Ebf1-mediated down-regulation of Id2 and Id3 is essential for specification of the B cell lineage

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Gene knockout experiments in mice have suggested a hierarchical model of early B cell commitment wherein E2A proteins (E47 and E12) activate early B cell factor (Ebf1), which in turn activates expression of the B cell commitment factor, Pax5. In IL-7 receptor alpha (IL-7Rα) knockout mice, B cell development is blocked before B-lineage commitment at the prepro-B cell stage in adult animals. In *IL-7R*$\alpha^{-/-}$ prepro-B cells, E47 is expressed and yet is insufficient to transcriptionally activate the putative downstream target gene, *Ebf1*. In this study, we show that further increases of E47 expression in *IL-7R*$\alpha^{-/-}$ prepro-B cells fails to activate *Ebf1*, but rather leads to a dramatic induction of the E2A inhibitory factors, *Id2* and *Id3*. In contrast, enforced expression of Ebf1 in *IL-7R*$\alpha^{-/-}$ bone marrow potently down-regulates *Id2* and *Id3* mRNA expression and restores B cell differentiation in vivo. Down-regulation of both Id2 and Id3 during B cell specification is essential in that overexpression of either Id2 or Id3 in wild-type bone marrow blocks B cell specification at the prepro-B cell stage. Collectively, these studies suggest a model where Ebf1 induction specifies the B cell fate by dramatically increasing activity of E47 at the posttranslational level.

B cell specification | E2A | Ebf1 | Id proteins | IL-7 receptor

The earliest steps in the development of antibody-secreting B lymphocytes from common lymphoid progenitor cells (CLP) in adult mouse bone marrow are orchestrated by the concerted activities of a number of B-lineage transcription factors and signals from growth factor and cytokine receptors. Gene knockout experiments in mice have shown the absolute requirement for the transcription factors PU.1 and Ikaros in the generation of CLP and the requirement for E2A and Ebf1 in the generation of the first specified B cell progenitors referred to as prepro-B cells (1–3). Prepro-B cells can be prospectively identified in murine bone marrow as $B220^+CD19^-IgM^-NK1.1^-$ cells that either lack or have just initiated rearrangement of the D_H-J_H gene segments of the Ig heavy chain (*IgH*) locus. These cells developmentally progress to $B220^+CD19^+IgM^-NK1.1^-$ progenitor B (pro-B/pre-B) cells upon induction of the B cell commitment factor, Pax5, which functions to maintain B cell identity by both positively regulating early B-lineage-associated gene expression and negatively regulating a large number of genes that promote development along alternative hematopoietic cell fates (4–7). B cell development is completely blocked at the pro-B cell stage in the absence of Pax5.

A number of studies have suggested that a complex hierarchical relationship exists between E2A, Ebf1, and Pax5 in early B cell development (8). The *E2A* gene encodes two alternative mRNA splicing variants, E47 and E12, which can homo- or heterodimerize via their helix–loop–helix domains (9). Both variants are able to rescue B cell development when expressed from a *pim-1* promoter/*IgH* enhancer transgene vector that was crossed onto an *E2A* knockout background (10). Observations showing that E2A is present (albeit at reduced levels) in *Ebf1* knockout animals (3) and that the *Ebf1* promoter is bound by E47 in vivo (11, 12) and can be transactivated by overexpression of E47 (13) or E12 (14) in non-B-lineage cell lines, suggest that E2A activity is essential upstream of Ebf1. Similarly, the *Pax5* promoter is bound by Ebf1 based on EMSA and Ebf1 can transactivate the *Pax5* promoter in transient co-transfection assays (15, 16). Ebf1 is also present in Pax5-deficient pro-B cells derived from *Pax5*$^{-/-}$ adult mice (17), suggesting that Ebf1 may participate in the activation of Pax5 expression. Complicating the simple hierarchical model where E2A induces expression of Ebf1, which then activates Pax5, are observations showing that up-regulation of E2A protein levels in B cell progenitors are dependent on Ebf1 (18). In addition, expression of Pax5 from the endogenous *Ikaros* locus resulted in activation of *Ebf1* and B cell development in the thymus (19) and in the bone marrow of E2A-deficient mice (20). Pax5 was also shown to directly bind one of two distinct *EBF* promoters in vivo and could transactivate this promoter in transient transfection assays (12). Collectively, these results demonstrate a complex regulatory circuitry controlling B cell specification and commitment from CLP.

The loss of either the IL-7 receptor alpha (IL-7Rα) chain or the IL-7 ligand also results in a complete block in adult B lymphopoiesis at the prepro-B cell stage, with either modest or no reduction in the numbers of CLP being present in *IL-7* knockout animals (21–24). In IL-7Rα− or IL-7-deficient mice, E2A proteins are expressed at wild-type levels and yet Ebf1 and Pax5 are absent, which suggests that other factors participate in Ebf1 induction in addition to E2A, that E2A requires posttranslational modification to activate Ebf1 expression, or that inhibitory proteins suppress the ability of E2A to activate Ebf1 expression in this context (23–25). The activation of Ebf1 seems to be the pivotal event in B cell fate specification in that Ebf1 can surprisingly rescue the B cell developmental program when overexpressed in the context of multiple gene knockout backgrounds that completely lack B-lineage cells including *PU.1* (8), *E2A* (26), *IL-7R*α (23), and *IL-7* (24).

To clarify the complex regulatory circuitry between E2A, Ebf1, and Pax5 in the specification and commitment of the B cell lineage, we have performed in vivo genetic complementation assays to address why Ebf1, but not E47, is sufficient to rescue the B cell developmental block in *IL-7R*α-knockout animals. Analysis of animals transplanted with *IL-7R*$\alpha^{-/-}$ cells expressing either a control retroviral vector or vectors expressing IL-7Rα, E47, Ebf1, or Pax5 showed that B cell development was rescued by Ebf1 in all cases and in 7/11 animals by Pax5. Expression of E47 did not activate *Ebf1* mRNA but rather led to increased expression of the E2A-inhibitory proteins Id2 and Id3. Expres-

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sion of Ebf1 resulted in near complete inactivation of both *Id2* and *Id3* in prepro-B cells. This suggests that one major function of Ebf1 in B-lineage specification is to down-regulate Id levels to allow for subsequent activation of E2A protein activity. Collectively, these results provide important new insights into the significance of Ebf1 induction as the defining molecular event that determines B-lineage cell fate.

Results

Ebf1 and Pax5 Can Rescue B Cell Development in *IL-7Rα*-Knockout Mice. To characterize the mechanism by which Ebf1 can specify the B cell fate, we first transduced *IL-7Rα*$^{-/-}$ bone marrow cells with retroviruses that co-expressed either E47, Ebf1, Pax5, or the IL-7Rα chain along with a blue-excited green fluorescent protein variant (Bex, hereafter referred to as GFP) (Fig. 1*A*). Transduced cells were then transplanted into lethally irradiated C57BL/6 recipient mice and analyzed for reconstitution beginning at 3 weeks post-transplantation (PT). As previously noted (23), B cell development in *IL-7Rα*$^{-/-}$ animals is blocked at the prepro-B cell stage (B220$^+$CD19$^-$NK1.1$^-$IgM$^-$), which was confirmed by analysis of GFP$^+$ control cells in bone marrow of animals reconstituted at 6 weeks PT (n = 15) (Fig. 1*B*). Expression of E47 failed to promote B cell development to the pro-B/pre-B cell stage (B220$^+$CD19$^+$NK1.1$^-$IgM$^-$) even though *E47* mRNA levels were increased in GFP$^+$ prepro-B cells by expression of the retroviral vector (Figs. 1 *B* and *C*). Expression of Ebf1 or Pax5 rescued B cell differentiation in *IL-7Rα*$^{-/-}$ bone marrow cells but failed to restore absolute numbers of pro-B or pre-B lymphocytes due to the absence of IL-7 signaling in rescued cells (Fig. 1*B*). In contrast to Ebf1, which rescued 100% of reconstituted animals (n = 14), Pax5 only rescued B cell development in 7/11 animals. The ability of Pax5 to rescue B cell development was statistically correlated with the absolute expression levels of Pax5 as determined by the mean fluorescence intensity (MFI) of GFP in prepro-B cells from rescued (MFI = 72.6) versus non-rescued (MFI = 41.7) animals (P = 0.006). We also noted a substantial increase in the frequency of B220$^+$CD19$^-$NK1.1$^-$IgM$^-$ cells that expressed Pax5 (Fig. 1*B*), which we attributed to Pax5 induction of B220 expression on myeloid-lineage cells that expressed Mac-1 and Gr-1 (27) (supporting information (SI) Fig. S1). Because of this, characterization of prepro-B cells in Pax5-rescued animals was always done in B220$^+$CD19$^-$NK1.1$^-$IgM$^-$Mac-1$^-$Gr-1$^-$ cells. Further analysis of mRNA expression levels by Real-time PCR using FACS-sorted prepro-B cells from reconstituted mice showed that both Ebf1 and Pax5 up-regulate the B cell-specific genes *mb-1*, *VpreB* and *λ5* (Fig. S2), further suggesting that rescued B220$^+$CD19$^+$ cells belong to the B lineage. Ebf1 also stimulated a high level of endogenous *Pax5* mRNA expression and increased *E47* mRNA approximately 1.7-fold compared with *E47* levels in GFP control cells (Fig. 1*C*). This suggests that Ebf1 stimulates a feedback loop that reinforces *E47* expression. In animals where Pax5 rescued B cell development, endogenous *Ebf1* mRNA levels were induced approximately 12-fold in prepro-B cells compared with *Ebf1* levels in GFP control prepro-B cells. *Ebf1* expression increased an additional10-fold in Pax5-rescued pro-B/pre-B cells to a level that was comparable to *Ebf1* expression levels in Ebf1-rescued prepro-B cells (Fig. 1*C*). This indicates that maximal *Ebf1* induction by Pax5 may be occurring with delayed kinetics. The fact that Pax5 can induce *Ebf1* expression when expressed at high enough levels suggests that, in addition to the Ebf1-*E2A* feedback loop, Pax5 may reinforce *Ebf1* activation through a positive feedback mechanism by binding the *Ebf1* proximal promoter (12, 20). These observations also raise the possibility that Pax5 activation could function as the primary B-lineage specifying event in some developmental contexts through its ability to activate *Ebf1*.

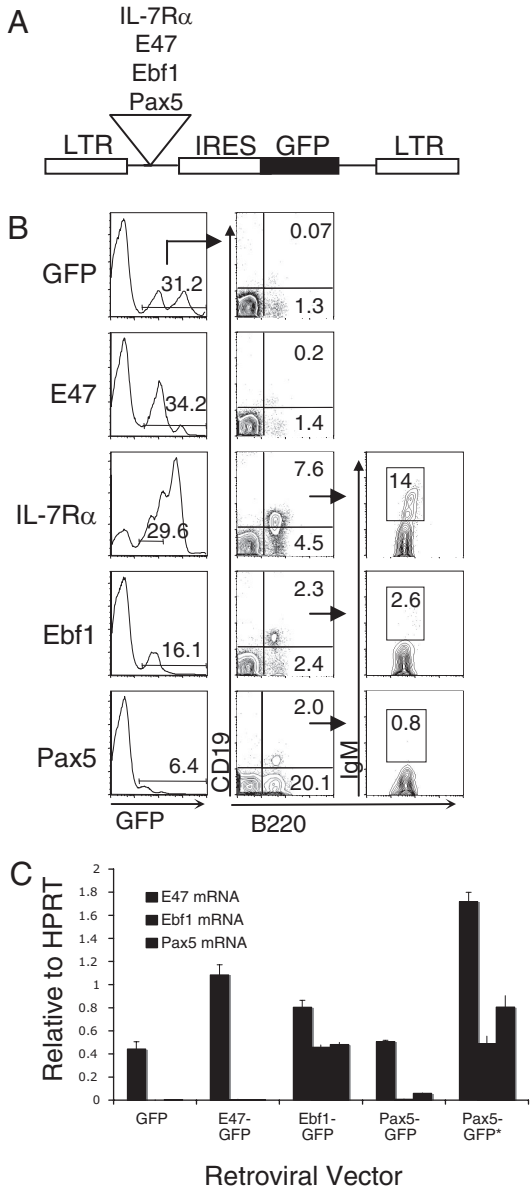

Fig. 1. Ebf1 and Pax5 rescue B cell development in IL-7Rα-deficient bone marrow. (*A*) MSCV retroviral construct coexpressing the cDNA of interest and GFP (IRES = internal ribosome entry site). (*B*) Flow cytometric analysis of bone marrow from 6-week PT mice (n = 5 each). Plots were gated on GFP$^+$NK1.1$^-$ cells. IgM expression was analyzed on B220$^+$CD19$^+$ cells. Numbers indicate percentages of total GFP$^+$ cells from representative animals. (*C*) Representative Real-time PCR analysis of *E47*, *Ebf1*, and *Pax5* expression in FACS-sorted prepro-B or pro-B/pre-B (*) cells isolated from recipient mice at 6 weeks PT (n = 3 independent FACS sorts for each retroviral vector). Data show relative expression compared to the internal *Hprt* control. Error bars indicate the standard deviation for quadruplicate reactions using mRNA isolated from one representative mouse per retroviral construct.

Ebf1 and Pax5 Promote D_H-J_H Rearrangement in *IL-7Rα*$^{-/-}$ Bone Marrow Cells. To determine whether Ebf1- or Pax5-rescued cells were undergoing recombination between the Ig heavy chain D_H-J_H gene segments, we FACS-sorted 500 GFP$^+$ prepro-B or pro-B/pre-B cells from animals reconstituted with retrovirus-expressing cells and used an upstream primer that anneals to the majority of the 10 D_H gene segments in C57BL/6 mice and a 3′-primer complementary to a sequence downstream of J_H4 (Fig. 2) (28). Using relatively short PCR extension times, this assay

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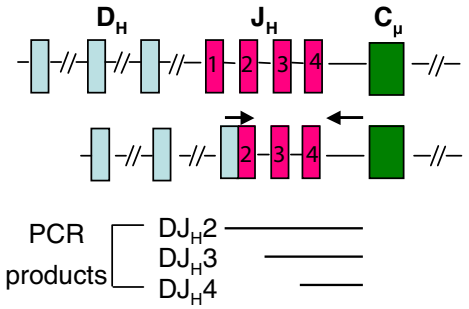

Fig. 2. Ebf1 promotes D_HJ_H rearrangement in *IL-7Rα*-deficient bone marrow. (*A*) *Ig* heavy chain locus before and after rearrangement. Arrows indicate locations of the 5′ degenerate primer within the D_H gene segments and the 3′ primer downstream of J_H4.

detects 3 major PCR products that account for D_H rearrangements to either J_H2, J_H3, or J_H4, as determined by direct sequencing of the PCR products (Fig. 2 and data not shown). Results summarized in Table 1 indicate that *IL-7Rα*-deficient prepro-B cells expressing the GFP control retrovirus show a relatively low incidence of rearrangement (minimally 1 event per 500 cells), with single bands being commonly detected. The low frequency of detection can be explained, in part, by the functional heterogeneity within the prepro-B cell subset, which is likely to be impure for bona fide B-lineage precursors. Similar low frequency rearrangement events were detected in E47- or Pax5-expressing prepro-B cells, which were not statistically different from controls. In contrast, Ebf1 substantially increased the overall frequency of recombination events in prepro-B cells compared with GFP control cells ($P < 0.01$, Student's t test). Pax5 stimulated recombination in rescued pro-B/pre-B cells ($P < 0.05$), which reinforces the view that Pax5 rescue of B cell development is occurring with delayed kinetics that correlate with the timing of maximal *Ebf1* induction by Pax5 (Table 1 and Fig. 1*C*).

***IgH* Transgenes Do Not Rescue B Cell Development in IL-7Rα-Deficient Animals.** Previous studies have shown that Ebf1 functions in coordination with E2A to regulate transcription of the recombinase genes, *Rag1* and *Rag2*, the surrogate light chains *λ5* and *VpreB*, and signaling molecules like *Igα*/*mb-1* in early progenitor B cell subsets (15, 29–32). To test whether the primary function of Ebf1 in B cell specification relates to its role in *Ig* gene rearrangement, we crossed two different *IgH* transgene mice (33) to animals deficient in either the IL-7 cytokine or the IL-7Rα chain. Analysis of B cell development in compound transgenic/knockout mice showed that expression of a fully rearranged heavy chain gene could not rescue the B cell developmental block in *IL-7*- or *IL-7Rα*-knockout mice (Fig. S3, n = 5 each). Since Ebf1 can rescue B cell development in *IL-7*- or

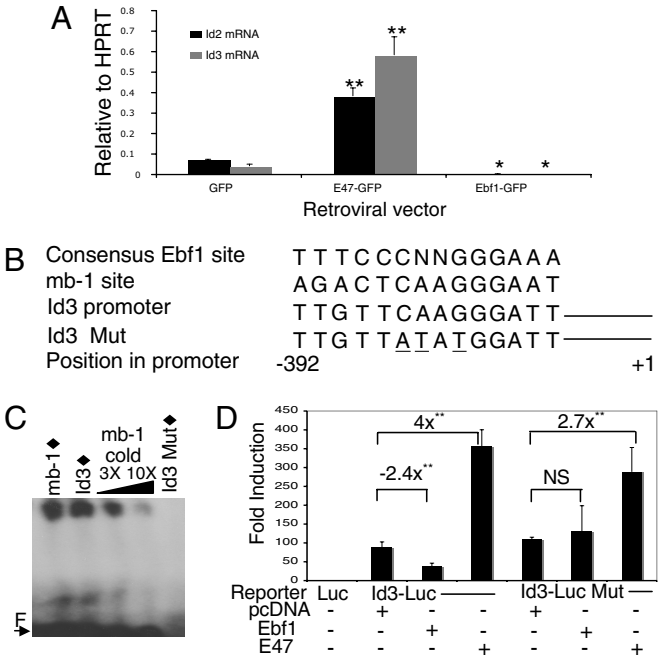

Fig. 3. E47 and Ebf1 differentially regulate *Id2*/*Id3* expression during early B cell development. (*A*) Real-time PCR analysis of *Id2* and *Id3* expression levels in FACS-sorted GFP$^+$ prepro-B cells from recipient mice at 3–6 weeks PT. Data shown are representative of one reconstituted mouse per retroviral construct out of three that were analyzed in quadruplicate. (*B*) Putative Ebf1 binding site at position −392 in the *Id3* promoter. Point mutations introduced into the site are underlined. (*C*) EMSA using 10 ng of recombinant Ebf1 protein and radiolabeled sequences (filled diamonds) shown in (*B*). Increasing amounts of unlabeled *mb-1* cold probe were used as competitor. ''F'' indicates free probe. (*D*) Luciferase assay using extracts from K562 cells co-transfected with either the wild-type or mutant *Id3* promoter-reporter and Ebf1, E47, or control pcDNA expression plasmids. Luciferase activity was normalized to a co-transfected *Renilla* expression plasmid. Results are shown as fold-induction over the promoterless luciferase vector in all experiments. Error bars indicate the SEM from four different experiments performed in triplicate. (*, $P < 0.05$; **, $P < 0.01$) NS = not significant.

IL-7Rα-knockout mice, these results indicate that Ebf1 has critical functions in addition to activation of *Ig* recombination in early B-lineage development.

Ebf1 Potently Downregulates *Id2* and *Id3* mRNA Levels in Prepro-B Cells. To address why E47 overexpression could not rescue B cell development in *IL-7Rα*$^{-/-}$ mice (Fig. 1*B*) or activate *Ebf1* transcription (Fig. 1*C*), we characterized expression of the E2A inhibitory factors, Id2 and Id3, in sorted prepro-B cells that expressed E47 (Fig. 3*A*). Of the 4 Id proteins expressed in mammalian cells (Id1, Id2, Id3, and Id4), Id2 and Id3 are the only

Table 1. D_HJ_H Rearrangement Products

*Prepro-B				Pro-B/Pre-B	
GFP	E47	Ebf1‡‡	Pax5	Ebf1‡‡	Pax5‡
0†	2	3,4	3,4	2,3,4	3,4
2,3	3,4	2,4	3,4	2,3,4	3,4
3,4	3	2,3,4	2	2,3,4	3,4
2	3	2,3,4	0	2,3,4	3,4
3	2,4	2,3,4	3	2,3,4	2,3,4

Table 1. *Prepro-B: B220$^+$CD19$^-$NK1.1$^-$IgM$^-$, Pro-B/Pre-B: B220$^+$CD19$^+$NK1.1$^-$IgM$^-$. † Numbers indicate J_H rearrangement product detected. (0 = only germline product detected). Student t test comparing the number of bands detected to the GFP control, ‡$P < 0.05$, ‡‡ $P < 0.01$. The number of rearrangement products in prepro-B cells expressing E47 or Pax5 were not statistically significant ($P = 0.3$ and $P = 0.5$, respectively)

family members expressed in B220$^+$ B-lymphocyte progenitors (34). Real-time PCR analysis confirmed that *Id2* and *Id3* mRNA are expressed in *IL-7Rα*-deficient prepro-B cells expressing the GFP control provirus and both factors are significantly induced by E47 (approximately 5- and 7-fold, respectively, compared with GFP control-expressing cells). In contrast to E47, Ebf1 dramatically downregulated both *Id2* and *Id3* mRNA to essentially undetectable levels in prepro-B cells ($P = 0.01$ and $P = 0.02$, respectively). The potent down-regulation of *Id2* and *Id3* suggests that Ebf1 induction would markedly enhance E2A protein activity, thus providing a molecular basis for co-activation of B cell specification by concerted E2A/Ebf1 activity.

Ebf1 Directly Binds and Negatively Regulates *Id3* Promoter Activity. Inspection of the *Id3* promoter sequence revealed a consensus binding site for Ebf1 positioned about 390 bp upstream of the transcription start site (Fig. 3*B*). EMSA analysis using recombinant Ebf1 protein (35) revealed that Ebf1 binds this sequence in vitro (Fig. 3*C*, lane 2). The band co-migrates with a shifted complex containing recombinant Ebf1 and the known Ebf1 binding site within the *mb-1* promoter and is competed away by a cold *mb-1* probe. To determine whether Ebf1 could regulate expression of an *Id3*-driven reporter gene through this sequence, we then co-transfected an Ebf1 expression plasmid and an *Id3* promoter-driven luciferase reporter construct into K562 cells. After normalization of luciferase activity to *Renilla* luciferase, the results showed that Ebf1 could significantly repress luciferase expression in a binding-site-dependent manner (Fig. 3*D*, $P < 0.001$). Co-transfection of two additional cell lines, BOSC23 and NIH 3T3, with the same constructs resulted in similar reductions in luciferase activity (3.2- and 3.6-fold, respectively, data not shown). Each of these lines express the endogenous *Id3* gene as demonstrated by Western blot analysis using an Id3-specific monoclonal antibody (clone 6–1, Cal Bioreagents) that recognizes both murine and human Id3 protein (Fig. S4). Conversely, E47 expression activated the *Id3* promoter-reporter 4-fold (Fig. 3*D*, $P < 0.001$), which was likely dependent on the presence of 6 consensus E-box sequences located within the upstream *Id3* promoter region. E47 also activated the mutant *Id3* promoter construct, which indicates that E47 activation of *Id3* is not dependent on Ebf1. A recent study showed that Ebf1 could directly bind the *Id2* promoter by ChIP analysis, which supports the hypothesis that Ebf1 may negatively regulate *Id2* gene expression (36).

Id2 and Id3 Down-Regulation Are Essential for B Cell Specification. To address whether Id2 and/or Id3 down-regulation is necessary for B cell specification, we overexpressed Id2 or Id3 in wild-type C57BL/6 bone marrow cells using a retroviral vector linked with GFP. Transduced cells were transplanted into lethally irradiated syngeneic recipient animals and B cell development within total bone marrow GFP$^+$ cells was then assessed at 6 weeks PT (Fig. 4*A*). Analysis of Id2- or Id3-expressing cells revealed that either factor blocked B cell development at the B220$^+$CD19$^-$NK-1.1$^-$IgM$^-$ prepro-B cell stage ($n \geq 4$ each). These results indicate that down-regulation of both Id2 and Id3 is an essential event in B-lineage specification.

Loss of Id2 or Id3 Fails to Rescue B Cell Development in *IL-7Rα*$^{-/-}$ Mice. In *IL-7Rα*-deficient prepro-B cells, *Id2*, *Id3*, and *E2A* are all expressed at high levels while *Ebf1* expression is absent. To test whether loss of Id2 or Id3 might allow E2A to stimulate endogenous *Ebf1* transcription and rescue B cell development in *IL-7Rα*-knockout mice in the absence of retroviral Ebf1 expression, we generated *IL-7Rα*$^{-/-}$*Id2*$^{-/-}$ and *IL-7Rα*$^{-/-}$*Id3*$^{-/-}$ double-deficient mice. Analysis of *IL-7Rα*$^{-/-}$*Id2*$^{-/-}$ or *IL-7Rα*$^{-/-}$*Id3*$^{-/-}$ mice showed that loss of individual Id proteins was not sufficient to promote B cell development in the context of *IL-7Rα*$^{-/-}$ bone marrow cells (Fig. 4*B*). We were unable to generate *IL-7Rα*$^{-/-}$*Id2*$^{-/-}$*Id3*$^{-/-}$ mice since the loss of both *Id2* and *Id3* results in an embryonic lethal phenotype (R. Benezra, personal communication). These results indicate that inactivation of both Id proteins by Ebf1 expression is necessary for B cell fate specification to occur in *IL-7Rα*-deficient animals.

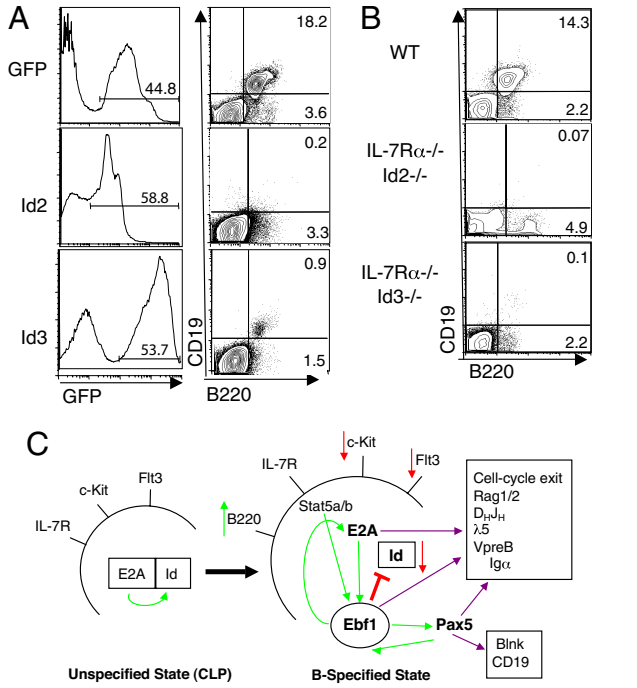

Fig. 4. Enforced expression of Id2 or Id3 inhibits B-lineage specification. (*A*) Flow cytometric analysis of bone marrow from mice reconstituted with GFP- ($n = 5$), Id2- ($n = 4$), or Id3-expressing ($n = 5$) cells at 3–6 weeks PT. Plots are gated on GFP$^+$ cells. (*B*) Loss of Id3 ($n = 3$) or Id2 ($n = 1$) does not rescue B cell development in *IL-7Rα*-knockout mice. (*C*) Model for Ebf1-induced activation of B cell specification from unspecified CLP. Red arrows indicate inhibition or down-regulation, while green arrows indicate activating events or up-regulation.

Discussion

The results described above demonstrate that Id2 and Id3 function as repressors of B-lineage fate specification and that Ebf1 overcomes this inhibition through direct or indirect down-regulation of *Id2* and *Id3* gene transcription. These data suggest a model (Fig. 4*C*) wherein activation of Ebf1 expression promotes a substantial increase in E2A activity at both the transcriptional level, through up-regulation of *E47* mRNA (Fig. 1*C*), and at the posttranslational level, by Ebf1 down-regulation of E2A inhibitory protein activity (Fig. 3*A*). High levels of E2A in cooperation with Ebf1 would then stimulate cell-cycle exit and activation of multiple E2A/Ebf1 target genes involved in sterile *IgH* transcription and recombination at D_H-J_H gene segments, as well as activation of *Pax5*. In support of this model, high levels of E2A protein activity are known to repress lymphocyte proliferation (37) and recombinase activity is negatively regulated in cycling cells (38). In addition, *E2A-GFP* knock-in mice crossed with *Ebf1*-deficient animals showed that up-regulation of E2A was dependent on Ebf1 at the onset of B cell specification (18). This scenario suggests a complex interplay between key regulatory factors that function to both reinforce early B cell developmental decisions through positive feedback loops that further up-regulate expression of differentiation-promoting factors and inactivation of inhibitory pathways that dampen activity of select factors like E2A in a temporally-specific manner. The primary

effector molecule driving B-lineage specification and development in this process seems to be Ebf1 through its ability to potently activate E2A, which would explain how Ebf1 can rescue B cell development in multiple gene knockout contexts that lack the earliest B-lineage cells.

One lingering question related to B cell specification is how Ebf1 becomes activated in the first place if increased levels of E47 only lead to increased expression of the E2A inhibitors, Id2 and Id3. The observation that *Id2*/*Id3* mRNA levels increase in E47-expressing prepro-B cells suggest that some level of E47 must be transcriptionally competent and free from Id protein inhibition to transcriptionally activate these genes, although this level of "free" E2A is not sufficient to activate *Ebf1* transcription. One possibility is that E47 may be posttranslationally modified by phosphorylation to stimulate homodimerization in a manner that is not inhibitable by Id proteins (25). Alternatively, E2A activation alone may not be sufficient to induce *Ebf1* without cooperation from other regulatory factors stimulated by IL-7 receptor signaling. Another attractive possibility is that *Ebf1* is induced by removal of repressive signals like Flt3 (39) or TGFβ signaling (34) that may restrain CLP from differentiation along the B cell pathway. Removal of repressive signals via daughter cell migration away from the CLP niche in the bone marrow may be sufficient to allow IL-7 receptor signaling to maximally activate *Ebf1* expression. Further work will be necessary to clarify these issues.

Methods

Mice. *IL-7R*α- and *IL-7*-deficient mice were all on a C57BL/6 background. Transgenic lines Vh81x and m167 were obtained from Dr. J. Kearney (University of Alabama-Birmingham, Birmingham, AL). All mice were bred and maintained at University of Alabama-Birmingham under a protocol approved by the Institutional Animal Care and Use Committee in compliance with all state and federal regulations governing the use of experimental animals.

Retroviral Transductions. All cDNA sequences were cloned into the EcoRI site of the murine stem cell virus (MSCV) retroviral vector upstream of an IRES element and GFP. cDNA constructs were kind gifts from L. Park (IL-7Rα chain, Amgen), C. Murre (E47, University of California San Diego, La Jolla, CA), R. Grosschedl (Ebf1, Max Planck Institute, Leipzig, Germany), M. Busslinger (Pax5, Institute of Molecular Pathology, Vienna, Austria) and X.H. Sun (Id2 and Id3, Oklahoma Medical Research Foundation, Oklahoma City, OK). Retrovirus preparation and transplantation was performed as described previously (40).

Flow Cytometry. Bone marrow extracted from the femurs and tibiae of mice 3–6 weeks PT were analyzed using labeled antibodies against B220(RA3–6B2)$^{\text{Pacific Blue}}$, CD19 (ID3)$^{\text{Texas Red}}$, NK1.1 (PK136)$^{\text{PE}}$, IgM$^{\text{APC}}$, Mac-1(M1/70)$^{\text{PE-Cy7}}$, and Gr-1(RB6–8C5)$^{\text{PE-Cy7}}$. Prepro-B (B220$^+$CD19$^-$NK1.1$^-$IgM$^-$) and pro-B/pre-B cell (B220$^+$CD19$^+$NK1.1$^-$IgM$^-$) populations were analyzed and sorted using LSRII (BD Biosciences) or a triple-laser Mo-Flo cell sorter (Cytomation).

Real-time PCR. GFP$^+$ prepro-B and pro-B/pre-B cells from transplanted mice were sorted and resuspended in RNA STAT-60 (Tel-Test B,). RNA was purified using the MicroRNAeasy kit (Qiagen). cDNA was made using the High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed with SYBR Green PCR Master Mix using the ABI Prism 7900HT sequence detection system (Applied Biosystems). Reactions containing 2 μl cDNA were performed in a 20 μl total volume. HPRT was used as an internal control. All primer sequences are available upon request.

D_H-J_H Rearrangement. Five hundred transduced prepro-B (B220$^+$CD19$^-$NK1.1$^-$IgM$^-$) or pro-B/pre-B (B220$^+$CD19$^+$NK1.1$^-$IgM$^-$) cells were sorted into complete DMEM media with 10% FBS. All FACS-sorted populations from mice reconstituted with Pax5-expressing cells were also negative for Mac-1 and Gr-1. Cells were spun at 2000 rpm for 5 min and then resuspended in 10 μl 1X PCR buffer (Roche) with 0.1% Triton-X. Nested PCR to detect D_H-J_H rearrangement products were performed using primers as previously described (28).

EMSA. The *Id3* probe was a 20 bp fragment containing the putative Ebf1 binding site in the *Id3* promoter (GTTGTTCAAGGGATTTATGA). The *Id3* mutant probe has three point mutations in the core of the Ebf1 site (GTTGT-*TATATG*GATTTATGA). The Ebf1 binding site within the *mb-1* promoter was used as a positive control. Probes were labeled with [^{32}P]-dCTP using Klenow. 10 ng of recombinant Ebf1 protein (provided by J. Hagman, National Jewish Medical Research Center, Denver, CO) was incubated with the labeled probe in the presence of 20 mM Hepes pH 7.9, 70 mM KCl, 1 mM DTT, 2.4 mM $MgCl_2$, 2% glycerol, 0.1% Nonidet P-40, and 0.1 mg/ml BSA for 30 min. Samples were separated on a 1X TGE/6% acrylamide gel at 4 °C.

Luciferase Reporter Assay. The *Id3* promoter region from −1319 to +14 was cloned into the pGL2-Basic vector (Promega). The Dual-Luciferase Reporter Assay System (Promega) was used to detect both firefly and *Renilla* luciferase activity in Lipofectamine 2000 (Invitrogen)-transfected K562, BOSC23, or NIH 3T3 cells. *Ebf1* and *E47* cDNAs were cloned into the EcoRI site of pcDNA3.1 (Invitrogen).

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