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Early B Cell Factor 1 Regulates B Cell Gene Networks by Activation, Repression, and Transcription-Independent Poising of Chromatin

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SUMMARY

The transcription factor early B cell factor-1 (Ebf1) is a key determinant of B lineage specification and differentiation. To gain insight into the molecular basis of Ebf1 function in early-stage B cells, we combined a genome-wide ChIP sequencing analysis with gain- and loss-of-function transcriptome analyses. Among 565 genes that are occupied and transcriptionally regulated by Ebf1, we identified large sets involved in (pre)-B cell receptor and Akt signaling, cell adhesion, and migration. Interestingly, a third of previously described Pax5 targets was found to be occupied by Ebf1. In addition to Ebf1-activated and -repressed genes, we identified targets at which Ebf1 induces chromatin changes that poise the genes for expression at subsequent stages of differentiation. Poised chromatin states on specific targets could also be established by Ebf1 expression in T cells but not in NIH 3T3 cells, suggesting that Ebf1 acts as a "pioneer" factor in a hematopoietic chromatin context.

INTRODUCTION

The differentiation of highly specialized, hematopoietic lineage cells from hematopoietic stem cells (HSCs) involves regulated expression of lineage-specific factors and concomitant loss of alternate lineage potential. In adult bone marrow, lymphopoiesis initiates with the generation of lymphoid-primed multipotent progenitors (LMPPs) that differentiate into common lymphoid progenitors (CLPs), a heterogeneous cell population containing cells that already express select markers of the B cell lineage (Inlay et al., 2009). From the CLP population, pre-pro-B cells, also termed Fraction A cells, are generated and further differentiate into pro-B cells. These cells, also known as Fraction B cells, are characterized by the upregulation of B lineage markers including CD19 and the immunoglobulin (Ig) surrogate light chains VpreB and λ5, initiation of Ig heavy chain gene rearrangement and proliferation in response to interleukin-7 (IL-7) (reviewed in Hardy et al., 2007; Murre, 2009). Pre-B cells express the pre-B cell receptor (pre-BCR) and further differentiate into immature B cells that have undergone Ig light chain gene rearrangement and migrate from the bone marrow to the spleen.

Each of these B cell differentiation steps is dependent on the coordinated expression of cell-type-specific transcription factors and activities of signaling pathways (reviewed in Mandel and Grosschedl, 2010). Genetic ablation and complementation studies have demonstrated key roles for transcription factors such as Ikaros, Pu.1, E2A, early B cell factor-1 (Ebf1), and Pax5 (reviewed in Busslinger, 2004; Hagman and Lukin, 2006; Singh et al., 2007). Prior to the differentiation of CLPs, Ikaros and Pu.1 induce the expression of components of signaling pathways, including Il7 and Flt3, which are necessary for the generation of pro-B cells (DeKoter et al., 2002; Yoshida et al., 2006). Several lines of evidence demonstrate the requirement for E2A (*Tcf3*) and Ebf1 (*Ebf1*) in the specification of the B cell lineage. Targeted inactivation of either *Tcf3* or *Ebf1* results in a similar block of early B cell differentiation, and a synergistic relationship of these two factors has been inferred from the analysis of compound heterozygous mice that display more severe phenotypes than single heterozygous mice (Bain et al., 1994; Lin and Grosschedl, 1995; O'Riordan and Grosschedl, 1999; Zhuang et al., 1994). Moreover, forced expression of Ebf1 in hematopoietic progenitors promotes B cell development at the expense of other lineages (Medina et al., 2004; Zhang et al., 2003). Pax5 has been shown to act as a determinant of B cell commitment, given that Pax5-deficient pro-B cells display uncharacteristic lineage plasticity and are able to generate non-lymphoid cell types (Nutt et al., 1999).

Because Ebf1 plays a pivotal role in early developmental processes of the B cell lineage and functions at the intersection of its initial specification and subsequent commitment, much work has focused on characterizing this transcription factor. Ebf1 is expressed in all stages of B lymphopoiesis with the exception of terminally differentiated plasma cells (Hagman et al., 1993). In addition, Ebf1 is expressed and has a functional role in neuronal and adipocyte lineages (Jimenez et al., 2007; Wang et al., 1997). Targeted inactivation of *Ebf1* results in the complete block at the pre-pro B cell stage, and induced expression of Ebf1 is sufficient to restrict HSCs to the B cell lineage or to overcome blocks in B cell differentiation resulting from the loss of factors such as Il7 (*Il7*), Ikaros (*Ikzf1*), PU.1 (*Sfpi1*), or E2A (*Tcf3*)

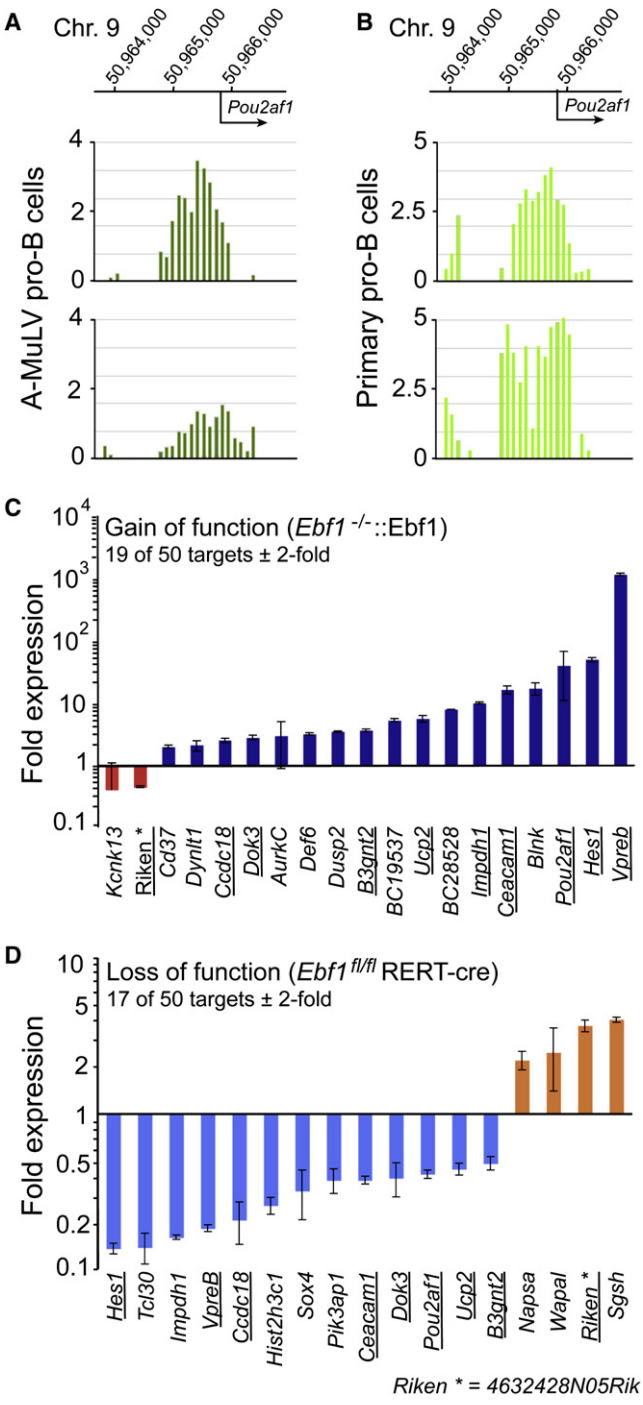


Figure 1. Ebf1 Directly Binds and Functionally Regulates Target Gene Promoters in B Cells

(A and B) SignalMap view of the location and enrichment of Ebf1-binding regions within the *Pou2af1* promoter by ChIP of A-MuLV-transformed pro-B cells (A) or primary pro-B cells (B).

(C) Transcriptional regulation of bound Ebf1 targets shown by qRT-PCR in a gain-of-function approach. *Ebf1*$^{-/-}$ pre-pro-B cells were infected with an Ebf1-IRES-EGFP or a control EGFP retrovirus, and a set of 50 Ebf1-bound genes was examined for expression 24 hr after transduction. Fold expression is displayed as values relative to the empty vector control.

(D) qRT-PCR analysis of Ebf1-bound targets regulated in a loss-of-function approach. Ebf1 was deleted by tamoxifen treatment of *Ebf*$^{fl/fl}$RERT-Cre pro-B cells and cells were collected after complete loss of Ebf1 protein at day 5 for analysis of the same 50 Ebf1-bound genes as in (C). Fold expression is displayed as values relative to heterozygote control cells. Genes common to both gain- and loss-of-function studies are underlined (C and D). See also Figure S1.

(Dias et al., 2005; Reynaud et al., 2008; Seet et al., 2004; Singh et al., 2007; Zhang et al., 2003). In part, Ebf1 acts through the regulation of downstream targets such as *Pax5*, *Cd79a* (formerly known as *mb-1*), *Blk*, *Vpreb1*, *Igll1(λ5)*, *Cd19*, *Pou2af1* (OCAB), and *Foxo1* (O'Riordan and Grosschedl, 1999; Sigvardsson et al., 2002; Zandi et al., 2008). Although Ebf1 functions to promote B lineage specification, Ebf1 is also an important factor in the repression of alternative cell fates. Ectopic Ebf1 expression promotes B cell development in multipotent progenitor (MPP) cells and antagonizes myeloid and T cell differentiation in *Pax5*$^{-/-}$ progenitor cells, whereby Ebf1 downregulates the expression of myeloid determinants including *Cebpa*, *Sfpi1*, and *Id2* (Pongubala et al., 2008; Thal et al., 2009). Finally, Ebf1 has been suggested to act as a "pioneer factor" in gene regulation by controlling the epigenetic status and resulting accessibility of target genes (Maier et al., 2004). In studies of the Ebf1 target gene *Cd79a*, Ebf1 has been shown to contribute to epigenetic regulation of the promoter through CpG demethylation and nucleosomal remodeling, thus allowing accessibility to additional transcriptional regulators (Maier et al., 2004).

Although it is clear that Ebf1 acts as a determinant of the specification and commitment of the B cell lineage through the direct and indirect regulation of several downstream targets, our current understanding is not sufficient to fully account for both loss- and gain-of-function phenotypes. To gain further insight into the underlying molecular mechanisms of Ebf1 function, we have utilized multiple genome-wide analyses aimed at identifying not only directly bound Ebf1 targets, but also targets that are functionally regulated by Ebf1.

RESULTS

Genome-wide Mapping of Ebf1 Binding in Promoter Regions by ChIP-on-Chip Analysis

To identify direct targets of Ebf1 in pro-B cells, we adopted a chromatin-immunoprecipitation (ChIP) strategy using an Ebf1 antibody and hybridization with DNA tiling arrays that represent ~1.7 × 10^4 promoter regions (−2kb to +0.5kb relative to the transcription start site) of RefSeq genes. We first examined the enrichment of the well-characterized Ebf1-binding sites in the *Cd79a* and *Igll1* promoters (Figure S1A available online). Quantitation of the immunoprecipitated fragments by quantitative PCR (qPCR) showed that these Ebf1-bound chromatin fragments were enriched more than 1000-fold relative to two randomly chosen intergenic control regions (Figure S1B). Duplicate ChIP-on-chip analyses with Abelson murine leukemia virus (A-MuLV)-transformed pro-B cells and primary pro-B cells showed robust and reproducible binding of Ebf1 to a subset of promoters including several known Ebf1 targets. For example, Ebf1 strongly bound upstream of the transcriptional start site (TSS) of the *Pou2af1* gene in both cell types (Figures 1A and 1B). In primary pro-B cells, we detected 207 Ebf1 binding events in the tiled promoters corresponding to 228 potential target genes, given that some promoter regions reside between two genes on

opposing DNA strands (Table S1). From this data set, 50 putative targets were selected for validation. Using qPCR analysis of the two ChIP-samples from the array hybridization and a third independent ChIP experiment, we could reproduce binding of Ebf1 to more than 90% of the 50 regions (Figure S1C).

Altered Expression of Ebf1-Bound Genes in Ebf1 Gain- and Loss-of-Function Experiments

To assess the functional relevance of the identified Ebf1-bound genes, we used both Ebf1 gain- and loss-of-function approaches in early B cells. For the induction of Ebf1 activity, we transduced *Ebf1*$^{-/-}$ pre-pro-B cells (Fraction A) with an Ebf1-expressing retrovirus, which resulted in Ebf1 expression similar to that of wild-type pro-B cells (Figure S1D). The cells were harvested 24 hr after infection, as previously described (Pongubala et al., 2008), and assessed for transcripts from Ebf1 target genes by qRT-PCR. In addition to the robust induction of known Ebf1 targets including *Igll1* (data not shown) and *VpreB*, 19 of the 50 test set genes (38%) showed a more than two-fold expression change compared to empty retrovirus-transduced controls, and most genes were activated by Ebf1 (Figure 1C).

In a complementary set of experiments, we isolated pro-B cells from mice carrying one or two floxed *Ebf1* alleles and a tamoxifen-inducible form of the cre recombinase (I.G. and R.G., unpublished data). Within 2 days of treatment with 4-hydroxy-tamoxifen, the *Ebf1* locus is efficiently inactivated, and within 5 days, Ebf1 protein is no longer detectable by immunoblot analysis (data not shown). At this time point, the expression of 50 putative Ebf1 target genes in the test set was compared between homo- and heterozygously-floxed cell cultures. The expression of 34% (17 of 50) of the Ebf1-bound genes was substantially changed, whereby most genes were downregulated upon loss of Ebf1 (Figure 1D). Comparison of the gain- and loss-of-function analyses reveals an overlap of ten genes (20% of the test set) that are reciprocally regulated in both experimental approaches. Therefore, these genes can be regarded as direct Ebf1 targets with high confidence.

Genome-wide Analysis of Ebf1 Binding in Pro-B Cells by ChIP Sequencing

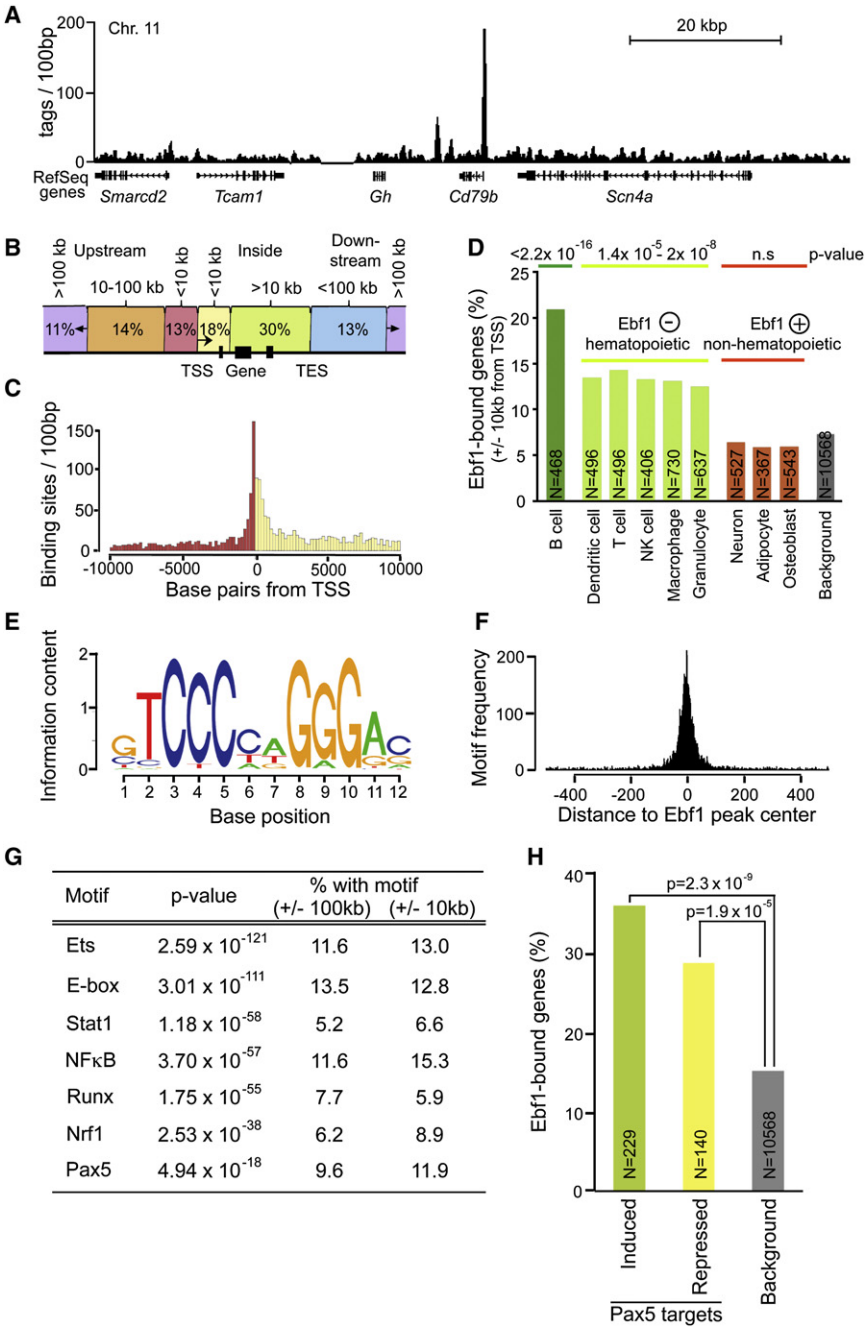
Because only a fraction of transcription factor binding occurs in promoter regions (Farnham, 2009), we analyzed anti-Ebf1-immunoprecipitated DNA from A-MuLV-transformed pro-B cells by deep-sequencing. Ebf1-bound and control input DNA were analyzed, generating sequence reads sufficient to cover nearly the entire genome. Comparison of Ebf1 and control data sets with the CCAT peak-calling algorithm (Xu et al., 2010) identified 9561 significant peaks at a false discovery rate (FDR) < 0.01 that correspond to 5025 genes. Similar results were obtained with two other peak-calling modules (Figure S2A). Moreover, sequencing of an independent, replicate Ebf1 ChIP experiment demonstrated the high reproducibility of our data (Pearson correlation = 0.79), given that more than 94% of the bound regions were also identified in the original ChIP-seq analysis (Figure S2B). More than half of the Ebf1-bound sites identified by ChIP-on-chip were found to be enriched in the ChIP-seq experiment (Figure S2C), which is consistent with the overlap reported for other comparisons of ChIP-on-Chip and Chip-seq binding data (e.g., for nuclear hormone receptors, see Cheung and Kraus, 2010). Notably, ChIP-seq peaks that overlap with Ebf1-bound regions in the ChIP-on-chip analysis showed higher enrichment (Figure S2D), suggesting that discordant binding in both assays is most likely due to differential identification of weakly-bound regions. To increase the stringency of detection and minimize false positive signals, we introduced a 3-fold enrichment threshold for the anti-Ebf1-ChIP peaks based on the comparison between sequenced tags in the Ebf1 library and the control library. Thus, the final data set included 5071 high-confidence peaks that correspond to 3138 genes (Table S2). Sharp peaks were identified at known Ebf1-binding sites, such as the promoter of *Cd79b*, in both ChIP-seq replicates (Figure 2A and Figure S2E). Binding of Ebf1 to the identified peak regions could consistently be verified by qPCR on four individual anti-Ebf1 ChIP samples from primary pro-B cell cultures (Figure S2F). Overall, the distribution of Ebf1-binding events throughout the genome showed a clear enrichment within and in close proximity to genes, given that only 11% of the bound regions lie more than 100 kb from the closest RefGene (Figure 2B). Inspection of the binding site distribution in the region around the TSS revealed preferential recruitment of Ebf1 to proximal promoter sites (Figure 2C). Throughout our analysis, we used two categories of potential Ebf1 targets. The first group (3138 genes) contains at least one Ebf1-binding site within 100 kb of the gene body, whereas the second group (1470 genes) contains at least one binding site within 10 kb of the TSS. Taken together, the data sets generated by both ChIP-on-chip and ChIP-seq identify a large number of Ebf1-bound sites near genes and suggest a role for Ebf1 in the regulation of these targets.

Binding Site Occupancy of Ebf1 Is Tissue Specific

In the B cell lineage, Ebf1 has been proposed to act as "pioneer" factor that binds to gene loci even in the context of "closed" chromatin to initiate locus activation (Maier et al., 2004). Because Ebf1 also plays important roles in other developmental processes such as adipogenesis and neurogenesis, we examined whether the regions bound by Ebf1 in B cells are specific for B lymphoid genes or whether neuronal and adipocyte-specific loci are also bound (Jimenez et al., 2007; Wang et al., 1997). Using publicly available microarray data, we assembled independent lists of genes that showed preferential expression in B cells, in other hematopoietic cell lineages that do not express Ebf1, and in non-hematopoietic Ebf1-expressing tissues. The incidence of Ebf1-bound regions close to genes in these lists was then compared to a random background gene set. From this comparison, B cell-specific genes were the most highly enriched of the three gene sets, with Ebf1 bound within 10 kb from the TSS of more than 20% of the genes ($p < 2.2 \times 10^{-16}$). Interestingly, other hematopoietic lineage-specific genes also contained a markedly enriched number of Ebf1-bound regions, whereas genes specific for nonhematopoietic Ebf1-expressing tissues were not enriched (Figure 2D). This finding indicates that Ebf1 binding is tissue specific and suggests that Ebf1 does not occupy binding sites in "silenced" chromatin.

Coenrichment of Binding Sites for Ebf1 and Other Hematopoietic Transcription Factors

The binding site for Ebf1 was previously defined as a palindromic repeat of the sequence 5′-ATTCCC-3′ with a spacer of two



Motif	p-value	% with motif (+/- 100kb)	(+/- 10kb)
Ets	2.59×10^{-121}	11.6	13.0
E-box	3.01×10^{-111}	13.5	12.8
Stat1	1.18×10^{-58}	5.2	6.6
NFκB	3.70×10^{-57}	11.6	15.3
Runx	1.75×10^{-55}	7.7	5.9
Nrf1	2.53×10^{-38}	6.2	8.9
Pax5	4.94×10^{-18}	9.6	11.9

Figure 2. ChIP Sequencing Reveals Direct Binding of Ebf1 to Genomic Regions Corresponding to Hematopoietic Genes

(A) Sequence tag profile of the genomic region surrounding the Ebf1 binding site in the *Cd79b* promoter. RefGenes are indicated, and average tag counts per 100 bp bins are plotted.

(B) Distribution of Ebf1-bound regions identified by ChIP-seq in relation to annotated gene loci. A schematic diagram of a gene locus displays the relative abundance of Ebf1-binding sites (TSS, transcription start site; TES, transcription end site).

(C) Frequency of Ebf1-bound sites per 100 bp in the regions 10 kb up- and down-stream of TSS.

(D) Association of Ebf1-bound regions with the expression of tissue-specific genes. For each cell type, preferentially expressed genes were identified based on the GNF Gene Expression Atlas. The significance of the correlation was determined by comparison to a random gene set (χ^2 test).

(E) Single most enriched DNA sequence motif identified with WEEDER in an analysis of the 1000 most highly enriched, Ebf1-bound sequences identified by ChIP-seq.

(F) Graphical representation of the frequency of the Ebf1-binding motif in relation to the center position of all Ebf1-binding peaks.

(G) Overrepresented transcription factor binding motifs within all Ebf1 binding regions (±150bp from peak center) were identified with position weighed matrices from the TRANSFAC database. p values represent the enrichment of each binding motif in comparison to the local background. Percentages represent a secondary analysis for calculating the proportion of gene-associated Ebf1-bound regions that also contain the given transcription factor binding motif (e < 0.0001).

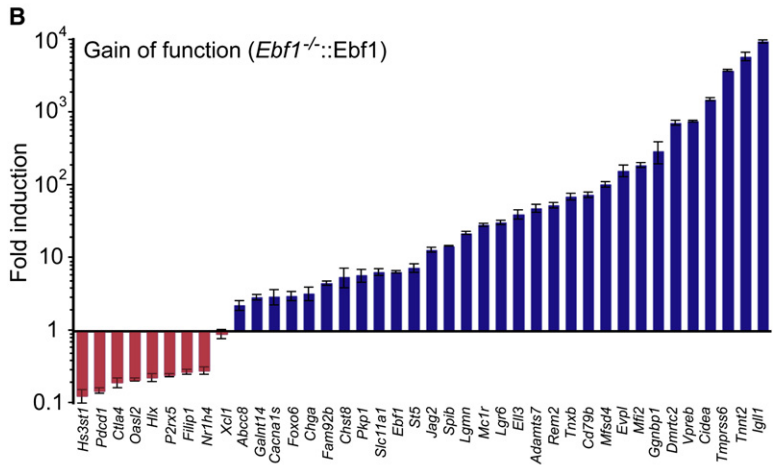
(H) Genes regulated by the transcription factor Pax5 were compiled from published studies. Percentages represent the proportion of the Pax5-regulated genes that lie within 100 kb of an Ebf1-bound region. p values represent a comparison to a random gene set (χ^2 test). See also Figure S2.

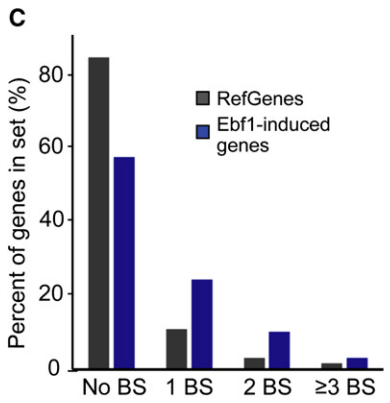
nucleotides between the two half-sites (Travis et al., 1993). A de novo search for enriched sequence motifs in the sequences underlying the Ebf1-bound peaks yielded a similar but shortened binding site with the two first nucleotides of the reported half-site appearing to be dispensable for in vivo binding (Figure 2E). The Ebf1 motif is found in 88% of the bound regions (e < 0.0005) with the motif instances strongly enriched around the center of the Ebf1 peaks (Figure 2F). This observation suggests that the palindromic sequence 5′-TCCCNNGGGA-3′ is the only relevant Ebf1-binding motif in vivo.

To identify factors that potentially collaborate with Ebf1 in the regulation of transcription, we scanned the Ebf1-bound regions for the presence of overrepresented transcription factor binding motifs using the TRANSFAC database (Matys et al., 2006). Seven motifs or groups of related binding motifs of transcription factors known to be involved in hematopoiesis were found to be strongly enriched in this sequence set (Figure 2G, Figure S2G, and Table S3). Notably, binding sites of all transcription factors that have been described as functional "collaborators" of Ebf1 are found among the enriched motifs. In particular, E2A has been shown to genetically and functionally interact with Ebf1 (O'Riordan and Grosschedl, 1999; Sigvardsson et al., 2002). Moreover, a functional interaction between Ebf1 and Runx1 was shown on the *Cd79a* promoter, on which Ets1 and Pax5 also collaborate in an Ebf1-dependent manner (Maier

A

Dataset		Mapped RefGenes	With binding site (+/-100kb)	With binding site (+/-10kb)	P-value (+/-100kb)	P-value (+/-10kb)
GoF	up-regulated	853	365	217	$< 2.2 \times 10^{-16}$	$< 2.2 \times 10^{-16}$
	down-regulated	465	101	46	1.4×10^{-4}	5.2×10^{-2}
LoF	down-regulated	792	278	147	$< 2.2 \times 10^{-16}$	$< 2.2 \times 10^{-16}$
	up-regulated	587	158	68	2.9×10^{-14}	2.3×10^{-4}
Background		10568	1595	778		





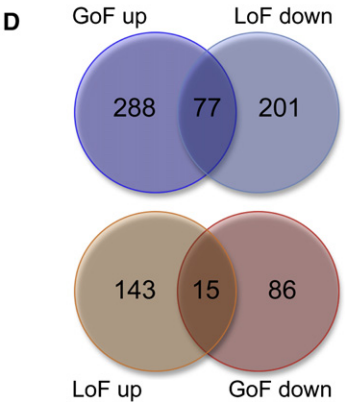


Figure 3. Direct Ebf1 Binding Is Strongly Associated with Transcriptional Changes upon Perturbation of Ebf1 Activity

(A) Comparison of the microarray data sets from Ebf1 gain- or loss-of-function studies with genes associated with Ebf1-bound regions identified by ChIP-seq.

(B) Validation of the gain-of-function transcriptome analysis. *Ebf1*$^{-/-}$ pre-pro-B cells were infected with empty or Ebf1-containing retroviruses in triplicate as described above. Expression of Ebf1 functional targets identified in the gain-of-function microarray that also contain Ebf1-binding sites within 100 kb of the gene were determined by qRT-PCR and are shown relative to empty vector-infected cells.

(C) Quantitation of the number of Ebf1-bound regions identified by ChIP-seq within 100 kb of genes upregulated in the gain-of-function experiment. Gray bars correspond to all RefGenes; blue bars correspond to those induced by Ebf1 expression.

(D) Overlap of Ebf1-bound genes (ChIP-seq peak within 100 kb of the gene) also identified as functional targets by Ebf1 gain- or loss-of-function analyses. Genes activated by Ebf1 are displayed in blue, repressed genes in red. See also Figure S3.

et al., 2003; Maier et al., 2004). These data suggest that Ebf1 cooperates not only with previously described, but also with additional transcription factors in target gene regulation.

Ebf1 Binding Correlates with Gene Regulation by Pax5

The enrichment of Pax5-binding sites within the Ebf1-bound regions prompted us to examine whether genes regulated by Pax5 are also enriched for Ebf1 binding. To this end, we compared published data sets of Pax5-activated and -repressed genes (Delogu et al., 2006; Pridans et al., 2008; Schebesta et al., 2007) with genes associated with Ebf1-bound genomic regions. Interestingly, Pax5-activated, and to a minor extent also Pax5-repressed, genes have a markedly increased frequency of Ebf1-occupied sites compared to a random gene set (Figure 2H and Table S4). This finding suggests that the network control of B lymphopoiesis by Ebf1 and Pax5 involves not only cross-regulation of these transcription factors (Decker et al., 2009; Roessler et al., 2007) but also their concerted binding and regulation of target genes.

Ebf1 Binding Correlates Strongly with Transcriptional Regulation

To link the identified Ebf1 binding sites to the function of Ebf1 in transcriptional regulation, we performed microarray expression analyses on *Ebf1*$^{-/-}$ pre-pro-B cells retrovirally transduced with Ebf1 (gain-of-function [GoF]) and Il7-cultured pro-B cells with conditionally inactivated *Ebf1* (loss-of-function [LoF]) (Figures S3A and S3B and Tables S5 and S6). These experiments provided us with three genome-wide data sets whose comparisons and overlaps could be used to identify direct, transcriptional targets of Ebf1. In a first step, the regulated RefGenes from each microarray experiment were matched with the ChIP-seq binding data. Genes containing an Ebf1-bound region within 100 kb of the gene body as well as the subset showing Ebf1 binding within 10 kb of the TSS show a substantial enrichment among the Ebf1-regulated genes identified by the microarray experiments (Figure 3A). More specifically, in comparing our gain-of-function results with the ChIP-seq data set, ~15% of Ebf1-occupied genes were regulated, whereas our loss-of-function experiment showed regulation of ~14% of the Ebf1-bound genes.

Although the correlation with activated genes is significantly stronger than with repressed targets ($p = 1.27 \times 10^{-3}$ / 4.06×10^{-4} for the LoF 100 kb/10 kb sets), a clear link exists between direct Ebf1 binding and transcriptional repression. For validation of the gain-of-function microarray experiment, the expression changes of the most strongly up- and downregulated genes that lie close to a mapped Ebf1-binding site were examined by qRT-PCR (Figure 3B). In this experiment, we could verify the repression of eight out of nine Ebf1 target genes and the

activation of all tested Ebf1-bound genes (Figure 3B). We also noted that genes containing more than one Ebf1-binding site within 100 kb are more likely to be regulated by Ebf1; such a result may be related to a cumulative or cooperative effect of multiple Ebf1 molecules bound to a particular gene locus (Figure 3C). Together, the collection of three genome-wide data sets reveals that approximately one-quarter of Ebf1-bound targets are also regulated by Ebf1 at the transcriptional level.

Overlap of Three Different Genome-wide Data Sets Defines High-Confidence Targets of Ebf1

Comparison of the gain- and loss-of-function microarray data shows only a modest overlap of 137 Ebf1-activated and 60 Ebf1-repressed genes whose expression is consistently altered in both experimental settings (Figure S3C). To identify Ebf1-bound genes among these high-confidence targets, we aligned the overlapping set of Ebf1-activated RefGenes (114 out of the 137 consistently activated genes) with the ChIP-seq data, resulting in a 68% match (77/114) (Figure 3D). Together with a similar alignment of Ebf1-repressed genes, 92 genes (77 activated and 15 repressed) were identified in all three target gene analyses, whereby 60 activated and 7 repressed genes contain occupied Ebf1-binding sites within 10 kb from the transcription start site, including genes identified in the initial ChIP-on-chip analysis (Table 1 and Table S7). This core gene set represents a "gold standard" collection of genes that are directly regulated by Ebf1.

Ebf1 Preferentially Regulates Components of Signal Transduction Cascades

The core set of Ebf1 targets can be grouped into seven broad functional categories according to available annotations (Table 1). Interestingly, approximately half of the genes are related to the cellular signal transduction machinery ranging from surface receptors to transcription factors. To confirm this observation, we performed a statistical analysis using annotations from the PANTHER database and found that three closely related biological processes are strongly overrepresented: intracellular signaling, protein modification, and protein phosphorylation (Figure S4A). In particular, components of B cell receptor signaling were enriched, and so were components of T cell receptor signaling, many of which are shared between both pathways (Figure S4B). Inspection of the more than 50 potential Ebf1 targets within the BCR signaling pathway showed a higher proportion of Ebf1-bound genes in the proximal part of the BCR signaling cascade (Figure S4C). Moreover, a large number of these Ebf1-bound genes are regulated in our functional analysis, most of them being activated in the gain-of-function experiment. Together, these results suggest that one of the cardinal functions of Ebf1 is the establishment of the B cell-specific signal transduction network.

Chromatin Structure at Promoters that Are Activated, Repressed, or Poised by Ebf1

The large number of in vivo binding sites for Ebf1 identified in this study represents a valuable resource for studies concerning the mechanisms of Ebf1 function. In a first approach, we grouped Ebf1-bound genes into three categories: genes activated by Ebf1, genes repressed by Ebf1, and genes that are bound by Ebf1 in early-stage B cells, but are not transcriptionally active until the mature B cell stage. For those genes not immediately activated by Ebf1, we predicted that Ebf1 functions to prepare the target genes for future expression and establish a "poised" state. For each of the three target categories, representative genes were chosen on the basis of their response in the gain- and loss-of-function experiments and on their expression patterns at different stages of B cell differentiation (Figures 4A–4C). In ChIP experiments, we used antibodies specific for covalent modifications of histone H3 that represent marks of accessible and transcribed chromatin (H3K4me3 and H3Ac) or silenced chromatin (H3K27me3). In this analysis, we also examined H3K4me2 modification, which has been associated with "poised" chromatin, similar to H3K4me3-H3K27me3 "bivalent" chromatin (Bernstein et al., 2006; Orford et al., 2008). We determined the chromatin state of these genes at the transcriptional start sites and the Ebf1-binding sites (if located more than 1 kb from the TSS) in Ebf1-deficient pre-pro-B cells, in Ebf1-positive pro-B cells, and in mature splenic B cells (Figures 4D–4F). As expected, the Ebf1-activated targets *Cd79a*, *Gfra2*, and *Pax5* gain H3K4 methylation and H3 acetylation marks upon the transition to the pro-B stage, correlating with their transcriptional activation (Figure 4D). The genes that show a marked downregulation in pro-B cells relative to pre-pro-B cells (Figure 4B), and that are repressed after retroviral transduction of Ebf1 into *Ebf1*$^{-/-}$ pre-pro-B cells, show a loss of activating chromatin marks and gain of H3K27 trimethylation between the pre-pro-B and pro-B cell stages (Figure 4E).

Finally, in a third set of genes, including *Cd40*, *Acp5*, and *Egr3*, we do not observe transcriptional activation in Ebf1-expressing cells until the mature B cell stage (Figure 4C). Interestingly, we detect two stages of chromatin alterations, one corresponding with the binding of Ebf1 in pro-B cells and the other with transcriptional activation at the mature B cell stage. Specifically, the chromatin at the Ebf1-binding sites gains H3K4 dimethylation during the transition of pre-pro-B cells to pro-B cells (Figure 4F). For *Acp5* and *Egr3*, in which the Ebf1-binding regions reside some 8 kb upstream of the TSS, these changes may reflect the chromatin "opening" at *cis*-regulatory elements in Ebf1-positive cell stages. The H3K4me3 and H3Ac activation marks only appear in mature B cells, coinciding with transcriptional activation (Figure 4F). We observe a similar pattern of histone modifications in the promoter region of the *Cd40* gene in which the Ebf1-binding site resides 435 bp upstream of the TSS. In the pro-B stage, a robust H3K4me2 signal is detected, then activation marks are found in mature B cells (Figure 4F). Together, these data show that direct target binding by Ebf1 is linked to chromatin modifications that either precede transcriptional activation, or correlate with gene activation or repression.

Ebf1 "Poises" Chromatin Structure Independent of Transcription

To analyze the direct effects of Ebf1 binding on the surrounding chromatin without the changes induced by transcriptional activation or cellular differentiation, we transduced a CD4^{+}CD8^{+} T cell progenitor line and the fibroblastic NIH 3T3 cell line with an Ebf1-encoding retrovirus or a control virus. Twenty-four hours after gene transfer, we performed a ChIP assay to monitor

Table 1. Genes that Are Consistently Activated or Repressed in the Ebf1 Gain- and Loss-of-Function Experiments and that Are Bound by Ebf1 within 10 kb of the Transcription Start Site in the ChIP-Seq and/or ChIP-on-Chip Studies

Gene Symbol	Accession No.
Transcription and Regulation	
Bhlha15	NM_010800
Cbx2	NM_007623
Ell3	NM_145973
Foxo6	NM_194060
Hes1	NM_008235
Hes5	NM_010419
Mterfd3	NM_028832
Parp1	NM_007415
Pold4	NM_027196
Pou2af1	NM_011136
Zfpm1	NM_009569
Receptors and Signal Transduction	
Bst1	NM_009763
Cd72	NM_001110320
Ceacam1	NM_001039185
Cmtm8	NM_027294
Dok3	NM_013739
Dtx1	NM_008052
Gpr56	NM_018882
Gpr97	NM_173036
Icosl	NM_015790
Igll1	AK137514
Notch3	NM_008716
P2rx3	NM_145526
Pde4a	NM_183408
Plekhg2	NM_001083912
Rgs12	NM_173402
Rinl	NM_177158
Sit1	NM_019436
Spred2	NM_033523
Trfr2	NM_015799
Vpreb1	NM_016982
Vpreb2	NM_016983
Kinases and Phosphatases	
Blk	NM_007549
Dusp7	NM_153459
Pkig	NM_001039390
Ptp4a3	NM_008975
Ptprs	NM_011218
Sbk1	NM_145587
Enzymes and Metabolism	
Agpat9	NM_172715
Aldh3b1	NM_026316
B3gnt2	NM_016888
Impdh1	NM_011829
Neu1	NM_010893
Pck2	NM_028994
Pitpnm2	NM_011256
Membrane Transport	
Kcna5	NM_145983
Mfsd4	NM_172510
Ucp2	NM_011671
Cytoskeleton and Motility	
Asb2	NM_023049
Atp8b3	NM_026094
Cyfip2	NM_133769
Itgb7	NM_013566
Micall2	NM_174850
Rsph9	NM_029338
Sema7a	NM_011352
Smtn	NM_001159284
Miscellaneous	
2010317E24Rik	NM_001081085
4632428N05Rik	NM_028732
Bcl7a	NM_029850
Ccdc18	NM_028481
Chchd10	NM_175329
Fam102a	NM_153560
Lrmp	NM_008511
Mreg	NM_001005423
Srl	NM_175347
Stxbp1	NM_001113569
Zc3h12d	NM_172785

Repressed targets are shown in bold, and all other genes are activated targets. See also Figure S4.

binding of Ebf1 at *Cd79a* and *Pax5*, genes that are activated by Ebf1 in pro-B cells, and at *Egr3* and *Cd40*, genes that are "poised" for expression in later stage B cells. We observed binding of exogenously expressed Ebf1 to the sites identified by ChIP-seq in the Beko cells. Notably, we detected no transcription or transcriptional activation of *Cd79a*, *Pax5*, and *Egr3*, whereas *Cd40* was activated to low expression by Ebf1 (Figures 5A and 5B). In NIH 3T3 cells, we detected no substantial binding of Ebf1, despite the similar expression of *Ebf1* and an active transcriptional state of *Egr3* in the NIH 3T3 cells (Figures 5C and 5D; data not shown). Together with the ChIP-seq analysis, which indicated that binding of endogenous Ebf1 in pro-B cells is enriched for T cell-specific genes but not nonhematopoietic genes, these results indicate that Ebf1 recognizes binding sites in a hematopoietic chromatin context, independent of an active transcriptional state.

Analysis of histone modifications revealed a marked increase in H3K4 dimethylation at the Ebf1-bound regions of all four genes specifically in Ebf1-expressing Beko cells (Figure 5E). Consistent with the lack of robust transcription from the four test genes, no enrichment of H3K4 trimethylation could be observed, whereas H3K4 di-and trimethylation was detected at a housekeeping

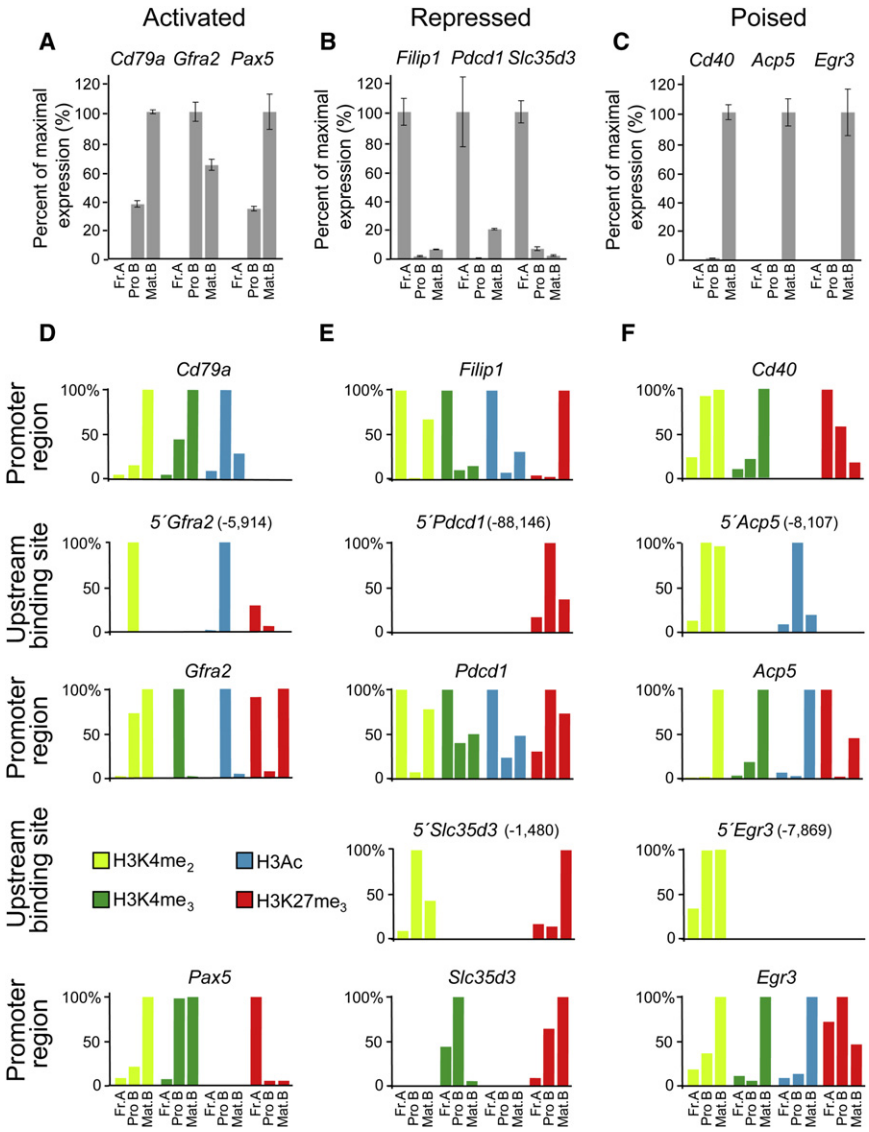


Figure 4. Ebf1 Activity Generates Active, Repressed, or Poised States of Gene Expression that Are Reflected by Alterations in Chromatin Status

(A–C) Transcript expression of select Ebf1-bound genes that are activated (A), repressed (B), or poised (C) for expression by Ebf1 were analyzed in three stages of B cell development. Percentages displayed are relative to the cell type with the maximum expression value for each gene. Error bars represent standard deviation of triplicate qRT-PCRs.

(D–F) ChIP analysis of four histone modifications in the cell types shown in (A). The promoter regions and the Ebf1-bound regions identified in this study for each gene shown in (A)–(C) were analyzed by qPCR. Percentages are relative to the cell type with the highest enrichment for a given modification in each bound region. The location relative to the transcriptional start site (in base pairs) is given for each Ebf1-bound region within an upstream regulatory element. Only modifications shown to have greater than 5-fold enrichment over a nonspecific control region are shown. *Fr.A*, *Ebf1*$^{-/-}$ pre-pro-B cells; *pro-B*, Il7 cultured pro-B cells; and *Mat.B*, B220^{+} splenic B cells. See also Figure S5.

gene, *Tpi*. In NIH 3T3 cells, we detected no substantial changes of H3K4 di- or trimethylation upon Ebf1 expression at the sites tested. The chromatin at the *Egr3* gene contained both modifications at the transcription start site (Figure 5F), which is consistent with its transcriptional activity. The upstream enhancer is not modified, which suggests a different and Ebf1-independent regulation in the fibroblastic cells. Taken together, these data suggest that Ebf1 binds to lineage-specific target sites in a hematopoietic chromatin context and induces changes in chromatin that poise the genes for subsequent transcriptional activation.

DISCUSSION

The combination of genome-wide analyses of Ebf1-bound and -regulated genes allows for a comprehensive view into the molecular basis of Ebf1 function in B lineage cells. Although Ebf1 has been identified as a determinant of B lymphocyte specification and differentiation, the number of known target genes has been limited (reviewed in Lukin et al., 2008). Our ChIP-seq analysis indicated that Ebf1 occupies ~4500 sites within 100 kb of annotated genes. Previous analysis of transcription factor (TF) binding in embryonic stem cells has shown that binding site numbers for a given TF can range between 1,126 for Smad1 and 39,609 for CTCF (Chen et al., 2008). Such large numbers of occupied binding sites do, however, raise the question of functional relevance and necessitate their interrogation by transcriptome analysis. Comparison of our ChIP-seq data with two independent, functional analyses showed that ~26% of the genes containing Ebf1-binding sites within 100 kb were also transcriptionally regulated. In addition to a small subset of genes that are conversely regulated in gain- and loss-of-function experiments, this comparison of genome-wide data sets also indicated that several genes require Ebf1 only for the initiation of expression. For example, *Cd79a* and *b* (encoding Igα and β), *Gcgr* (coding for the glucagon receptor), and others are markedly upregulated in the gain-of-function experiment, but are not affected by the inactivation of Ebf1. Another interesting set of genes was identified by comparing Ebf1-bound genes with published data sets in which differential gene expression was examined in pro-B cells and mature B cells versus CLPs (Mansson et al., 2008). In particular, for several Ebf1-occupied genes, expression is detected at later stages of differentiation, but not in Ebf1-positive pro-B cells. Thus, these genes represent targets on which Ebf1 appears to play a role in "poising" chromatin for expression.

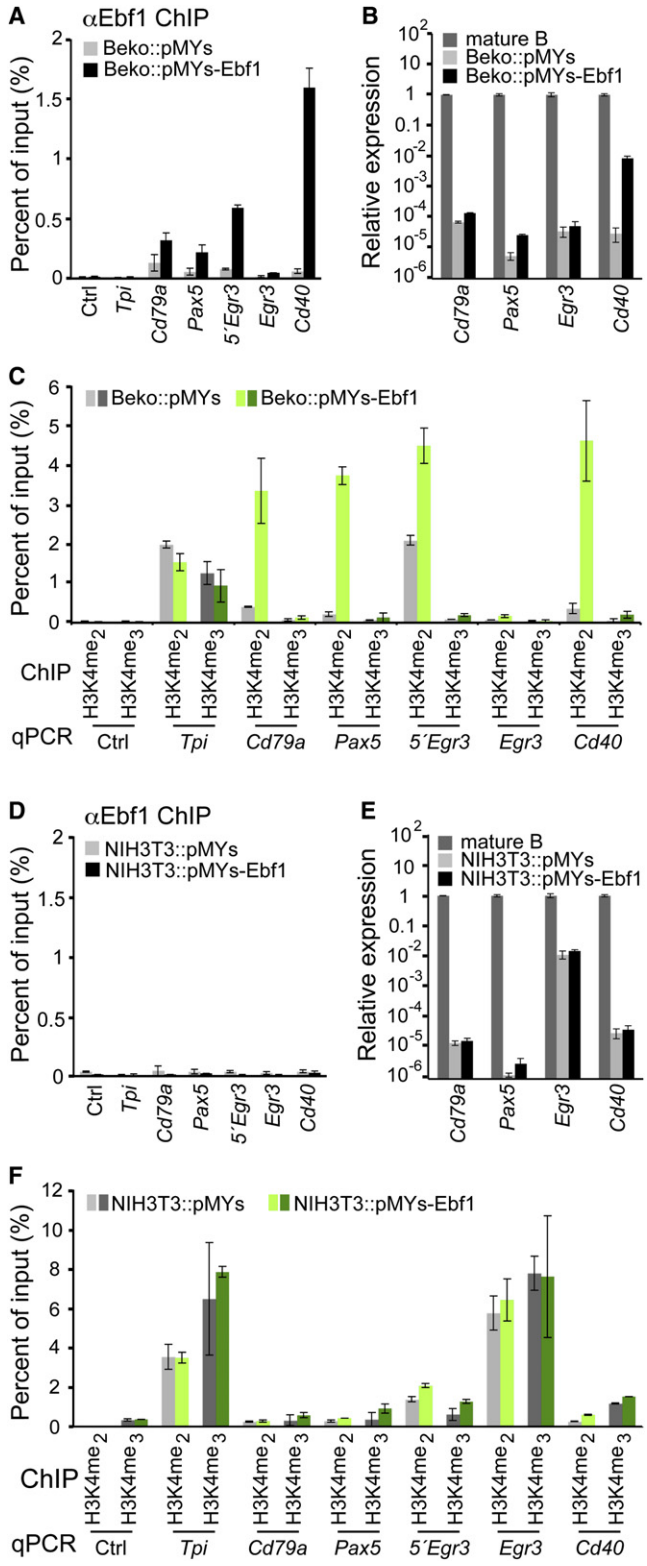


Figure 5. Ebf1-Binding Requires a Hematopoietic Chromatin Context and Directly Leads to Local Chromatin Activation

Beko T cells (A, B, and E) and NIH 3T3 cells (C, D, and F) were infected with an Ebf1-IRES-GFP expressing retrovirus or control virus and sorted for GFP expression 24 hr after infection. (A) and (C) show analysis of Ebf1-binding to an intergenic control region, the *Tpi* promoter, and four Ebf1 target genes by anti-Ebf1 ChIP and qPCR. 5′ Egr3 represents the Ebf1 binding region 7869 bp upstream of the TSS, whereas the other amplicons are located in the proximal promoter regions. Error bars represent standard deviation of duplicate ChIP experiments. (B) and (D) show qRT-PCR expression analysis of Ebf1 targets in Beko (B) and NIH 3T3 cells (D) retrovirally transduced with Ebf1 or an empty vector control. Raw cycle values of the qRT-PCR were normalized to *Tpi* expression and are displayed as fold expression compared with mature splenic B cells. Error bars represent standard deviation of experimental triplicates. (E) and (F) show quantification of H3K4 di- (light green) and trimethylation (dark green) of the regions analyzed in (A) by ChIP and qPCR. Empty virus controls are shown in light and dark gray. Error bars represent standard deviation of duplicate ChIP experiments.

In previous studies, targeted gene inactivation of Ebf1 and retroviral transduction of hematopoietic progenitor cells indicated that Ebf1 regulates the earliest steps of B lymphopoiesis, including specification (Lin and Grosschedl, 1995; Pongubala et al., 2008). However, the initially identified target genes do not fully account for the presumed roles of Ebf1 in this process. In the hematopoietic system, both transcription factors E2A and Ebf1 are markedly upregulated in the B lymphoid progenitor population of the CLP compartment (Inlay et al., 2009). Ebf1 has been shown to induce the expression of the *Tcf3* gene and conversely, E2A can activate the *Ebf1* gene (Kee and Murre, 1998; Zhuang et al., 1994). In our ChIP-seq analysis, we identified three Ebf1-binding sites in the *Tcf3* gene and conditional inactivation of *Ebf1* results in a marked downregulation of *Tcf3*. Likewise, the transcription factor Bcl11a has been identified as a determinant of B cell differentiation that acts upstream of Ebf1 (Liu et al., 2003). We found three Ebf1-binding sites in the *Bcl11a* locus and detected an increase of *Bcl11a* expression in the gain-of-function experiment. Thus, Ebf1, E2A, and Bcl11a appear to act in a concerted manner to establish the early B cell program in which pairs of regulatory factors are interconnected by feedback loops that stabilize developmental lineage decisions. Another interconnection in this network control is provided by the reciprocal regulation of the *Ebf1* and *Pax5* genes and the binding of Ebf1 to a relatively large portion of Pax5 targets (Decker et al., 2009; Roessler et al., 2007; this study).

An important aspect of B cell differentiation is the commitment step in which alternative cell fates are repressed. Pax5 has been shown to act as the major determinant of this process as $Pax5^{-/-}$ cells gain developmental plasticity and are able to differentiate into alternative lineages (Nutt et al., 1999; Urbánek et al., 1994). Recently, Ebf1 has also been implicated in the repression of alternative cell fates as it was found that Ebf1 can repress the *Cebpa, Sfpi1*, *Id2*, and *Id3* genes independently of Pax5 (Pongubala et al., 2008; Thal et al., 2009). Our combined ChIP-sequencing and microarray experiments provide additional insight into the potential function of Ebf1 in lineage restriction. Ebf1 was found to directly repress the homeobox gene *Hlx*, encoding a protein that collaborates with Tbet to promote Th1 differentiation (Mullen et al., 2002). Moreover, we identified *Pdcd1*, *Ctla4*, and *Icosl* as genes that are directly repressed by Ebf1. *Pdcd1* and *Ctla4* encode members of the CD28 family of receptors that are expressed on T lymphocytes and regulate T cell activation and tolerance (Greenwald et al., 2005). Likewise, the ligand of the Icos receptor regulates T helper cell differentiation and humoral immune responses (Greenwald et al., 2005).

In addition to Ebf1-mediated repression of *Id2*, which has been implicated in the restriction of alternative lineage choices (Pongubala et al., 2008; Thal et al., 2009), we identified *Gfi1b* as an Ebf1-repressed gene. *Gfi1b* encodes a transcriptional repressor that is under cross-regulatory feedback control with its relative *Gfi1*, which has been shown to promote B lineage fate choices (Spooner et al., 2009; Vassen et al., 2007). Consistently, *Gfi1*, which itself is not bound by Ebf1, is downregulated upon loss of Ebf1 in pro-B cells, presumably via upregulation of the Ebf1-bound *Gfi1b* gene. Thus, Ebf1 is an important regulatory component of multiple transcriptional networks that specify the B lineage fate.

The most prominent cluster of Ebf1-bound and -regulated genes includes components of the (pre)BCR signaling pathway. Previously, several genes, including *Cd79a* and *Cd79b*, *VpreB*, *Igll1*, and *Blnk*, were identified as Ebf1 targets (reviewed in Lukin et al., 2008). Our genome-wide analysis allowed for the identification of an additional 21 Ebf1-occupied and -regulated genes common to this pathway. Moreover, ~75 genes implicated in various aspects of (pre)BCR signaling are bound by Ebf1. These Ebf1-regulated genes include components of the (pre)BCR receptor complex and receptors that modulate and fine-tune BCR signals, such as Cd72 and Cd19 (reviewed in Brezski and Monroe, 2008). In addition to several important BCR downstream components, such as Blk and Plcγ, Ebf1 regulates other signaling events that are initiated by BCR stimulation. In particular, the genes encoding components of Ca^{2+} signaling, such as the IP3 receptor, PKC, and Dok3, a negative regulator of Jnk activation and Ca^{2+} mobilization (reviewed in Mashima et al., 2009), are direct Ebf1 targets. Another Ebf1-regulated and BCR-linked signaling cascade includes the PI3 kinase pathway. This pathway has been recently shown to regulate BCR-mediated cell survival, whereby constitutively active PI3 kinase was found to rescue the survival of cells in which the BCR has been conditionally inactivated (Srinivasan et al., 2009). Notably, Ebf1 directly regulates genes encoding components that link the PI3 kinase pathway to BCR signaling, including Cd19 and the genes encoding BCAP (*Pik3ap1*) and Bank1 (*Bank1*), which were up- and downregulated, respectively. *Pik3ap1* encodes an adaptor protein that potentiates Akt signaling by regulating PI3K localization, whereas *Bank1* encodes a negative regulator of Akt activation (Aiba et al., 2006; Okada et al., 2000). In our analysis, we noticed that the coincidence of Pax5 regulation and Ebf1 binding is most extensive for genes involved in (pre)BCR signaling, the key signaling pathway in lymphocytes. This observation may reflect an "overwiring" of this pathway and explain why many of the genes are not affected upon conditional Ebf1 inactivation

Ebf1 has been implicated in epigenetic regulation by initiating DNA demethylation at the *Cd79a* gene (Maier et al., 2004). Moreover, Ebf1 has been found to associate with components of the Swi/Snf2 nucleosome-remodeling complex (Gao et al., 2009). The inability of Ebf1 to occupy known adipocyte- or neuronal-specific target genes in pro-B cells indicates that Ebf1, despite its suggested role as "pioneer factor," requires a permissive chromatin context for binding. This notion was confirmed by the binding of Ebf1 to B cell-specific genes upon expression in hematopoietic T lineage cells but not in fibroblastic NIH 3T3 cells. The permissive chromatin context could be generated by Ebf1 binding with other cell type-specific transcription factors and concomitant recruitment of chromatin-modifying complexes or by the establishment of a hematopoietic chromatin "signature" in HSCs. The latter view is consistent with the promiscuous expression of genes in HSCs, which has been attributed to a "multi-lineage priming" of genes (Hu et al., 1997; Miyamoto et al., 2002).

The developmental dynamics in the chromatin state of several Ebf1-target genes that are activated, repressed, or poised for expression by Ebf1 revealed an increase in activating chromatin marks and a decrease in repressive marks on Ebf1-activated genes. Conversely, genes that are expressed in Ebf1-deficient pre-pro-B cells, but are repressed in Ebf1-positive pro-B and mature B cells, show Ebf1-associated decreases in activating histone marks and increases of repressive marks. Because these chromatin changes are associated with alterations in gene expression, our analysis does not allow for conclusions about a potential role of Ebf1 in epigenetic regulation. However, an interesting Ebf1-bound gene set, including *Cd40* and *Egr3*, is expressed in mature B cells, but not in pro-B cells. In these genes, Ebf1-binding is associated with the appearance of H3K4 dimethyl marks independent of gene expression. The poised chromatin state can be recapitulated in T lineage cells upon ectopic expression of Ebf1, showing that its establishment does not require B cell differentiation. Notably, the two Ebf1 target genes *Cd79a* and *Pax5*, which are rapidly activated at the onset of Ebf1 expression in early-stage B cells, exhibit a poised chromatin state in T cells that have been transduced with an Ebf1-expressing retrovirus. Taken together, our data reveal three distinct events involved in gene regulation by Ebf1. First, binding of Ebf1 is dependent on a permissive hematopoietic chromatin context, given that no binding of Ebf1 to its B cell gene targets is observed in fibroblastic cells. Second, binding of Ebf1 induces changes in chromatin structure including histone modifications that "poise" target gene loci in a transcription-independent manner. In particular, H3K4 dimethylation has been associated with "poised" chromatin (Orford et al., 2008), and our data suggest that Ebf1 is a transcription factor that is involved in targeting this chromatin mark to specific genes. Lastly, collaboration with other transcription factors and cofactors allows Ebf1 to direct the ultimate transcription state of target genes throughout B cell differentiation.

EXPERIMENTAL PROCEDURES

Chromatin Immunoprecipitation and ChIP-on-Chip

Anti-Ebf1 chromatin-IP (ChIP) was performed essentially as previously described (Pongubala et al., 2008). Detailed ChIP-protocols are included in the Supplemental Experimental Procedures. DNA from αEbf1 chromatin IP experiments was amplified in two steps with the whole-genome amplification and reamplification kits (Sigma) and purified via a Qiaprep spin column (QIAGEN). The resulting material and equally treated input control DNA were labeled and hybridized to mm8 RefSeq promoter tiling arrays (Nimblegen) by ImaGenes (Berlin). Data was analyzed using SignalMap (Nimblegen), and the implemented FindPeaks algorithm was used for peak calling. A peak of FDR < 0.05 in one experiment with an overlapping peak of FDR < 0.1 in the duplicate experiment was scored as an Ebf1-bound region.

Deep Sequencing and Peak Calling

Libraries were prepared from 10 ng of Ebf1 ChIP DNA and input DNA, respectively, in accordance with the manufacturer's protocol. ChIP-seq data were

generated with Illumina GA single-read sequencing. Sequenced tags were mapped to the mouse genome (mm8, NCBI) with ELAND (Illumina) and only uniquely mapped tags (~26 × 10^6 per library) were considered. For identification of Ebf1-binding regions, tags with the same mapping coordinates were counted as one to address possible amplification biases. The sequence tag density generated from the input library was used as background. Regions enriched in the Ebf1 ChIP sample were identified with CCAT (Xu et al., 2010). Peaks with FDR < 0.01 were considered significant. Additionally, a 3-fold enrichment cut-off was imposed. Gene coordinates were obtained from the UCSC RefGene table (mm8), and binding sites were mapped to the nearest genes (up to a maximal distance of 100 kb). Information regarding the additional bioinformatic analysis is provided in the Supplemental Experimental Procedures.

Retroviral Expression of Ebf1

Retrovirus was produced by transient transfection of Plat-E cells with retroviral constructs encoding Ebf1-EGFP (pMYs-Ebf1-IRES-EGFP) or EGFP alone (pMYs-IRES-EGFP). The supernatant was collected 24 hr and 48 hr after transfection and supplemented with 10 μg/ml polybrene. For spin infection, the target cells were resuspended in retroviral supernatant at 0.7 × 10^6 cells/ml. Cells were spun for 2.5 hr at 2600 rpm in 24-well plates. After infection, the cells were resuspended in fresh media and cultured for 24 hr. Cells were sorted for EGFP expression prior to analysis.

Conditional Deletion of Ebf1 in Fetal Liver Pro-B Cells

The floxed *Ebf1* allele was generated by introduction of loxP sites flanking exons 2 and 3 in ESCs by homologous recombination. These cells were used for generating $Ebf1^{+/fl}$ mice. $Ebf1^{+/fl}$ mice were intercrossed and the resulting $Ebf1^{fl/fl}$ mice were crossed with RERT-Cre mice, expressing a tamoxifen-inducible ERT-Cre recombinase fusion protein under the control of a ubiquitous promoter (Guerra et al., 2003), as well as with a Bcl2 transgenic mouse strain. Further details of the $Ebf1^{fl/fl}$ mice will be described elsewhere. Total cell preparations from livers of 16.5 days postcoitum (dpc) embryos were cultured in the presence of OP-9 bone marrow stromal cells and Il7 until a pure pro-B cell population was obtained. For inactivation of *Ebf1*, the B cells were treated with 2 μM 4-hydroxy-tamoxifen for 24 hr, washed, and cultured for an additional 4 days.

ACCESSION NUMBERS

The ChIP-seq data set is available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE19971. The data sets of the loss-and gain-of function microarray analyses are available under accession numbers GSE21454 and GSE21455, respectively. The ChIP-on-chip data set is available under accession number GSE21469.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, seven tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2010.04.013.

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