to confer B lineage signatures, including expression of a trio of B cell core transcription factors, E2A, Ebf1, and Pax5. Based on the absence of *Ebf1* transcripts in E2A-deficient B cell progenitors, E2A has been regarded as the principal transcription factor regulating *Ebf1* gene expression (Ikawa et al., 2004). However, our results suggest an equally essential role for Runx1 during B lymphopoiesis in the activation of the *Ebf1* locus. Although E2A was shown to bind the *Ebf1* distal α promoter (Roessler et al., 2007), we show here that Runx1 binds to the proximal β promoter. It will be of interest to study how these two promoters cooperate to regulate *Ebf1* expression during B cell differentiation. Interestingly, the finding of reduced *Ebf1* transcripts in pro-B cells of *E2a*$^{F/F}$*; mb1-cre* mice (Kwon et al., 2008) and in peripheral mature B cells of *Cbfβ*$^{F/F}$*;mb1-cre* mice (unpublished data) suggests that after *Ebf1* expression is initiated, both E2A and Runx1–Cbfβ complexes are still required to maintain *Ebf1* expression. This idea is further supported by the recent genome-wide search for sites bound by E2A that revealed that RRSs frequently collocate with E2A binding sites (Lin et al., 2010). However, our results also showed that high amounts of exogenous Ebf1 could partially bypass activation of the *Ebf1* gene and its maintenance in Runx1-deficient cells, suggesting the possibility of an auto–feed-forward regulation at the *Ebf1* locus, which might contribute to further enforce specification to B lineage upon Ebf1 induction. Indeed, such an autoregulatory loop has been previously suggested (Roessler et al., 2007). It will be of great importance to further examine whether Ebf1 autoregulation operates under physiological conditions and, if so, its relevance to B cell development.

It has been proposed that the cascading activation of E2A, Ebf1, and Pax5 in that order is the most important sequence of events for programming B cell development. However, Ebf1 and Pax5 were also shown to be able to act as upstream factors for *E2a* and *Ebf1* gene expression, respectively (Fuxa et al., 2004; Zhuang et al., 2004). Thus, it is becoming evident that cross-regulatory networks among these three factors, rather than a simple one-directional hierarchy, contribute to imprint the B lymphoid signature. Our results place the Runx1–Cbfβ complex as another essential component on this transcription factor network. It will be important in future studies to understand the function of Runx1–Cbfβ complexes in differentiation and function of mature B cells.

MATERIALS AND METHODS

Mice and cells. *Runx1*^{F}, *Runx3*^{F}, *Cbfβ*^{F}, and *mb1-cre* (provided by M. Reth, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany) mice have been previously described (Taniuchi et al., 2002; Hobeika et al., 2006; Naoe et al., 2007). Mouse colonies were maintained in the specific pathogen–free animal facility of the Research Center for Allergy and Immunology at the RIKEN Yokohama Institute. Mouse experiments were approved by the RIKEN Institute and performed according to the institutional guidelines for animal care. B cells were prepared from spleen or bone marrow (femurs and tibiae). To enrich specific populations of B cells, MACS magnetic beads (Miltenyi Biotec) were used according to the manufacturer's instruction, and in some cases a FACS Aria (BD) was used to further purify cells after magnetic separation. A mouse IL-3–dependent pro-B cell line Ba/F3 was maintained in IL-3–containing RPMI medium.

Flow cytometry. Flow cytometry was performed by using either FACSCalibur or FACSCanto II (BD), and data were analyzed using FlowJo software (Tree Star). The following antibodies were purchased from BD and used for staining and cell sorting: B220, CD19, CD43, IgM, IgD, CD24 (HSA), BP-1, and MacI.

PCR analysis of VDJ recombination. Genomic DNA was isolated either from sorted pro-B cells (HSA^+ $BP\text{-}1^-$, HSA^+ $BP\text{-}1^+$, and HSA^{hi} $BP\text{-}1^+$) or from purified $B220^+$ cells of in vitro culture by using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. PCR cycle conditions were based on the literature (Schlissel et al., 1991) and adjusted to be in the linear amplification range by using serial dilution (threefold) of template DNA. Sequences of primers used are provided as supplemental text.

Quantitative real-time PCR. Total RNA was purified by using TRIZOL reagent and subjected to first-strand cDNA synthesis by using the SuperScript III system (Invitrogen). Real-time PCR was performed by using TaqMan Universal PCR Master Mix (Applied Biosystems) and the 7300 Real Time PCR System (Applied Biosystems) according to the manufacturer's instruction. To distinguish exogenous from endogenous Ebf1 transcripts, a forward primer was used that only anneals to the 5′ untranslated region sequence of the expression vector.

ChIP assay. Purified $B220^+$ cells (5×10^6 cells) from bone marrow were fixed and immunoprecipitated with 10 µg anti-Cbfβ or normal rabbit IgG as described previously (Setoguchi et al., 2008). After DNA purification, enriched DNA sequences were assessed by PCR for analytic ChIP assay. Sequences of primers are provided as supplemental text. The PCR reaction conditions were 35 cycles for 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C. Also, the same enriched DNA was used to examine Runx1–Cbfβ complex binding around promoter regions of *E2a*, *Ebf1*, and *Pax5* by ChIP-on-chip assays with custom-designed arrays. For analytic ChIP assay of histone modification, we used micro-ChIP assay (Dahl and Collas, 2008) because of the limited numbers of $B220^+$ cells in *Runx1*$^{F/F}$*;mb1-cre* mice.

In vitro B cell differentiation and retroviral transduction. Hematopoietic progenitors were enriched from bone marrow cells by negative purification using Dynabeads (Invitrogen) with Ter119, Mac1, Gr-1, Thy1.2, B220, and CD19 according to the manufacturer's instruction. Enriched hematopoietic progenitors were transduced with retroviruses containing GFP alone, Ebf1-IRES-GFP, or Pax5-IRES-GFP on TSt-4 stromal cell cultures supplemented with 10 ng/ml of each SCF, TPO, Flt3L, and IL-7 (Ikawa et al., 2010). 3 d later, GFP^+ cells were sorted by FACS Vantage (BD) and were further cultured on TSt-4 cells in the presence of SCF, IL-7, and Flt3L for up to 3 wk. Retrovirus was produced by transfection of the Platinum-E retroviral packaging cell line (Cell Biolabs, Inc.) with retroviral vectors by using Fugene6 (Promega). After 48 h of transfection, the virus-containing supernatant was used for spin transduction of target cells in the presence of 5 µg/ml polybrene (2,500 rpm for 90 min at 30°C).

Plasmid construction and luciferase reporter assay. The pMSCV-Ebf1-IRES-GFP vector has been described previously (Maier et al., 2004). A p1.7 vector harboring the 1.7-kb *Ebf1* proximal β promoter fragment (provided by R. Grosschedl, Max Planck Institute of Immunobiology and Epigenetics) in pGL3-basic plasmid (Promega) was previously described (Roessler et al., 2007). Fragments harboring specific mutations in Runx binding sites (from TGCGGTC to TCGCCTC at −1520 for p1.7-mut1; from TGGGGTT to TCCCCTT at −889 for p1.7-mut2) were generated by overlapping PCR and used to replace the wild-type fragment in the p1.7 vector. Each luciferase reporter was transiently transfected into the Ba/F3 cell line by electroporation (280 V, 960 µF). The pGL3 *renila* luciferase control vector (Promega) was cotransfected to monitor transfection efficiency in different samples and experiments. After 26–36 h of culture, cells were lysed and assayed for luciferase activity using the Dual Luciferase Assay System (Promega) according to the manufacturer's instruction. The amount of *pyralis* luciferase activity was normalized against transfection efficiency that was assessed by *renila* luciferase activity from the cotransfected control plasmid.

Online supplemental material. Sequences of primers used are shown as supplemental text. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20112745/DC1.

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