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 Vh to DJH 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 Vh to DJH 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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regulates expressions of many genes only required for B cell development, including mb1, VpreB, A3, 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). Accordingly, a partial or a complete loss of 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 VH-DJH 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 Runx1F/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 Runx3F/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 Runx1F/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 Runx1F/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+ B220hi) and a fivefold reduction in pre-B cells (CD43+ B220int). The pro–B cells (CD43+ B220int) 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− 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 Runx1F/F; mb1-cre mice compared with control mice (Fig. 1, C and D). This demonstrates that Runx1 is necessary for efficient transition from pro–pro–B to pre–B 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− BP-1−) using a DNA-PCR assay to further examine the perturbed B cell development. As shown in Fig. 1, E, rearrangement of both Vh538 and Vh7183 gene segments to a rearranged DJH was easily detected in the pro–B cell population from control mice, whereas such rearrangements were absent in pro–B cells from Runx1F/F; mb1-cre mice. Dj5 to JH rearrangement was also impaired in Runx1-deficient cells, albeit to a lesser extent. Given that Dj5 to JH rearrangement initiates in pro–B cells and Vh to DJH rearrangement starts from pro–B cells, this genetic analysis indicates that inactivation of Runx1 impairs early B cell development from the pro–pro–B to pro–B transition, consistent with the analysis using surface marker expression.

In CbβF/F; mb1-cre mice, although there were very few CD43− B220int immature B cells in the bone marrow, the frequency of the pro–B cell (CD43− B220int) population was reduced to only one third of that in control mice (Fig. 1 B), indicating that the B cell developmental arrest in CbβF/F; mb1-cre mice was at a later stage than that in Runx1F/F; mb1-cre mice. Given the putative obligatory function of Cbβ in the function of all Runx proteins, this result suggests that there is a delayed loss of Cbβ protein after Cbβ 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 CbβF/F; mb1-cre than in CbβF/F; mb1-cre mice (Fig. 1 F). Thus, it is likely that a gradual loss of Cbβ

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protein after its gene inactivation results in arrest of B cell development at a relatively later stage in Cbfβ<sup>F/F</sup>;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<sup>F/F</sup>;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.
were further fractionated into three subpopulations (HSA+ BP-1−, HSA+ BP-1+, and HSAhi BP-1+ of CD43+ B220int 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 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
further confirmed using an analytical ChIP assay (Fig. 2 C). Thus, although there are four putative RRSs within the 2.0-kb Ebf1 proximal promoter (Fig. 2 D), only RRS1 and RRS2 were bound by Runx1–Cbfβ complex 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 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 Runx1F/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 Runx1F/F;mb1-cre mice generated almost only Mac1+ 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 Dμ to JH rearrangement but also Vμ to DJH 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-deficient 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-deficient mice generated almost only Mac1+ 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.

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 E2aF/F; mb1-cre mice (Kwon et al., 2008) and in peripheral mature B cells of CbfβF/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 enhance 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. Runx1F/F, Runx3F/F, CbfβF/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; Hobefika 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 FACS-Calibur or FACS Canto 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 Mac1.
PCR analysis of VDJ recombination. Genomic DNA was isolated either from sorted pre-B cells (F8A<sup>+</sup> BP-1<sup>-</sup> , F8A<sup>+</sup> BP-1<sup>-</sup> , and F8A<sup>+</sup> BP-1<sup>-</sup> ) or from purified B220<sup>+</sup> cells of in vitro culture by using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. PCR cycle conditions were based on the literature (Söffig 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 instructions. To distinguish exogenous Ebf1 transcripts, a forward primer was used that only anneals to the 5′ untranslated region sequence of the expression vector.

ChIP assay. Purified B220<sup>+</sup> cells (5 × 10<sup>6</sup> cells) from bone marrow were fixed and immunoprecipitated with 10 µg anti-ChIP 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–ChIP 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<sup>+</sup> cells in Runx1<sup>−/−,mb1-cre</sup> 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 instructions. 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<sup>+</sup> 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 vector (provided by R. Grosschedl, Max Planck Institute of Immunobiology and Epigenetics) in pGL3-basic plasmid (Promega) was previously described jem.org/cgi/content/full/jem.20112745/DC1.

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 TGGCGTC to TCCGCTC at −1520 for p1.7–mut1; from TGGGTTG to TCCCGTT 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 mb1-cre mouse. Each luciferase reporter was transiently transfected into the mb1-cre mouse.

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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