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# Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5

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**Alternative lineage restriction and B cell fate commitment require the transcription factor Pax5, but the function of early B cell factor (EBF) in these processes remains mostly unexplored. Here we show that in the absence of EBF, 'expandable' and clonal lymphoid progenitor cells retained considerable myeloid potential. Conversely, ectopic expression of EBF in multipotential progenitor cells directed B cell generation at the expense of myeloid cell fates. EBF induced *Pax5* and antagonized expression of genes encoding the transcription factors C/EBPα, PU.1 and Id2. Notably, sustained expression of EBF in *Pax5*$^{-/-}$ hematopoietic progenitor cells was sufficient to block their myeloid and T lineage potential *in vivo*. Furthermore, in *Pax5*$^{-/-}$ pro–B cells, higher EBF expression repressed alternative lineage genes. Thus, EBF can restrict alternative lineage 'choice' and promote commitment to the B cell fate independently of Pax5.**

Hematopoiesis represents a leading developmental system for the analysis of gene-regulatory networks that orchestrate cell fate 'choice' and lineage commitment in complex metazoan systems[1–3]. The immune system is particularly well suited for elucidation of the transcriptional circuits underlying cell fate determination, as key progenitor cells and discrete developmental intermediates can be isolated, experimentally manipulated and assessed for their developmental competence. The analysis of early B cell development is highly advanced from this standpoint, as contingent regulatory networks comprising signaling molecules and transcription factors are being assembled that account for the generation of B lineage progeny from multipotent progenitors (MPPs)[2,4].

Published work suggests that B lymphocytes develop from lymphoid-primed multipotent progenitors (LMPPs) in the bone marrow that also give rise to myeloid progeny such as macrophages and granulocytes[5–7]. However, the molecular components regulating B cell fate 'choice' at the expense of myeloid cell fates remain to be delineated. The transcription factors PU.1 and C/EBPα represent chief determinants of myeloid cell fate[8–10]. A lower concentration of PU.1 is also needed to establish competence for the B cell fate[11,12]. The transcription factors E2A, EBF and Pax5 are essential for specification of and commitment to the B cell fate[13–16]. Specification of the B cell fate involves activation of the early B lineage genes *Cd79a* (*mb-1*), *Cd79b* (*B29*), *Igll1* (*λ5*) and *Vpreb1* (*VprebB*), which encode components of the pre–B cell receptor, and variable-to–diversity and joining ($V_H$-$DJ_H$) DNA rearrangements at the immunoglobulin heavy-chain (*Igh*) locus. The gene *Tcfe2a* (called '*E2A*' here) encodes two basic helix-loop-helix proteins, E12 and E47, generated by differential splicing[17]. EBF (also called EBF-1 or Olf-1) is an atypical helix-loop-helix zinc finger protein expressed exclusively in B lineage cells in the hematopoietic system[18]. Targeted inactivation of *E2A* or *Ebf1* leads to blockade of B cell development at the stage of onset of the expression of early B lineage genes and DNA rearrangements at the *Igh* locus[13–15]. E2A and EBF seem to function synergistically to activate the transcription of several early B lineage genes[19,20]. However, it has not been determined if one or both can initiate B cell fate 'choice' at the expense of alternative myeloid cell fates.

Unlike disruption of *E2A* and *Ebf1*, targeted mutation of *Pax5* blocks B cell development at the pro–B cell stage[16]. *Pax5*$^{-/-}$ pro–B cells properly express most early B lineage genes and undergo $D_H$-$J_H$ and proximal $V_H$-$DJ_H$ gene rearrangements[21,22]. However, unlike their wild-type counterparts, *Pax5*$^{-/-}$ pro–B cells generate other hematopoietic cell types after transplantation *in vivo* or culture *in vitro*[23,24]. Pax5 also maintains B cell identity by actively repressing lineage-inappropriate genes[25,26]. Thus, Pax5 has been considered the main factor required for the restriction of alternative hematopoietic cell fates and commitment to the B lineage[27]. However, such analysis has overlooked key functions of EBF in these processes. Studies have

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shown that EBF, but not Pax5, restores the B cell development of progenitor cells deficient in PU.1 (*Sfpi1*$^{-/-}$; called '*PU.1*$^{-/-}$' here), E2A (*E2A*$^{-/-}$) or the interleukin 7 receptor (IL-7R) α-chain (*Il7ra*$^{-/-}$) and of lymphoid progenitor cells isolated from IL-7-deficient mice[12,28–30]. Furthermore, Pax5 expression is regulated by EBF, and Pax5-mediated activation of B lineage genes such as *Cd19* and *Cd79a* is contingent on EBF[12,31]. Finally, common lymphoid progenitors (CLPs) that have little myeloid potential do not have appreciable expression of Pax5 (ref. 32). This suggests that an 'upstream' regulator must restrict myeloid lineage options before the onset of Pax5 expression. Thus, we analyzed the functions of EBF in alternative lineage restriction and B cell fate commitment with a particular emphasis on determining if some of these functions are independent of Pax5.

We did loss-of-function and gain-of-function experiments involving EBF using wild-type, *Ebf1*$^{-/-}$ or *Pax5*$^{-/-}$ hematopoietic progenitor cells. We found that *Ebf1*$^{-/-}$ lymphoid progenitor populations were expandable and their clones retained not only T cell and natural killer (NK) cell developmental potential but also myeloid developmental potential. Restoration of EBF expression in these progenitor cells inhibited myeloid differentiation and induced B cell development accompanied by $V_H$-$DJ_H$ rearrangement. Correspondingly, ectopic expression of EBF in wild-type MPPs promoted B cell generation at the expense of myeloid cell fates. EBF induced *Pax5* expression and antagonized the expression of genes encoding the alternative cell fate determinants C/EBPα, PU.1 and Id2. Notably, sustained expression of EBF in *Pax5*$^{-/-}$ hematopoietic progenitor cells restricted their alternative lineage potential (myeloid and T cell) *in vivo*. Finally, in *Pax5*$^{-/-}$ pro–B cells, higher expression of EBF repressed myeloid and T lineage genes, including subsets activated by PU.1 or repressed by Pax5. EBF directly targeted some of those genes. Our results establish that EBF is the main B cell fate determinant that initiates alternative lineage restriction and demonstrate a previously unknown function for EBF in cell fate commitment that is independent of Pax5.

## RESULTS

### *Ebf1*$^{-/-}$ lymphoid progenitor cells retain myeloid potential

In the absence of EBF, B cell development is blocked at the stage of a lymphoid progenitor cell expressing Flt3, IL-7R and B220 (ref. 12). To assess the function of EBF in the restriction of alternative lineage potentials, we isolated *Ebf1*$^{-/-}$ fetal hematopoietic progenitor cells and propagated them in culture with OP42 stromal cells and the cytokines stem cell factor (SCF), Flt3 ligand (Flt3L) and IL-7. In these B lymphoid–promoting conditions, *Ebf1*$^{-/-}$ progenitor cells proliferated extensively and could be readily established as cell lines and clonal derivatives. We used flow cytometry to characterize the developmental status of these cells. As expected from analysis of their *in vivo* counterparts[12], they expressed Flt3 and IL-7R and were responsive to both Flt3L and IL-7 signaling (**Fig. 1a** and data not shown). Additionally, they expressed c-Kit, Sca-1, CD34, CD43 and B220 but not CD19 (**Fig. 1a** and **Supplementary Fig. 1** online). As described earlier for their freshly isolated counterparts, *Ebf1*$^{-/-}$ progenitor cell populations expanded *in vitro* had not appreciably activated the early program of B lineage gene expression, with the exception of *Cd79b* (**Supplementary Fig. 2a** online). The mutant cells had $D_H$-$J_H$ but not $V_H$-$DJ_H$ rearrangements at *Igh* loci (**Fig. 1b**). On the basis of their cell surface phenotype, gene expression pattern and rearrangement status of *Igh* loci, the *Ebf1*$^{-/-}$ cells seem to represent lymphoid progenitor cells that are defective in undergoing B cell fate specification.

We tested the B cell developmental potential of *Ebf1*$^{-/-}$ lymphoid progenitor cells by restoring their expression of EBF with a retroviral construct of EBF and green fluorescent protein (EBF-GFP)[12]. This resulted in the generation of CD19$^+$ B lineage precursors that had

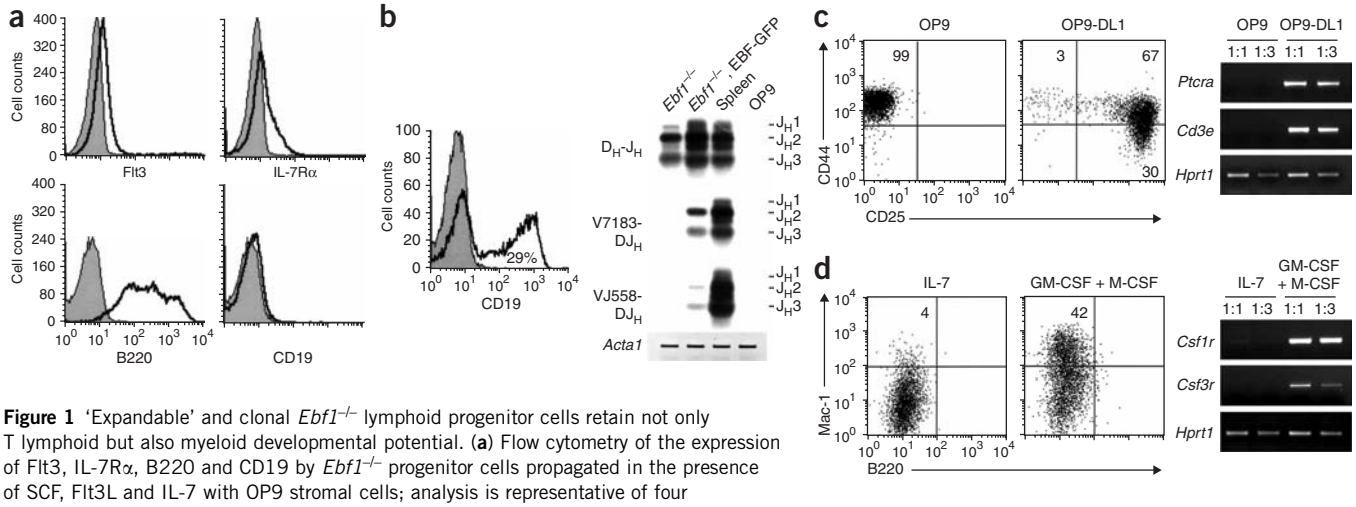


**Figure 1** 'Expandable' and clonal *Ebf1*$^{-/-}$ lymphoid progenitor cells retain not only T lymphoid but also myeloid developmental potential. (**a**) Flow cytometry of the expression of Flt3, IL-7Rα, B220 and CD19 by *Ebf1*$^{-/-}$ progenitor cells propagated in the presence of SCF, Flt3L and IL-7 with OP9 stromal cells; analysis is representative of four independently derived *Ebf1*$^{-/-}$ progenitor cell lines. Filled histograms, isotype control; open histograms, specific antibody staining. (**b**) Development of CD19$^+$ B lineage progeny (left) and rearrangement of *Igh* loci (right) by *Ebf1*$^{-/-}$ progenitor cells transduced with control GFP or EBF-GFP retrovirus, then sorted by GFP expression and maintained for 6 d in the presence of SCF, Flt3L and IL-7. Left: filled histogram, control GFP; open histogram, EBF-GFP (number in plot indicates percent CD19$^+$ B lineage progeny). Right, Southern blot analysis with a $D_HFL16$-$J_H4$ probe: $J_H1$, $J_H2$ and $J_H3$ indicate rearrangements to the respective *Igh* J-region segments; *Acta1* (encoding α-actin) was used as a loading control; genomic DNA from splenocytes (Spleen) and OP9 stromal cells (OP9) serve as a positive and negative control, respectively. (**c**) Flow cytometry of the generation of T lineage (CD25$^+$) precursors by *Ebf1*$^{-/-}$ progenitor cells plated on OP9 or OP9-DL1 stroma and cultured for 7 d in the presence of Flt3L and IL-7 (left); and RT-PCR of T lineage genes in CD25$^+$ cells isolated by flow cytometry (right). Transcripts encode pTα (*Ptcra*), CD3ε (*Cd3e*) and HPRT (*Hprt1*). (**d**) Flow cytometry of the generation of myeloid (Mac-1$^+$) lineages by *Ebf1*$^{-/-}$ progenitor cells plated on OP9 stromal cells and cultured for 6 d in the presence of IL-7 or GM-CSF plus M-CSF in combination with SCF and Flt3L (left); and RT-PCR of myeloid lineage genes in Mac-1$^{hi}$ cells sorted by flow cytometry (right). Transcripts encode M-CSFR (*Csf1r*), G-CSFR (*Csf3r*) and HPRT (*Hprt1*). Numbers in quadrants (left, **c**,**d**) indicate percent cells in each; ratios above lanes (right, **c**,**d**) indicate dilution of cDNA template used for amplification. Data are representative of three (**a**,**c**,**d**) or two (**b**) independent experiments.

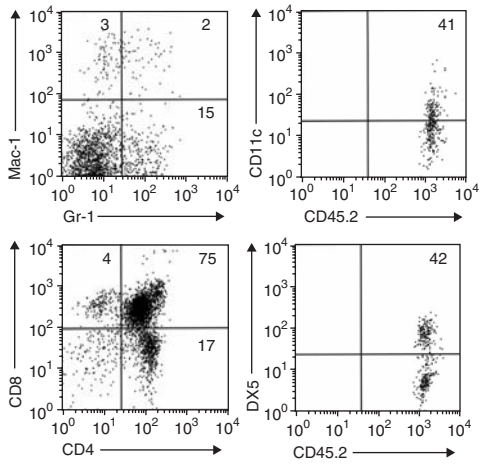


**Figure 2** *Ebf1*$^{-/-}$ lymphoid progenitor cells maintained *in vitro* in B lymphoid conditions show T cell, NK cell and myeloid developmental potential *in vivo*. Flow cytometry of cells from lethally irradiated mice (CD45.1) 5 weeks after injection with a mixture of *Ebf1*$^{-/-}$ progenitor cells (CD45.2) and wild-type syngeneic (CD45.1) bone marrow cells; thymocytes were analyzed for the presence of donor-derived T lineage (CD4$^+$, CD8$^+$) cells, and bone marrow cells were analyzed for the presence of donor-derived myeloid (Mac1$^+$, Gr-1$^+$), NK (DX5$^+$) and dendritic (CD11c$^+$) cells. All plots are pregated on donor-derived (CD45.2$^+$) cells. Numbers in quadrants indicate percent cells in each. Data are representative of three independent transplantation experiments.

undergone $V_H$-$DJ_H$ rearrangement (**Fig. 1b**). Thus, EBF not only regulates the early program of B lineage gene expression but is also required for B lineage–specific recombination events at the *Igh* locus. To examine T lineage developmental potential, we plated *Ebf1*$^{-/-}$ lymphoid progenitor clones on OP9 stromal cells expressing the Notch ligand DL1 (OP9-DL1 cells) and allowed them to differentiate as described before[33]. In these conditions, *Ebf1*$^{-/-}$ progenitor cells generated CD44$^+$CD25$^+$ (double-negative stage 2) and CD44$^-$CD25$^+$ (double-negative stage 3) T lineage precursors (**Fig. 1c**). These cells were properly specified, as they expressed the T lineage–specific genes *Ptcra* and *Cd3e* (**Fig. 1c**). The *Ebf1*$^{-/-}$ progenitor cells had appreciable expression of the T lineage regulator GATA-3 that was further upregulated during their differentiation into T cell precursors (**Supplementary Fig. 2a**,**b**). This *Gata3* expression probably contributes to their ability to readily differentiate along the T pathway. Thus, *Ebf1*$^{-/-}$ lymphoid progenitor cells are capable of giving rise to both T lineage and B lineage precursors, and the latter requires restoration of EBF expression.

To determine if *Ebf1*$^{-/-}$ progenitor cells were lymphoid restricted, we assessed the ability of clonal derivatives to undergo myeloid differentiation by culturing them in the presence of SCF, Flt3L, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF). In these conditions, a substantial fraction of *Ebf1*$^{-/-}$ progenitor cells differentiated into Mac-1$^{hi}$ cells and had morphology characteristic of granulocytes and macrophages (**Fig. 1d** and **Supplementary Fig. 3** online). These Mac-1$^{hi}$ cells expressed the key myeloid cytokine receptor genes *Csf1r* and *Csf3r*, which encode M-CSFR and G-CSFR, respectively (**Fig. 1d**). *Ebf1*$^{-/-}$ progenitor cells had higher expression of the myeloid determinant PU.1 than did wild-type pro–B cells (**Supplementary Fig. 2a**). Furthermore, expression of *PU.1* and *Cebpa* (encoding C/EBPα) was further induced during the differentiation of *Ebf1*$^{-/-}$ progenitor cells into myeloid progeny (**Supplementary Fig. 2b**). These results raised the possibility that EBF may be needed to inhibit the myeloid developmental options of lymphoid progenitor cells and that it may do so by antagonizing the upregulation of *PU.1* and *Cebpa*.

To initially test the ability of EBF in restricting myeloid lineage options, we transduced cloned *Ebf1*$^{-/-}$ progenitor cells with a control retroviral vector (GFP) or the EBF-GFP retroviral vector and plated them in the presence of myeloid lineage cytokines. As expected, *Ebf1*$^{-/-}$ progenitor cells transduced with the control virus generated Mac-1$^{hi}$ progeny within 2 d (**Supplementary Fig. 3**). In identical conditions, expression of EBF in *Ebf1*$^{-/-}$ progenitor cells led to an 85% reduction in the generation of Mac-1$^{hi}$ myeloid progeny (data not shown). EBF expression also diminished the responsiveness of the progenitor cells to myeloid cytokines, as cell recovery was reduced by 50% (data not shown). Thus, restoration of EBF expression in *Ebf1*$^{-/-}$ progenitor cells restricts their myeloid developmental potential.

### *Ebf1*$^{-/-}$ progenitor cells reconstitute several lineages

To test developmental capacity *in vivo*, we individually mixed three independent lines of *Ebf1*$^{-/-}$ progenitor cells (CD45.2$^+$), maintained *in vitro* in B lymphoid conditions, with wild-type bone marrow cells (CD45.1$^+$) and transferred the cells into lethally irradiated host mice (CD45.1$^+$). At 5 weeks after transplantation, we isolated bone marrow, spleens and thymi from recipient mice and analyzed the generation of various hematopoietic lineages. We distinguished cells derived from *Ebf1*$^{-/-}$ progenitor cells versus wild-type syngeneic bone marrow cells on the basis of CD45.2 expression. *Ebf1*$^{-/-}$ progenitor cells generated Mac-1$^+$Gr-1$^+$ myeloid cells, CD11c$^+$ dendritic cells and DX5$^+$ NK cells in the bone marrow (**Fig. 2**). In the thymus, *Ebf1*$^{-/-}$ progenitor cells generated CD4$^+$CD8$^+$ double-positive and single-positive progeny (**Fig. 2**). Notably, clonal lines of *Ebf1*$^{-/-}$ progenitor cells recapitulated the lymphoid and myeloid developmental capacities of their parental counterparts (**Supplementary Fig. 4** online). Moreover, *Ebf1*$^{-/-}$ progenitor cells gave rise to very few erythrocyte precursors, detected on the basis of expression of CD71 and Ter119 (data not shown). Thus, *Ebf1*$^{-/-}$ progenitor cells, despite long-term culture in the presence of B lineage–inducing signals, generate T cells, NK cells, dendritic cells and myeloid cells *in vivo*.

### EBF is the limiting B cell fate determinant in MPPs

The loss-of-function studies described above indicated that *Ebf1*$^{-/-}$ lymphoid progenitor cells are blocked at the earliest events in B cell development and show both lymphoid (T cell and NK cell) and myeloid developmental potential. Those analyses suggested that EBF is required not only for B cell fate specification but also for restriction of alternative cell fates. We pursued a complementary gain-of-function approach to determine if EBF is able to direct B cell fate at the expense of myeloid cell fates in MPPs. We used flow cytometry–purified MPPs (Lin$^-$c-Kit$^{hi}$Sca-1$^{hi}$CD27$^+$) isolated from the bone marrow of wild-type mice for these experiments. CD27 expression distinguishes MPPs from long-term self-renewing hematopoietic stem cells (HSCs)[34]. We sorted transduced cells for GFP expression and plated equal numbers (250–500) on OP42 stromal cells in the presence of SCF, Flt3L and IL-7 (ref. 35). We analyzed cultures for their development of B lymphoid (CD19$^+$) and myeloid (Mac-1$^+$) progeny at two intervals (days 7 and 14). In these culture conditions, MPPs transduced with the control virus (GFP) generated mainly Mac-1$^+$ myeloid progeny by day 7 (**Fig. 3a**). Continuous culture in IL-7 resulted in the eventual emergence of CD19$^+$ B cell precursors by day 14 (data not shown). In contrast, EBF-GFP–transduced MPPs gave rise to a large proportion

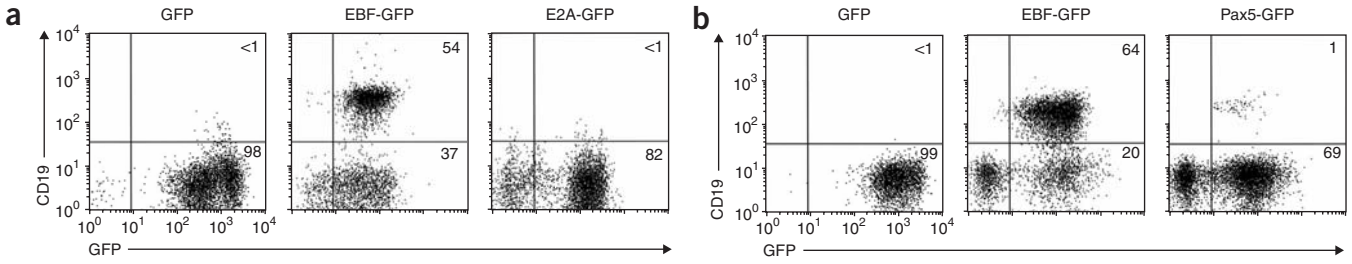


**Figure 3** Higher expression of EBF in MPPs induces B cell development. (**a**) Generation of B lineage (CD19$^+$) precursors by wild-type bone marrow–derived MPPs (Lin$^-$c-Kit$^{hi}$Sca-1$^{hi}$CD27$^+$) stimulated for 12–16 h in the presence of SCF, IL-3, IL-6, then transduced by spin infection with retrovirus, maintained for 24 h in the presence of SCF, IL-3, IL-6, IL-7 and GM-CSF and sorted by flow cytometry on the basis of GFP expression; equivalent numbers (500 cells) were plated for differentiation on OP42 stromal cells in the presence of the B lymphoid–promoting cytokines SCF, Flt3L and IL-7 and were collected after 7 d for analysis. (**b**) Flow cytometry of MPPs isolated and stimulated as described in **a** and transduced with retroviruses by coculture with GP+E-86-derived packaging cells; GFP$^+$ cells were sorted by flow cytometry and equivalent numbers (250 cells) were plated for differentiation and analysis as described in **a**. Numbers in quadrants indicate percent cells in each. Data are representative of three independent experiments.

of CD19$^+$ B lineage precursors by day 7 (**Fig. 3a**), which predominated over myeloid progeny by day 14 (data not shown).

Because E2A functions in concert with EBF to specify the B cell fate, we tested if higher E2A expression in MPPs also resulted in enhanced generation of B cell precursors at the expense of myeloid progeny. We transduced MPPs with GFP-expressing retroviral vectors encoding the E12 or E47 isoform of E2A. These retroviral constructs express biologically active proteins, as they are able to complement *E2A*$^{-/-}$ progenitor cells[30]. Unlike EBF, neither E2A isoform enhanced the generation of B cell precursors from MPPs (**Fig. 3a** and data not shown).

The CD19$^+$ cells arising in EBF-GFP–transduced cultures had the morphology of pro–B cells and expressed the B lineage–specific genes *Igll1* and *Cd79a* (**Supplementary Fig. 5a,b** online). Not all EBF-GFP–transduced MPPs generated B lineage progeny, as shown by the presence of GFP$^+$CD19$^-$Mac-1$^+$ cells (data not shown). This result may have been due to variation in the rate of accumulation of EBF expressed from the retroviral vector in MPPs. As a consequence, a subset of MPPs may have responded to endogenous myeloid determinants and differentiated before EBF could antagonize these developmental options. Transduction of EBF into MPPs also resulted in the rapid generation of B lineage progeny even in the presence of exogenously added myeloid cytokines GM-CSF and M-CSF (data not shown). In these conditions, control MPPs gave rise exclusively to myeloid progeny even in prolonged culture (14 d; data not shown). Thus, higher expression of EBF in MPPs stimulates the generation of B cell precursors even in the presence of myeloid cytokines. These results collectively suggest that EBF, not E2A, is the limiting B cell fate determinant in MPPs.

## EBF is more potent than Pax5 in B cell generation

EBF has been shown to regulate *Pax5* expression[12,20]. To analyze whether induction of B cell development by EBF is mediated mainly through activation of *Pax5*, we transduced MPPs with EBF or Pax5 and assessed the efficiency of pro–B cell generation after 7 and 14 d in culture. The virus encoding Pax5 had sufficient expression of a

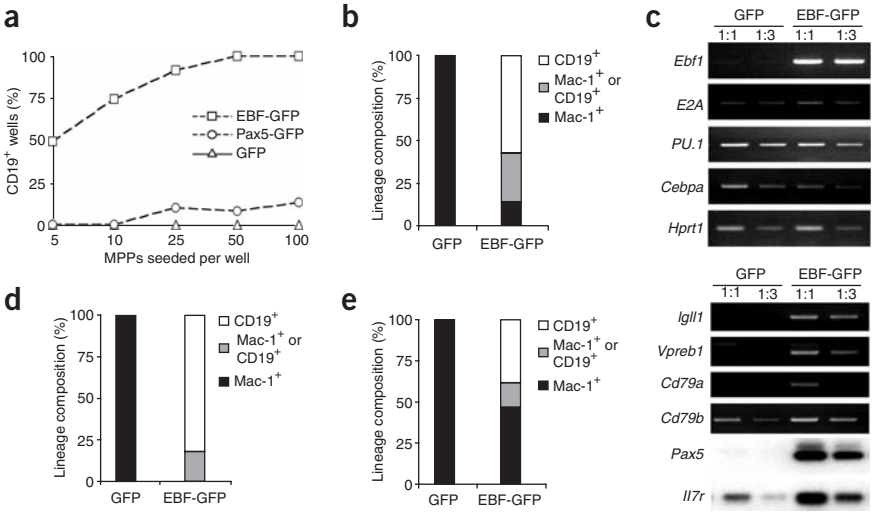


**Figure 4** EBF promotes B cell fate in MPPs at the expense of myeloid cell fate options. (**a**) Generation of B lineage (CD19$^+$) precursors by wild-type MPPs (Lin$^-$c-Kit$^{hi}$Sca-1$^{hi}$CD27$^+$) transduced with EBF or Pax5, sorted (in triplicate) directly onto OP42 stromal cells (density, horizontal axis) and maintained for 7 d in the presence of the lymphoid-promoting cytokines SCF, Flt3L and IL-7. (**b**) B lymphoid and myeloid lineage progeny of MPPs transduced with control GFP or EBF-GFP and plated at a density of 5 cells per well. (**c**) PCR analysis of gene expression by MPPs (Lin$^-$c-Kit$^{hi}$Sca-1$^{hi}$CD27$^+$) transduced with control GFP or EBF-GFP retrovirus; mRNA was isolated from GFP$^+$ MPPs 48 h after the onset of infection. Transcripts encode EBF (*Ebf1*), E2A, PU.1, C/EBPα (*Cebpa*), λ5 (*Igll1*), VpreB (*Vpreb1*), mb-1 (*Cd79a*), B29 (*Cd79b*), Pax5 (*Pax5*), IL-7Rα (*Il7r*) and HPRT (*Hprt1*). Serial dilutions (above lanes) of cDNA were used for PCR with *Hprt1* as a loading control; products were visualized by ethidium bromide staining, except *Pax5* and *Il7r* (detected by Southern blot). (**d**,**e**) Generation of CD19$^+$ and/or Mac-1$^+$ progeny by single MPPs (Lin$^-$c-Kit$^{hi}$Sca-1$^{hi}$Flt3$^{lo}$) transduced with control GFP or EBF-GFP retrovirus, then directly sorted onto OP9 stromal cells in 96-well plates and cultured with SCF, Flt3L and IL-7 (lymphoid conditions; **d**) or with SCF and M-CSF (myeloid conditions; **e**) and collected after 18 d or 12 d, respectively. Data in **b**,**d**,**e** represent the percent wells containing CD19$^+$Mac-1$^-$ B lymphoid and/or CD19$^-$Mac-1$^+$ myeloid progeny. Data represent four (**a**,**b**), three (**c**) or two (**d**,**e**) independent experiments.

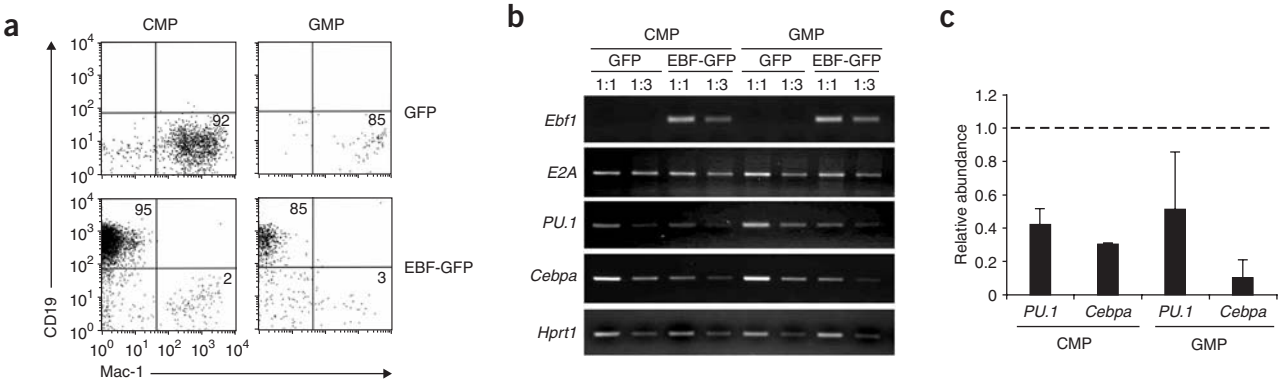


**Figure 5** EBF induces the generation of B cell precursors from myeloid progenitor cells. (**a**) Flow cytometry of the expression of CD19 and Mac-1 by bone marrow CMPs (IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRIII/II$^{lo}$) or GMPs (IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRIII/II$^{hi}$) sorted by flow cytometry, transduced by spin infection with control GFP or EBF-GFP retrovirus and then maintained for 2 d in Opti-MEM containing SCF, IL-3, Flt3L, IL-7; GFP$^+$ cells were isolated by flow cytometry and plated ($5 \times 10^3$ cells) on OP42 stromal cells in the presence of lymphoid-promoting cytokines and analyzed after 7 d of culture. Numbers in quadrants indicate percent CD19$^+$Mac-1$^-$ cells (top left) or CD19$^-$Mac-1$^+$ cells (bottom right). (**b**) PCR of serial dilutions (1:1 or 1:3; above lanes) of cDNA (after normalization with *Hprt1*) from cells transduced with control GFP or EBF-GFP retrovirus, analyzed 48 h after the onset of infection. (**c**) Quantitative PCR analysis of *PU.1* and *Cebpa* in CMPs and GMPs after transduction with EBF-GFP. *Hprt1* transcripts were used for normalization. Values represents transcript abundance in EBF-GFP-transduced cells relative to that in their GFP-transduced counterparts, set as 1.0 (dashed line). Data are representative of three independent experiments (error bars, s.d.).

functional protein that complemented *Pax5*$^{-/-}$ pro–B cells and induced expression of the target gene *Cd19* (**Supplementary Fig. 5c**). EBF was much more potent than Pax5 in inducing B cell development, as its expression in MPPs yielded at least 100-fold more B lineage progeny than did expression of Pax5 (**Fig. 3b** and data not shown). These data suggest that promotion of B cell generation from MPPs by EBF is not mediated solely through activation of Pax5 expression.

## EBF regulates B lymphoid versus myeloid cell fate

To rigorously test if EBF promotes B cell fate at the expense of myeloid cell fate options, we did limiting-dilution analysis with EBF-GFP–transduced MPPs and assessed their efficiency of generation of CD19$^+$ progeny on days 7 and 14 (**Fig. 4**). On day 7, control MPPs generated exclusively Mac-1$^+$ myeloid progeny at plating densities ranging from 100 cells per well to 5 cells per well (**Fig. 4a**). CD19$^+$ B cell progeny were generated from MPPs transduced with control GFP virus by day 14 only at plating densities above 100 cells per well (data not shown). In contrast, EBF-GFP–transduced MPPs generated CD19$^+$ cells even at the lowest cell density (5 cells per well) by day 7. Limiting-dilution analysis of EBF-GFP–transduced MPPs showed a frequency of 1 in 8 for B cell generation (**Supplementary Fig. 6a** online). Consistent with results reported above (**Fig. 3b**), Pax5-transduced MPPs generated CD19$^+$ cells at a much lower frequency than did EBF-GFP–transduced MPPs (**Fig. 4a**). Notably, when we plated EBF-GFP–transduced MPPs at a density of 5 cells per well, of the wells in which MPPs expanded and differentiated, 57% contained exclusively CD19$^+$ cells, 29% contained both B lineage (CD19$^+$) and myeloid (Mac-1$^+$) progeny, and the remaining 14% contained only Mac-1$^+$ cells (**Fig. 4b**). Higher EBF expression did not appreciably affect the plating efficiency of MPPs (**Supplementary Fig. 6b**). These results suggest that EBF 'dictates' B cell fate in an MPP at the expense of myeloid cell fates.

To unequivocally establish that EBF directs individual MPPs along the B cell pathway at the expense of myeloid options, we analyzed single-cell cultures of sorted MPPs transduced with control or EBF virus. For these experiments we used Flt3$^{lo}$ Lin$^-$Sca-1$^+$c-Kit$^+$ cells, to avoid both HSCs and LMPPs (Flt3$^{hi}$ Lin$^-$Sca-1$^+$c-Kit$^+$ cells)[5]. When transduced with EBF-GFP and cultured in SCF, Flt3L and IL-7, most individual MPPs gave rise to B cell progeny or mixed cultures containing both CD19$^+$ and Mac-1$^+$ cells. That finding was in contrast to results obtained with cells transduced with control GFP, which gave rise to exclusively Mac-1$^+$ colonies (**Fig. 4d** and **Supplementary Table 1** and **Fig. 7** online). Even when individual EBF-GFP–transduced MPPs were plated in myeloid-promoting conditions (SCF and M-CSF), half of the wells contained B lineage progeny (**Fig. 4e** and **Supplementary Table 1**). As noted above (**Fig. 3a**,**b**), myeloid cells expressing EBF can be generated from MPPs, and this may reflect a requirement for EBF accumulation and action before that of the competing myeloid determinants such as C/EBPα and PU.1. Nevertheless, these data demonstrate that EBF can direct individual MPPs along the B pathway at the expense of alternative myeloid options.

To gain insight into the molecular pathways by which EBF induces B cell development and antagonizes myeloid cell fate options, we assessed the expression of key B and myeloid lineage genes shortly (48 h) after transduction of MPPs. As expected, EBF-GFP–transduced MPPs had higher expression of EBF transcripts than did their counterparts transduced with control GFP (**Fig. 4c**). The abundance of EBF transcripts was similar to that in pro–B cells (data not shown). Higher expression of EBF in MPPs resulted in induction of *Pax5* and the early B lineage genes *Igll1*, *Vpreb1*, *Cd79a* and *Cd79b*, as well as upregulation of *Il7ra* (**Fig. 4c**). These results demonstrate that EBF activates expression of *Pax5* in MPPs during the course of B cell fate specification. Although EBF is not required for the initial expression of *Il7ra*[12], our results suggest that EBF may function in a feedback loop to augment *Il7ra* expression. A subset of early B lineage genes (*Igll1*, *Vpreb1* and *Cd79a*) are most strongly induced by EBF, and they represent direct targets[36]. Notably, expression of *Cebpa*, which encodes the myeloid cell fate determinant C/EBPα, was lower in MPPs after expression of EBF (**Fig. 4c**). In contrast, we found no substantial changes in expression of *PU.1* or *E2A* in the EBF-GFP–transduced MPPs. These results raised the possibility that EBF could inhibit myeloid cell fate options of an MPP by antagonizing expression of *Cebpa* (discussed below).

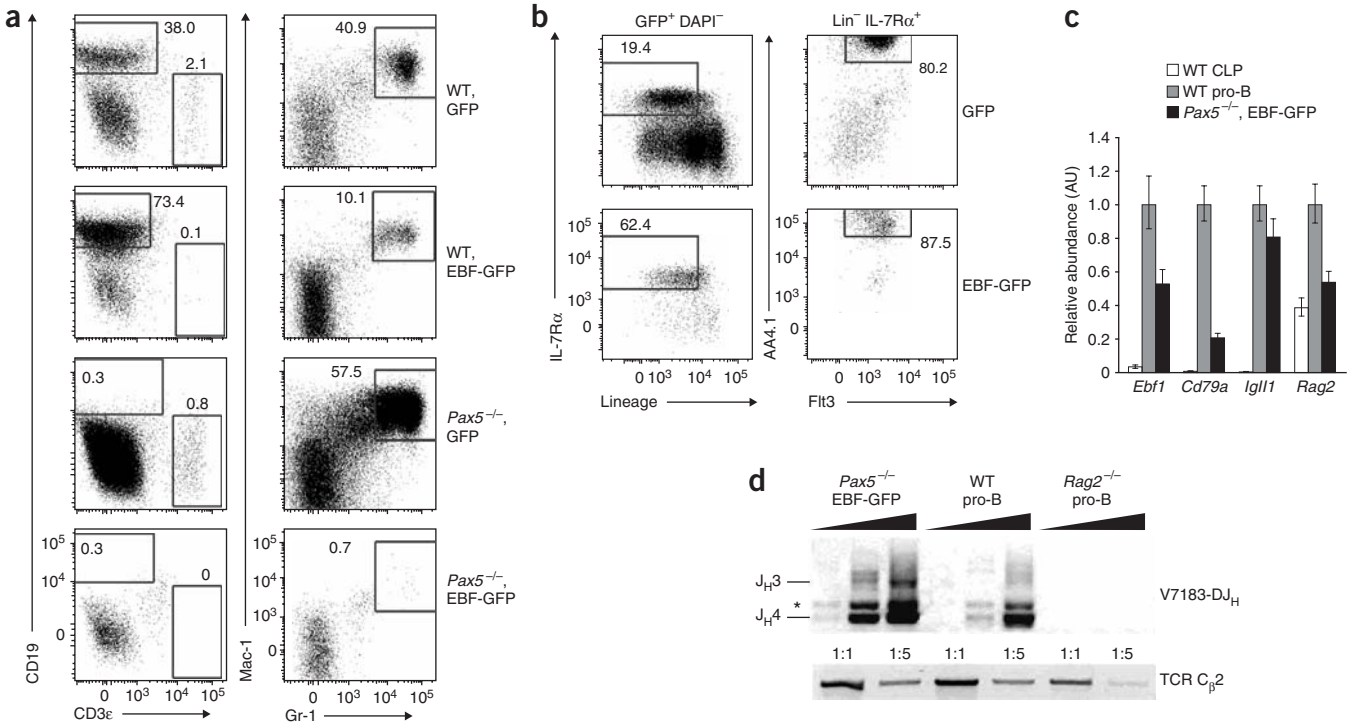


**Figure 6** EBF inhibits non–B cell fates independently of Pax5. Fetal liver progenitor cells from *Pax5*$^{-/-}$ mice or wild-type (WT) littermate control mice (both CD45.2$^+$) were transduced with control GFP or EBF-GFP retrovirus, then transferred intravenously into irradiated B6.CD45.1 congenic mice; 6 weeks after transfer, host bone marrow cells were analyzed for donor-derived (CD45.2$^+$) lineages. (**a**) Flow cytometry of donor-derived B lineage (CD19$^+$), T lineage (CD3ε$^+$) and myeloid lineage (Mac-1$^+$Gr-1$^+$) progeny among cells gated on viable (DAPI$^-$) GFP$^+$ CD45.2$^+$ cells. (**b**) Flow cytometry of B lymphoid progenitor cells (Flt3$^+$AA4.1$^+$) among bone marrow cells derived from *Pax5*$^{-/-}$ progenitor cells transduced with control GFP or EBF-GFP virus (GFP$^+$CD45.2$^+$), gated on Lin$^-$IL-7Rα$^+$ cells. Numbers adjacent to outlined areas indicate percent cells in gated regions (**a**,**b**). (**c**) Quantitative PCR analysis of gene expression by B lymphoid progenitor cells (Lin$^-$IL-7Rα$^+$Flt3$^+$AA4.1$^+$) derived from EBF-GFP–transduced *Pax5*$^{-/-}$ hematopoietic progenitor cells; cDNA from wild-type CLPs (Lin$^-$IL-7Rα$^+$c-Kit$^{lo}$AA4.1$^+$Flt3$^+$) and wild-type pro–B cells (pro-B; B220$^+$CD43$^+$AA4.1$^+$CD19$^+$) serves as a control. Transcript abundance is presented relative to the value in pro–B cells, set as 1; error bars, 95% confidence interval after normalization to 18S rRNA from triplicate samples. AU, arbitrary units. (**d**) *Igh* DNA recombination analysis of B lymphoid progenitor cells (Lin$^-$IL-7Rα$^+$Flt3$^+$AA4.1$^+$) derived from EBF-GFP–transduced *Pax5*$^{-/-}$ hematopoietic progenitor cells; DNA from wild-type or recombination-activating gene 2–deficient (*Rag2*$^{-/-}$) pro–B cells (AA4.1$^+$B220$^+$CD43$^+$CD19$^+$) serves as a control. Bottom, amplification of the *Tcrb* locus (TCR-Cβ2) with fivefold dilutions of genomic DNA, used as a loading control; wedges indicate serial dilution of genomic DNA used for PCR. Data are representative of two experiments (**c**,**d**) or three host mice per experimental group in two experiments (**a**,**b**).

## EBF can 'reprogram' myeloid progenitor cells

Given that EBF antagonizes myeloid cell fates in the context of an MPP, we sought to determine if EBF could redirect myeloid progenitor cells along the B pathway. To test this possibility, we isolated common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs) from bone marrow[37] and transduced them with the control GFP or EBF-GFP retrovirus, then sorted the resulting GFP$^+$ cells and cultured them on OP42 stromal cells in the presence of SCF, Flt3L and IL-7. CMPs or GMPs transduced with the control GFP virus had limited capacity to proliferate in B lymphoid–promoting conditions, yet a few cells differentiated into Mac-1$^+$CD19$^-$ progeny (**Fig. 5a**). Notably, at these plating densities, no CD19$^+$ progeny were generated at day 7 or 14 (**Fig. 5a** and data not shown). In contrast, ectopic expression of EBF in CMPs or GMPs induced the generation of CD19$^+$ progeny (**Fig. 5a**). EBF expression in CMPs resulted in a 50% reduction in overall cell yield (**Supplementary Fig. 8** online). This reduction may reflect EBF antagonism of the myeloid gene expression program, particularly cytokine receptors such as M-CSFR and GM-CSFR, whose expression is dependent on PU.1 (ref. 38; discussed below). EBF promoted B cell generation from CMPs more efficiently than from GMPs. We used limiting-dilution assays to analyze the frequency of B cell generation by EBF in CMP and GMP populations. EBF-GFP–transduced CMPs generated CD19$^+$ precursors at a frequency of 1 in 800, which was approximately fourfold higher than the frequency for B cell generation from EBF-expressing GMPs (data not shown). As noted above, similar limiting-dilution analysis of EBF-GFP–transduced MPPs showed a much higher frequency of B cell generation (1 in 8; **Supplementary Fig. 6a**). These results demonstrate that EBF can efficiently induce B cell development from MPPs but its ability to do so decreases in progressively restricted myeloid progenitor cells. This difference may be a consequence of EBF's competing less effectively with myeloid cell fate determinants and activating repressed B lineage genes in the myeloid progenitor cells.

## EBF attenuates expression of *PU.1* and *Cebpa*

To analyze the mechanism by which EBF mediates the 'reprogramming' of myeloid progenitor cells, we assessed the expression of key myeloid regulatory genes shortly (48 h) after transduction of CMPs and GMPs with the EBF-GFP retrovirus. As in MPPs, transduction of EBF-GFP into CMPs or GMPs resulted in downregulation of *Cebpa* expression (**Fig. 5b**,**c**). *Cebpa* expression was reduced most by EBF expression in GMPs (10% that of controls). Unlike in MPPs, EBF expression in CMPs and GMPs downregulated *PU.1* expression (**Fig. 5b**,**c**). Consistent with those findings, transduction of CMPs with

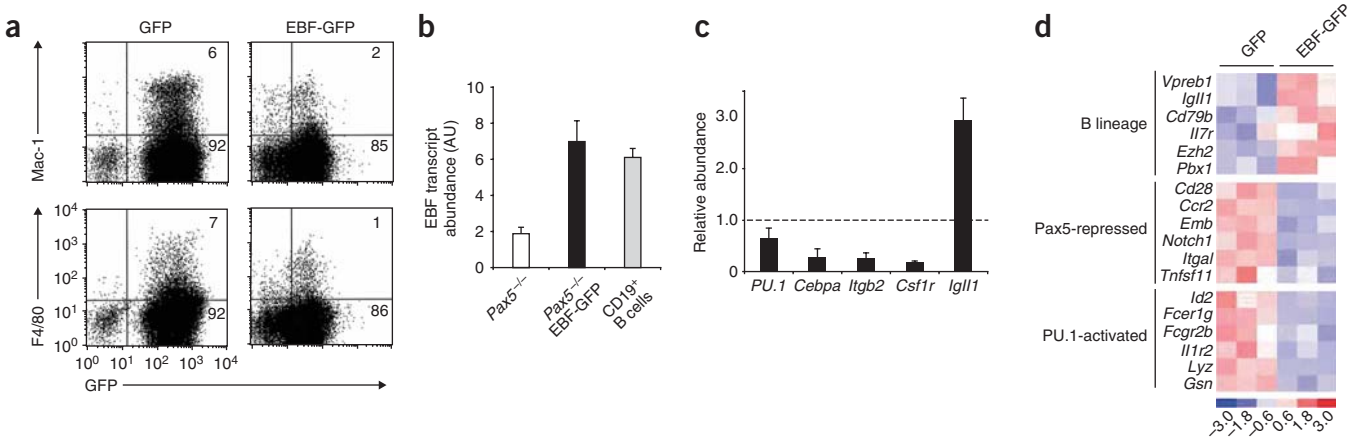


**Figure 7** Constitutive expression of EBF blocks the myeloid lineage differentiation of *Pax5*$^{-/-}$ pro–B cells. (**a**) Generation of myeloid (Mac-1$^{+}$F4/80$^{+}$) cells by *Pax5*$^{-/-}$ pro–B cells transduced with control GFP or EBF-GFP retrovirus, sorted by flow cytometry after 2d, plated in myeloid differentiation conditions, and then collected after 5 d. Numbers in quadrants indicate percent cells in each. (**b**) Quantitative PCR analysis of EBF transcripts in *Pax5*$^{-/-}$ pro–B cells transduced with EBF-GFP; untransduced *Pax5*$^{-/-}$ pro–B cells and CD19$^{+}$ B lineage cells serve as controls. (**c**) Quantitative PCR analysis of transcript expression in *Pax5*$^{-/-}$ pro–B cells transduced with control GFP or EBF-GFP retrovirus and sorted by flow cytometry on the basis of GFP expression; transcript abundance in EBF-GFP–transduced samples is presented relative to that of control GFP–transduced samples (dashed line). *Hprt1* transcripts were used as internal controls in **b**,**c**. (**d**) DNA microarray analysis of mRNA isolated from *Pax5*$^{-/-}$ pro–B cells transduced with control GFP or EBF-GFP retrovirus. Gene clusters show EBF-activated B lymphoid genes and EBF-downregulated myeloid and T lineage genes. Middle cluster, genes repressed by Pax5; lower cluster, genes activated by PU.1. Below, linear quantitative scale for gene expression. Data are representative of three independent experiments (**a**) or are from three independent experiments (**b**–**d**; error bars, s.d.)

EBF-GFP resulted in impaired induction of Mac-1 in a concentration-dependent way (**Supplementary Fig. 9** online). The CD11b subunit of Mac-1 is encoded by *Itgb2*, a PU.1-regulated gene[39]. CMPs and GMPs have higher expression of PU.1 than do MPPs[40]. Furthermore, the development of myeloid cells requires higher PU.1 expression than does the development of B lineage cells[41]. We suggest that EBF antagonizes the upregulation of PU.1 that is necessary for myeloid differentiation (discussed below). These results demonstrate that EBF is capable of antagonizing the expression of both of the myeloid determinants C/EBPα and PU.1. The 'reprogramming' of myeloid progenitor cells by EBF is probably a consequence of down-regulation of these key cell fate determinants. However, this process is inefficient, as it was not accompanied by robust activation of *Il7ra* (data not shown).

## EBF restricts lineage options of *Pax5*$^{-/-}$ progenitor cells

Given that ectopic expression of EBF but not Pax5 in MPPs robustly promoted B cell generation and inhibited myeloid differentiation, we considered the possibility that EBF can restrict myeloid development options independently of Pax5. To directly test that possibility, we assessed whether constitutive expression of EBF could inhibit the myeloid as well as T lineage developmental potential of *Pax5*$^{-/-}$ progenitor cells. We transduced fetal liver progenitor cells from wild-type or *Pax5*$^{-/-}$ embryos (day 15) with the control GFP or EBF-GFP retrovirus and then transplanted them intravenously into irradiated CD45.1 congenic host mice. We analyzed bone marrow, spleens and thymi of recipient mice 6 weeks after transplantation for the presence of donor-derived (CD45.2$^{+}$) GFP$^{+}$ cells. Control mice transplanted with GFP-transduced wild-type HSCs showed efficient reconstitution of the B lineage, T lineage and myeloid lineage, as shown by their generation of GFP$^{+}$ cells expressing CD19, CD3ε and Mac-1, respectively (**Fig. 6a** and **Supplementary Figs. 10** and **11** online). Transduction of EBF-GFP into wild-type HSCs promoted the generation of B lineage cells (CD19$^{+}$) in the bone marrow, whereas it inhibited the development of Mac-1$^{+}$Gr-1$^{+}$ myeloid precursors (**Fig. 6a**). These results are consistent with an earlier finding that transduction of EBF into HSCs promotes B lymphopoiesis[42]. However, our experiments additionally provided *in vivo* evidence that EBF inhibited myeloid differentiation (**Fig. 6a** and **Supplementary Fig. 10**). As reported before[42], transduction of EBF-GFP in HSCs also resulted in failure of thymic T cell development (**Supplementary Fig. 11**). This led to a loss of mature T cells in spleen and bone marrow (**Fig. 6a** and **Supplementary Fig. 10**). The failure of T cell development after EBF expression could have been due to impaired survival and/or homing of prethymic progenitor cells. As expected[23], control GFP–transduced *Pax5*$^{-/-}$ progenitor cells readily generated myeloid and T lineage progeny but not CD19$^{+}$ B cells (**Fig. 6a** and **Supplementary Figs. 10** and **11**). Notably, EBF expression in *Pax5*$^{-/-}$ progenitor cells resulted in an inhibition of both myeloid lineage and T lineage development (**Fig. 6a** and **Supplementary Figs. 10** and **11**). Most donor-derived cells gave rise to CD19$^{-}$ B lineage progeny that were blocked for further development because of their lack of Pax5 (discussed below). These experiments demonstrate that EBF restricts the developmental potential of multipotent progenitor cells by a Pax5-independent mechanism.

## EBF regulates B cell fate independently of Pax5

We next determined if transduction of EBF-GFP into *Pax5*$^{-/-}$ progenitor cells resulted in the accumulation of B lineage progenitor cells that were blocked for further differentiation along the B pathway as a consequence of lacking Pax5. We further evaluated the bone marrow cells from the host mice described above (**Fig. 6a**) for the presence of B lymphoid progenitor cells. Mice transplanted with EBF-GFP–transduced *Pax5*$^{-/-}$ progenitor cells had a higher proportion of Lin$^{-}$IL-7Rα$^{+}$ cells (**Fig. 6b**). Most of these cells coexpressed Flt3 and AA4.1. To extend the characterization of the IL-7R$^{+}$AA4.1$^{hi}$ population that accumulated in the bone marrow after reconstitution with EBF-GFP–transduced *Pax5*$^{-/-}$ progenitor cells, we sorted these cells and examined their expression of B lineage genes as well as their status of DNA rearrangements at the *Igh* loci. These cells expressed the early

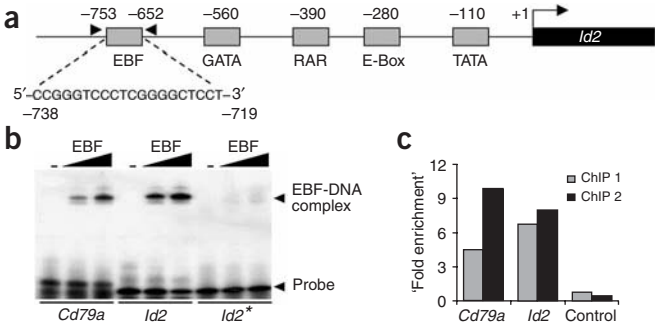


**Figure 8** EBF binds to the *Id2* promoter region both *in vitro* and *in vivo*. (**a**) *Id2* promoter, including the EBF-binding site and its sequence, as well as additional transcription factor motifs (GATA, RAR, E-box, TATA) in the promoter sequence. Triangles indicate positions of primers used to amplify the promoter region in chromatin immunoprecipitation experiments. (**b**) Electrophoretic mobility-shift assay of purified recombinant EBF (30 or 100 ng; wedges) and the *Id2* EBF-recognition site. *Cd79a*, binding site from the *Cd79a* (mb-1) promoter serves as a positive control to assess relative affinity; *Id2**, confirmation of binding specificity with a mutant probe in which half of the palindromic EBF-binding site is inverted. Data are representative of two independent experiments. (**c**) Chromatin immunoprecipitation analysis of PD36 pre–B cells: EBF-bound chromatin fragments were immunoprecipitated with anti-EBF; primers in **a** were used to amplify the *Id2* promoter region. 'Fold enrichment' indicates the ratio of DNA amplification from anti-EBF immunoprecipitation versus 'no-antibody' immunoprecipitation. The *CD79a* (mb-1) promoter region serves as a positive control; a region lacking an EBF-binding site on a different chromosome serves as a negative control. Data are from two independent experiments (ChIP 1 and ChIP 2).

B lineage genes *Cd79a* and *Igll1*, both of which are EBF targets (**Fig. 6c**). Consistent with the fact that *Cd79a* is a target of Pax5, its transcript abundance was lower in these *Pax5*$^{-/-}$ cells than in wild-type pro–B cells. Furthermore, after transplantation, EBF-GFP–transduced *Pax5*$^{-/-}$ multipotent progenitor cells expressed recombination-activating gene 2 and had proximal $V_H7183$-$DJ_H$ rearrangements known to occur in the absence of Pax5 (**Fig. 6c**,**d**). Notably, EBF transcript abundance in these cells was similar to that in wild-type pro–B cells but greater than that in wild-type CLPs (**Fig. 6c**). Therefore, sustained EBF expression in *Pax5*$^{-/-}$ hematopoietic progenitor cells, in a physiological range, can block their alternative lineage potentials while inducing B lineage specification. These data demonstrate that EBF not only induces the generation of B lineage progenitor cells but also restricts alternative lineage potentials in the absence of Pax5.

### EBF can repress myeloid genes independently of Pax5

*Pax5*$^{-/-}$ pro–B cells can differentiate into myeloid and T lineage cells[23,24]. During the differentiation of these cells into alternative lineages, EBF expression is downregulated. Given that EBF was able to restrict myeloid cell fates of early progenitor cells independently of Pax5 (**Fig. 6a**), we reasoned that sustained expression of EBF in *Pax5*$^{-/-}$ pro–B cells might also inhibit their ability to differentiate into myeloid cells. To test this, we constitutively expressed EBF in *Pax5*$^{-/-}$ pro–B cells by retroviral transduction. We allowed equivalent numbers of *Pax5*$^{-/-}$ pro–B cells transduced with control GFP or EBF-GFP retrovirus to differentiate for 5 d in myeloid conditions. EBF expression impaired the development of Mac-1$^+$ and F4/80$^+$ myeloid cells (**Fig. 7a**). Cumulative EBF expression in *Pax5*$^{-/-}$ pro–B cells was similar to that in CD19$^+$ B-lineage cells (**Fig. 7b**). EBF also blocked the upregulation of Mac-1 expression in a concentration-dependent way (**Supplementary Fig. 9**). These results establish that EBF functions to inhibit myeloid cell differentiation independently of Pax5.

*Pax5*$^{-/-}$ pro–B cells inappropriately express many myeloid lineage genes[26]. We tested if EBF was able to repress these genes in the absence of Pax5. Constitutive expression of EBF in *Pax5*$^{-/-}$ pro–B cells resulted in downregulation of the expression of *Itgb2* and *Csf1r* and, as expected, induction of *Igll1* (**Fig. 7c**). Consistent with analysis of MPPs and myeloid progenitor cells, EBF expression in *Pax5*$^{-/-}$ pro–B cells resulted in the attenuation of expression of *Cebpa* and *PU.1* (**Fig. 7c**). To comprehensively analyze changes in lineage-specific patterns of gene activity regulated by EBF, we did genome-wide expression analysis. As anticipated, EBF enhanced the expression of a set of early B lineage genes that function in B cell development (**Fig. 7d**). This analysis also identified a large set of genes whose expression was downregulated by EBF (**Fig. 7d** and **Supplementary Table 2** online). Notably, many of these genes (*Cd28*, *Ccr2*, *Emb*, *Notch1*, *Itgal* and *Tnfsf11*) seem to be also repressed by Pax5 (ref. 26). In addition, sustained expression of EBF resulted in the downregulation of a set of genes (*Id2*, *Fcer1g*, *Fcgr2b*, *Il1r2*, *Lyz* and *Gsn*) that are activated by PU.1 during myeloid differentiation[10]. We confirmed the downregulation of these genes by EBF in *Pax5*$^{-/-}$ pro–B cells by quantitative PCR (data not shown). Thus, EBF represses alternative lineage genes independently of Pax5.

### *Id2* and *Cebpa* are EBF targets

We examined the promoter regions of alternative lineage genes that undergo EBF-mediated repression in *Pax5*$^{-/-}$ progenitor cells for putative EBF-binding sites. Among these, the *Id2* and *Cebpa* promoter regions contained presumptive EBF-binding sites related to known sites in B lineage targets (**Fig. 8a** and **Supplementary Fig. 12a**,**b** online). EBF bound to each of these sites, as shown by gel-shift assay (**Fig. 8b** and **Supplementary Fig. 12c**). The EBF-binding site in the *Cebpa* promoter region was of somewhat lower affinity than the comparable sites in *Id2* and *Cd79a*. To determine if EBF binds to these target promoters *in vivo*, we did chromatin immunoprecipitation analysis. EBF crosslinking to the *Id2* promoter was readily detectable in PD36 pro–B cells (**Fig. 8c**). However, those cells did not have discernable binding of EBF to the *Cebpa* promoter region. To extend that chromatin immunoprecipitation analysis, we used *Ebf1*$^{-/-}$ progenitor cells expressing an epitope-tagged variant of EBF. In these cells, EBF binding was detectable on both the *Id2* and *Cebpa* promoters (**Supplementary Fig. 12d**). Consistent with the lower affinity of the EBF-binding site in the *Cebpa* promoter, crosslinking of EBF to this site was less efficient. Nevertheless, these results demonstrate that EBF binds to *Id2* and *Cebpa*, which undergo repression during B cell fate specification.

## DISCUSSION

The nature of the transcription factors that initiate B cell fate 'choice' by antagonizing alternative cell fate determinants has been unclear. Here we have shown that EBF promotes a B cell fate by antagonizing myeloid developmental options. This result was established by enforced expression of EBF in MPPs and clonal analysis and was further supported by the generation of B cells from CMPs and GMPs after ectopic expression of EBF. An earlier study involving transduction of EBF into HSCs and their transplantation noted that EBF promoted B cell development but failed to demonstrate a function for EBF in myeloid cell fate antagonism[42]. That may have been due to a

difference in the expression or kinetics of accumulation of EBF in MPPs. Furthermore, that study did not analyze the developmental potential of individual EBF-expressing MPPs in conditions in which such cells could generate both myeloid and B lineage precursors. Here we have shown EBF antagonized the expression of *Cebpa* in MPPs and attenuated the expression of *Cebpa* and *PU.1* in CMPs and GMPs. Although PU.1 is also required for early B cell development, a high PU.1 concentration is inhibitory for B lymphopoiesis[41]. We suggest that EBF attenuates the expression of *PU.1* during the course of B cell fate specification. Furthermore, we propose that in the context of bone marrow LMPPs, induction of EBF in a subset of such cells and its antagonism of *PU.1* and *Cebpa* inhibits myeloid cell fate options, thereby generating lymphoid-restricted progenitor cells such as CLPs.

*Ebf1*$^{-/-}$ progenitor cells propagated *in vitro* with the cytokines SCF, Flt3L and IL-7 resembled lymphoid progenitor cells, as they expressed IL-7R and had $D_H$-$J_H$ rearrangements. However, after transplantation, *Ebf1*$^{-/-}$ progenitor cells reconstituted not only lymphoid (T cell and NK cell) but also myeloid lineages. Wild-type CLPs show poor myeloid differentiation potential and do not have appreciable Pax5 expression[32,43]. As proposed above, EBF expression in CLPs could antagonize the upregulation of *PU.1* and *Cebpa* expression, thereby attenuating their myeloid options. Consistent with our findings, C/EBPα expression is inversely correlated with EBF expression in LMPPs and CLPs[43].

EBF antagonized the expression of *Cebpa* in many cellular contexts, including MPPs, CMPs, GMPs and *Pax5*$^{-/-}$ pro–B cells, and it antagonized the expression of *PU.1* in the last three. Furthermore, higher expression of EBF did not affect the plating efficiency of MPPs. Therefore, it is unlikely that the EBF-mediated antagonism of myeloid determinants was simply a consequence of selective elimination of differentiating myeloid precursors within 48 h of retroviral transduction. Ectopic EBF expression can be tolerated in differentiated myeloid cells. Thus, our data are compatible with the interpretation that above a threshold concentration, EBF is capable of antagonizing expression of *Cebpa* and *PU.1*, thereby inhibiting myeloid lineage options. Notably, we have also shown that the *Cebpa* promoter contained an EBF site that was bound *in vitro* and *in vivo*. Although this site had lower affinity for EBF than did *Cd79a*, our data are consistent with the possibility that EBF may directly repress *Cebpa*. Similar analysis of the *PU.1* promoter did not demonstrate EBF-binding sites. Notably, ectopic expression of EBF in a *PU.1*$^{-/-}$ progenitor cell line expressing a conditionally activatable PUER fusion protein[10] inhibited PU.1-dependent macrophage differentiation (J.M.R.P. and H.S. unpublished data). Furthermore, EBF downregulated a set of myeloid genes activated by PU.1. These results raise the possibility that EBF antagonizes the activity of PU.1 protein, thereby disrupting expression of its target genes, including *PU.1*, which is part of an autoregulatory loop suggested to contribute to *PU.1* expression[44].

*Ebf1*$^{-/-}$ progenitor cells seemed to differ from their *E2A*$^{-/-}$ counterparts as well as their *Pax5*$^{-/-}$ counterparts in their ability to reconstitute various hematopoietic lineages. Unlike *Pax5*$^{-/-}$ cells[45], *Ebf1*$^{-/-}$ progenitor cells reconstituted myeloid as well as lymphoid lineages within 4 weeks of transplantation. In contrast to reconstitution of the myeloid and T lineages, reconstitution of the erythroid lineage by *Ebf1*$^{-/-}$ progenitor cells is inefficient. The erythroid lineage is more efficiently reconstituted by *E2A*$^{-/-}$ progenitor cells[46]. Notably, E2A has been shown to antagonize *Gata1* expression. Consistent with those results, *E2A*$^{-/-}$ progenitor cells have appreciable *Gata1* expression[46], whereas *Ebf1*$^{-/-}$ progenitor cells have low *Gata1* expression. Such findings suggest that *E2A*$^{-/-}$ progenitor cells may be more efficient in generating erythrocytes than are *Ebf1*$^{-/-}$ progenitor cells because they have more GATA-1. These results collectively provide molecular insight into a stepwise process of B cell fate restriction leading to the generation of B lineage precursors. E2A function in the context of an MPP may result in the generation of an LMPP that has lost erythroid and megakaryocytic developmental potentials but retains myeloid and lymphoid potential[5,47,48]. Initial induction of EBF in an LMPP would then restrict myeloid developmental potential by antagonizing the expression and/or upregulation of *PU.1* and *Cebpa*, leading to the generation of a CLP.

We have shown here that sustained expression of EBF in *Pax5*$^{-/-}$ hematopoietic progenitor cells inhibited their myeloid and T lineage developmental options *in vivo* and also inhibited the myeloid differentiation capacity of *Pax5*$^{-/-}$ pro–B cells *in vitro*. Therefore, we propose that the developmental plasticity of *Pax5*$^{-/-}$ pro–B cells reflects a failure to sustain sufficient EBF. We suggest that in the absence of IL-7 signaling, *Pax5*$^{-/-}$ pro–B cells fail to maintain *Ebf1* expression[28,29] and therefore can differentiate into other cell types in response to alternative developmental signals[23,24]. Consistent with our proposal, Pax5 functions in a feedback loop to stabilize EBF expression[49,50]. We predict that conditional deletion of *Ebf1* in B lineage cells would lead to phenotypes similar to that noted after induced Pax5 deletion[25,26].

The function of Pax5 as an activator in B lineage cells is contingent on EBF[12]. For example, Pax5 is unable to induce expression of CD19 and *Cd79a* in *Ebf1*$^{-/-}$ progenitor cells. Consistent with that functional analysis, EBF seems to have molecular features that enable it to initiate developmental alterations in chromatin structure and to relocalize target genes in relation to heterochromatin[31,51]. Notably, unlike EBF, Pax5 seems to inefficiently inhibit the differentiation of *Ebf1*$^{-/-}$ progenitor cells into myeloid progeny (data not shown). In fact, 'precocious' expression of Pax5 in HSCs fails to interfere with the development of myeloid lineages[52]. That study[52] suggests that Pax5 promotes B cell generation at the expense of T cells by repressing Notch1. Given that EBF can inhibit the generation of T cells even in the absence of Pax5 and that EBF expression is sustained by Pax5 as part of a positive feedback loop[32,50], we propose that the Pax5-mediated block in T cell generation is EBF dependent. Consistent with that possibility, T lineage cells misexpressing Pax5 express EBF[49]. Thus, we suggest that both promotion of B lineage and inhibition of myeloid and T lineage developmental options by Pax5 are contingent on EBF.

EBF binds to the *Id2* promoter and represses expression of *Id2*. Downregulation of *Id2* in CLPs by EBF would augment E2A activity[19]. Sustained expression of an Id protein in transgenic mice has been shown to block early B cell development[53]. We therefore propose that B cell fate specification may be mediated by the sequential actions of EBF involving repression of *Id2* and other alternative lineage genes, followed by activation of *Pax5* and expression of the early program of B lineage genes.

It is noteworthy that EBF downregulates a subset of myeloid and T cell genes, such as *Cd28*, *Ccr2*, *Emb*, *Notch1*, *Itgal* and *Tnsf11*, considered to be Pax5 targets[25,26]. Of those, the *Notch1* promoter contains an EBF-binding site that is occupied *in vivo* (S. Firner, T.T. and R.G., unpublished observations). The other genes may contain EBF-binding sites in distal regulatory elements. Our results are compatible with the possibility that EBF and Pax5 can independently target these genes for repression but do so more efficiently when acting in a concerted way. In such a scenario, the molecular functions of EBF and Pax5 in repressing alternative lineage genes would parallel those of T-bet and Runx3, transcription factors that induce a T helper type 1 cell fate in part by repressing T helper type 2 cytokine genes in a synergistic way[54,55].

The transcription factor LRF has been suggested to function 'upstream' of EBF in regulating B cell fate at the expense of the T cell fate in the bone marrow[56]. Conditional deletion of the gene encoding LRF in HSCs results in the anomalous activation of Notch target genes and the generation of aberrant B220$^+$ DN T cell–like cells. We suggest that LRF functions to inhibit basal Notch signaling in bone marrow MPPs before their migration to the thymus[57,58], where they generate T lineage cells in response to robust Notch signaling. Because EBF can downregulate *Notch1* expression, we propose that it functions along with LRF to antagonize the sensing of basal Notch signals in bone marrow, thereby preventing inappropriate activation of the T lineage developmental program.

On the basis of those considerations and published data on the developmental control of EBF expression, we propose a regulatory circuit that uses EBF as a chief determinant of B cell versus myeloid cell fate in the bone marrow. *EBF* induction is controlled by at least three developmental 'inputs' (PU.1$^{lo}$, E2A, IL-7R), as its expression is impaired by mutations in the genes encoding each of those molecules[12,28–30]. Signaling through Flk2-Flt3 may also contribute to the expression of *EBF*, as combined loss of Flt3L and IL-7Rα results in a complete blockade of fetal and adult B lymphopoiesis[59]. We propose that these inputs are integrated by *cis* regulatory sequences in *EBF*. Consistent with that possibility, it has been shown that EBF expression is regulated by a promoter that is responsive to E2A and STAT5 (refs. 49,50). Such integration of multiple inputs would ensure activation of *EBF* in a stringent way, given its potent ability to initiate the B cell developmental program at the expense of alternative cell fates.

## METHODS

**Animals and antibodies.** Bone marrow from C57BL/6 male mice (6–8 weeks of age; Jackson Laboratories) was used for the purification of various progenitor populations. Unless otherwise indicated, antibodies were from BD Biosciences. All experiments involving mice used protocols approved by Institutional Animal Care and Use Committees of the University of Chicago and the University of Pennsylvania.

**Isolation of hematopoietic progenitor cells.** For *Ebf1*$^{-/-}$ progenitor cells, *Ebf1*$^{-/-}$ fetal liver cells were isolated from embryos at day 14.5 as described[12]. Lin$^-$B220$^+$ cells were plated on a stromal layer (OP42 or OP9 cells) and were maintained in Opti-MEM (Gibco-BRL) containing 4% (vol/vol) FCS, β-mercaptoethanol (50 μM), penicillin (10 U/ml) and streptomycin (10 μg/ml) and supplemented with SCF (10 ng/ml), Flt3L (10 ng/ml) and IL-7 (5 ng/ml). Four independent *Ebf1*$^{-/-}$ progenitor cell lines were established. Limiting dilution was used to isolate clones.

For MPPs, freshly isolated bone marrow cells were incubated with biotinylated antibodies to CD3ε (145-2C11), CD4 (H129.19), CD5 (53-7.3), CD8 (53-6.7), CD11b/Mac-1 (M1/70), CD19 (1D3), B220 (RA3-6B2), IgM, Gr-1 (Ly-6G) and Ter119 (Ly-76). Lineage-positive (Lin$^+$) cells were removed with a MACS cell separation system (Miltenyi Biotec) according to the manufacturer's recommendations. After depletion, Lin$^-$ cells were stained with allophycocyanin-conjugated antibody to c-Kit (anti-c-Kit), fluorescein isothiocyanate–conjugated anti-Sca-1 and biotinylated anti-CD27 (visualized by streptavidin-conjugated phycoerythrin-indotricarbocyanine). MPPs were sorted by flow cytometry as c-Kit$^{hi}$Sca-1$^{hi}$CD27$^+$ or c-Kit$^{hi}$Sca-1$^{hi}$Flt3$^{lo}$.

**CLPs, CMPs and GMPs.** Bone marrow samples were depleted of Lin$^+$ cells and CLPs were isolated on the basis of their expression of Flt3 and AA4.1. This population is c-Kit$^{lo}$ and therefore is defined as Lin$^-$IL-7R$^+$c-Kit$^{lo}$AA4.1$^+$Flt3$^+$ (ref. 57). Myeloid progenitor cells, CMPs and GMPs were isolated as described[37] with slight modification. Total bone marrow cells were incubated with monoclonal anti-c-Kit conjugated to microbeads (CD117 Microbeads; Miltenyi Biotech) and c-Kit$^+$ cells were obtained by two rounds of positive selection with a MACS cell separation system (Miltenyi Biotech). The c-Kit$^+$ cells were labeled with allophycocyanin-conjugated anti-c-Kit (2B8), phycoerythrin-conjugated anti-FcgRIII/II (2.4G2), fluorescein isothiocyanate–conjugated anti-CD34 (RAM34), and biotin-conjugated anti-Sca-1 (E13-161.7) and anti-IL-7R (A7R34; eBioscience). Biotinylated antibodies were visualized by phycoerythrin-indotricarbocyanine. CMPs were sorted as Lin$^-$IL-7Rα$^-$Sca-1$^-$c-Kit$^+$CD34$^+$ FcγRIII/II$^{lo}$; GMPs were purified as Lin$^-$IL-7Rα$^-$Sca-1$^-$c-Kit$^+$CD34$^+$ FcγRIII/II$^{hi}$. A MoFlo cell sorter (DakoCytomation) or FACSAria (BectonDickinson) was used for all progenitor isolation.

**Retroviral transduction.** After being sorted by flow cytometry, MPPs were transduced by coculture or spin infection. GP+E-86 packaging cell lines were used for coculture infection as described[41]. Progenitors were plated onto irradiated (2,500 rads from a cesium source) GP+E-86 packaging cells producing retrovirus encoding GFP alone (MIGR1), EBF-GFP (Mig-EBF) or Pax5-GFP (Mig-Pax5) in Opti-MEM containing 2.5% (vol/vol) FBS, β-mercaptoethanol (50 μM), penicillin (10 U/ml) and streptomycin (10 μg/ml; complete medium) supplemented with polybrene (10 μg/ml), SCF (50 ng/ml), IL-3 (5 ng/ml), IL-6 (10 ng/ml), IL-7 (5 ng/ml) and GM-CSF (10 ng/ml), and were maintained in culture for 2 d. For spin infection, retroviral supernatants were generated by transient transfection of PLAT-E cells with retroviral constructs encoding GFP (MIGR1), EBF-GFP (Mig-EBF) or E12-GFP (S003-E12) as described[12]. During spin infection, progenitor cells were resuspended in retroviral supernatants of equivalent titers supplemented with polybrene (10 μg/ml) and were centrifuged for 2.5 h at 1,400*g*. After being resuspended, cells were transferred onto irradiated stromal cells (OP42) and were maintained for 2 d in the presence of multilineage cytokines as described above. In some experiments, MPPs were stimulated overnight with SCF (50 ng/ml), IL-3 (5 ng/ml) and IL-6 (10 ng/ml). The next day, cells were spin infected and were maintained in the presence of multilineage cytokines as described above. After 24 h, cultures were collected for isolation of transduced cells on the basis of GFP expression.

CMPs, GMPs, *Ebf1*$^{-/-}$ progenitor cells and *Pax5*$^{-/-}$ pro–B cells were infected by spin infection with control GFP or EBF-GFP retroviral supernatants as described above. After infection, CMPs and GMPs were maintained on OP42 stromal cells in the presence of SCF (50 ng/ml), IL-3 (5 ng/ml) Flt3L (50 ng/ml) and IL-7 (5 ng/ml). *Ebf1*$^{-/-}$ progenitor cells were maintained on OP9 stromal cells in the presence of SCF (10 ng/ml), Flt3L (10 ng/ml) and IL-7 (5 ng/ml). *Pax5*$^{-/-}$ pro–B cells were cultured on ST2 cells as described[22] in the presence of IL-7 (5 ng/ml). After 2 d, transduced cells were sorted by flow cytometry on the basis of GFP expression and then were allowed to differentiate in lymphoid or myeloid conditions or were subjected to gene expression analysis.

**Assay of lineage potential of hematopoietic progenitor cells.** *Ebf1*$^{-/-}$ progenitor cells were differentiated into B lineage precursors by restoration of EBF expression and were cultured for 7 d on a stromal layer (OP9 or OP42 cells) in the presence of Opti-MEM (Gibco-BRL) containing SCF (10 ng/ml), Flt3L (10 ng/ml) and IL-7 (5 ng/ml). The myeloid differentiation capacity of *Ebf1*$^{-/-}$ progenitor cells was assessed by plating for a period of 2–6 d on OP9 stroma and the media described above containing 4% (vol/vol) FCS, but supplemented with SCF (10 ng/ml), Flt3L (10 ng/ml), GM-CSF (10 ng/ml) and M-CSF (10 ng/ml). The early T lineage differentiation capacity of *Ebf1*$^{-/-}$ progenitor cells was analyzed by culture on OP9-DL1 stroma as described[33]. All cultures were supplied with fresh media every 3 d and were collected on day 7. After differentiation in B lymphoid, myeloid or T lymphoid conditions, cultures were collected at various times and analyzed by flow cytometry.

The differentiation capacity of MPPs after retroviral transduction was assessed *in vitro* as described[35]. Transduced progenitor cells were sorted by flow cytometry on the basis of GFP expression and were plated onto irradiated OP42 stromal cells and cultured in complete medium supplemented with lymphoid- or myeloid-promoting cytokines. In B lymphoid–promoting conditions, medium was supplemented with SCF (50 ng/ml), Flt3L (50 ng/ml) and IL-7 (5 ng/ml). After 2 d, cells were supplied with fresh medium containing SCF and IL-7 and cultured for 3 d and then were maintained in medium containing IL-7 alone. In myeloid-promoting conditions, medium was supplemented with GM-CSF (10 ng/ml); after 2 d of culture, cells were maintained in the presence of M-CSF (10 ng/ml).

The B cell differentiation capacity of CMPs and GMPs transduced with control GFP or EBF-GFP retrovirus was assessed in lymphoid conditions as described above. The myeloid differentiation capacity of CMPs transduced with

EBF-GFP retrovirus was assessed in the presence of GM-CSF (10 ng/ml). After 4 d, cells were collected and analyzed for the generation of myeloid progeny by staining for Mac-1 expression. The myeloid differentiation capacity of *Pax5*$^{-/-}$ pro–B cells was determined by plating of cells for 5 d on γ-irradiated ST2 cells in DMEM containing 2% (vol/vol) FCS supplemented with recombinant cytokines, IL-7 (0.5 ng/ml) and M-CSF (25 ng/ml). After differentiation, cultures were collected and analyzed for the expression of Mac-1, Gr-1 or F4/80.

**Limiting dilution analysis.** Transduced MPPs (Lin$^-$c-Kit$^{hi}$Sca-1$^{hi}$CD27$^+$) were directly sorted by flow cytometry onto irradiated OP42 stromal layer (5–100 cells per well) in triplicate, were cultured in lymphoid conditions as described above and were analyzed after 7 or 14 d.

For single-cell analysis, MPPs (Lin$^-$c-Kit$^{hi}$Sca-1$^{hi}$Flt3$^{lo}$) were transduced with control GFP or EBF-GFP retrovirus by spin infection. After 30 h, single GFP$^+$ cells were directly sorted into 96-well plates containing OP9 stromal cells and were maintained in the presence of SCF (10 ng/ml), Flt3L (10 ng/ml) and IL-7 (10 ng/ml; lymphoid conditions) or SCF and M-CSF (myeloid conditions). Cultures maintained in lymphoid and myeloid conditions were collected after 18 d and 12 d, respectively, and were analyzed for the generation of CD19$^+$Mac-1$^-$ and/or CD19$^-$Mac-1$^+$ cells.

**Adoptive transfer of *Ebf1*$^{-/-}$ or *Pax5*$^{-/-}$ progenitor cells.** *Ebf1*$^{-/-}$ progenitor lines or clones derived from them were expanded and resuspended in PBS containing 0.5% (vol/vol) FCS. *Ebf1*$^{-/-}$ progenitor cells (CD45.2$^+$; 5 × 10$^6$ cells) were mixed with wild-type bone marrow cells (CD45.1$^+$; 2 × 10$^5$ cells) and were injected into the retro-orbital sinuses of lethally irradiated (1,200 rads) CD45.1$^+$ recipient host mice. *Pax5*$^{-/-}$ hematopoietic progenitor cells were isolated from fetal livers of *Pax5*$^{-/-}$ embryos at day 15 of gestation. *Pax5*$^{-/-}$ progenitor cells were stimulated for 18 h with 10 ng/ml of IL-3, IL-6 and SCF before transduction by spin infection with GFP (MigR1) or EBF-GFP (Mig-EBF) retrovirus. After 24 h, cells were injected into irradiated CD45.1 host mice. Mice were killed 4–6 weeks after transplantation and the reconstitution of various hematopoietic lineages was analyzed by flow cytometry.

**Gene expression analysis.** Poly(A) mRNA or total RNA was isolated from cells with a Micro mRNA purification kit (Amersham Biosciences) or TRIzol reagent (Invitrogen), respectively, and then was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen). Values obtained were normalized to values obtained with amplification of *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase). Two to three independent cDNA samples were used for semiquantitative PCR analysis. PCR-amplified products were visualized by ethidium bromide staining, except for *Il7ra* and *Pax5*, which were analyzed by Southern blot. Expression of genes in transduced cells was also assessed by quantitative PCR with the Mx4000 system (Stratagene).

RNA from EBF-GFP–transduced *Pax5*$^{-/-}$ B cell progenitor cells or CLPs (Lin$^-$IL-7R$^+$c-Kit$^{lo}$AA4.1$^+$Flt3$^+$) was isolated after cells were sorted directly into lysis buffer. After reverse transcription, gene expression was analyzed on an ABI7300 with 18S RNA as a control. Taqman probes and Taqman Gene expression analysis master mix were used according to the manufacturer's directions (ABI). Sequences of primers used for semiquantitative and quantitative PCR are available on request.

**Rearrangement of *Igh* loci.** CD19$^+$ B lineage progeny generated from *Ebf1*$^{-/-}$ progenitor cells after restoration of EBF expression, or from IL-7R$^+$AA4.1$^{hi}$ cells generated from *Pax5*$^{-/-}$ progenitor cells transduced with EBF and sorted by flow cytometry, were resuspended in PCR lysis buffer (10 mM Tris, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.5% (vol/vol) Nonidet-P40, 0.5% (vol/vol) Tween-20 and 40 μg/ml of proteinase K) and were lysed by incubation for 6–8 h at 50 °C. Proteinase K was heat-inactivated for 10 min at 95 °C and genomic DNA samples were used immediately for PCR. Values obtained by PCR were normalized relative to the PCR amplification of *Acta1* (encoding α-actin)[60] or the constant region of *Tcrb* (encoding the T cell receptor β-chain)[57].

For the detection of *Igh* rearrangements, samples were amplified by PCR in 35 cycles of 1 min at 95 °C, 1 min at 63 °C and 1.5 min at 72 °C, followed by 10 min at 72 °C, with the DHL 5′ primer[61] for detection of $D_H$-$J_H$ rearrangements or with the 7183 5′ primer[61] for detection of $V_H$-$DJ_H$ rearrangements, in combination with the JH4A3′ primer[62]. PCR-amplified products were separated by 1.3% agarose gel electrophoresis and then were analyzed by Southern blot with a $^{32}$P-labeled probe spanning the $J_H3$-$J_H4$ region of the *Igh* locus. *Igh* rearrangements in IL-7R$^+$AA4.1$^{hi}$ cells were assessed with oligonucleotides as described[63].

**DNA microarray analysis.** *Pax5*$^{-/-}$ pro–B cells were infected by spin infection and were maintained for 2 d in lymphoid culture conditions, then were collected and sorted by flow cytometry on the basis of GFP expression. Total RNA was isolated with TRIzol reagent (Invitrogen) and was further purified on RNeasy columns (Qiagen). RNA concentration and purity was determined with an ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity was assessed with an Agilent 2100 Bioanalyser (Agilent Technologies). Biotin-labeled cRNA was generated and was hybridized to the Mouse Genome 430 2.0 Array according to the manufacturer's instructions (Affymetrix). Data were analyzed with a dChip Analyzer 2006 using Affymetrix CEL files. A PM-only model was used for generating gene signal intensities. The invariant-set approach was used for normalization[64]. Thresholds for selecting important genes were set at a relative difference of greater than 1.4. Changes in gene expression patterns for *Pax5*$^{-/-}$ pro–B cells transduced with EBF-GFP versus those transduced with GFP were evaluated with a paired *t*-test and were considered statistically significant with a *P* of less than 0.05.

**Electrophoretic mobility-shift assay.** Recombinant EBF protein (amino acids 26–422) was expressed as a six-histidine-tagged fusion protein in *Escherichia coli* and was purified by affinity chromatography. Purified protein (30 or 100 ng) was incubated for 20 min at 20 °C with 2 fmol of 5′-end-radiolabeled double-stranded oligonucleotides in a final volume of 20 μl gel-shift buffer (20 mM HEPES, pH 7.6, 5% (vol/vol) glycerol, 75 mM NaCl, 2.5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mg/ml of BSA and 4 ng/μl of dI:dC). Reaction products were separated by 6% native PAGE containing 5% (vol/vol) glycerol; gels were analyzed by autoradiography. Oligonucleotides used as probes are as follows (underlining indicates EBF-binding sites; lower case indicates mutated nucleotides): *Id2*WT, 5′-CAGAGGAGCCCCGAGGGACCCGGTG-3′ and 5′-CACCGGGTCCCTCGGGGCTCCTCTG-3′; *Id2*mut, 5′-CAGAGGAGCCCCGAcccACCCGGTG-3′ and 5′-CACCGGGTgggTCGGGGCTCCTCTG-3′; and *Cd79a*, 5′-GAGAGAGACTCAAGGGAATTGTGGCCAGCG-3′ and 5′-CGCTGGCCACAATTCCCTTGAGTCTCTCTC-3′.

**Chromatin crosslinking and immunoprecipitation assays.** PD36 cells (5 × 10$^6$) were fixed for 5 min at 37 °C with 1% (vol/vol) formaldehyde, after which the reaction was 'quenched' with 100 mM glycine. Cells were washed three times with PBS and were lysed in 600 μl lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA and 1% (wt/vol) SDS) with protease inhibitors. DNA was sheared by sonication to an average size of 500 base pairs and was then diluted 1:10 with dilution buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 200 mM NaCl and 0.5% (vol/vol) Nonidet-P40). A 1.5-ml aliquot of the diluted chromatin was incubated with 6 × 10$^7$ Dynabeads sheep anti-rabbit IgG with or without prior binding of 10 μg rabbit anti-EBF1 (ref. 50) to the beads. Immunoprecipitation reactions were incubated for 16 h at 4 °C before being washed four times with wash buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 0.1% (wt/vol) SDS, 1% (vol/vol) Nonidet-P40 and 500 mM NaCl) and then washed twice with Tris-EDTA (10 mM Tris, pH 8.0, and 1 mM EDTA). Chromatin was eluted twice with 50 μl Tris-EDTA containing 2% (wt/vol) SDS and was reverse-crosslinked by incubation of the chromatin immunoprecipitation samples for 16 h at 65 °C. DNA was purified with the Qiaquick PCR purification kit (Qiagen) nd was analyzed by quantitative PCR with the following primers: *Id2*, 5′-GAGGCCTTCTCGGAAACTCAGT-3′ and 5′-GGAGTTCCGAATGCACCG-3′; control, 5′-GGTCTTAGACAGCTGGCTTTTTTTAA-3′ and 5′-GGTGCACTGTAGAAAGTTTAGCGTTA-3′.

**Accession codes.** GEO: microarray data, GSE9878.

*Note: Supplementary information is available on the Nature Immunology website.*

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**AUTHOR CONTRIBUTIONS**

J.M.R.P. designed and did experiments, analyzed data and wrote the manuscript; D.L.N. and D.W.L. designed and did experiments and analyzed data; K.L.M., T.T, M.T. and E.B. did experiments; D.A. and R.G. designed and supervised research and contributed to the writing; and H.S. designed and supervised research and wrote the manuscript.

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