Compound haploinsufficiencies of *Ebf1* and *Runx1* genes impede B cell lineage progression

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Early B cell factor (EBF)1 is essential for B lineage specification. Previously, we demonstrated the synergistic activation of *Cd79a* (*mb-1*) genes by EBF1 and its functional partner, RUNX1. Here, we identified consequences of *Ebf1* haploinsufficiency together with haploinsufficiency of *Runx1* genes in mice. Although numbers of "committed" pro-B cells were maintained in *Ebf1*$^{+/-}$*Runx1*$^{+/-}$ (*ER*^{het}) mice, activation of B cell-specific gene transcription was depressed in these cells. Expression of genes encoding Aiolos, *κ0* sterile transcripts, CD2 and CD25 were reduced and delayed in *ER*^{het} pro-B cells, whereas surface expression of BP-1 was increased on late pro-B cells in *ER*^{het} mice. Late pre-B and immature and mature B cells were decreased in the bone marrow of *Ebf1*$^{+/-}$ (*E*^{het}) mice and were nearly absent in *ER*^{het} mice. Although we did not observe significant effects of haploinsufficiencies on *IgH* or *Igκ* rearrangements, a relative lack of *Igλ* rearrangements was detected in *E*^{het} and *ER*^{het} pre-B cells. Together, these observations suggest that B cell lineage progression is impaired at multiple stages in the bone marrow of *E*^{het} and *ER*^{het} mice. Furthermore, enforced expression of EBF1 and RUNX1 in terminally differentiated plasmacytoma cells activated multiple early B cell-specific genes synergistically. Collectively, these studies illuminate the effects of reduced *Ebf1* dosage and the compounding effects of reduced *Runx1* dosage. Our data confirm and extend the importance of EBF1 in regulating target genes and *Ig* gene rearrangements necessary for B cell lineage specification, developmental progression, and homeostasis.

B cell development | B lymphopoiesis | immunoglobulin gene rearrangements | transcription factor dosage | transcriptional networks

Multiple lines of evidence suggest that early B cell factor (EBF) 1 (also known as EBF/O/E-1/COE1) is centrally important in B lineage specification (1). In EBF1 knockout mice, B cell development is arrested at an early progenitor stage (2). EBF1-deficient mice fail to rearrange *Ig heavy* (*IgH*) and *light* (*IgL*) chain genes and do not express essential proteins necessary for B cell development, e.g., Ig-α (*Cd79a*/*mb-1*), Ig-β (*Cd79b*/*b29*), CD19 (*Cd19*), λ5 (*Igll1*), VpreB1 (*Vpreb1*), Rag1 (*Rag1*), and Pax5 (*Pax5*). Evidence of EBF1's primary role in B cell lineage specification includes its ability to drive the B cell fate at the expense of other cell lineages (3). Furthermore, B cell lineage development is promoted by EBF1 in the absence of upstream regulators including IL-7, IL-7 receptor α, PU.1, Ikaros, and E2A (4–9). Recent evidence suggests that EBF1 controls B lymphopoiesis in at least two ways: (*i*) by "pioneering" the activation of genes that are essential for the B lineage-specific program (10, 11) and (*ii*) by reinforcing B lineage commitment with Pax5 (12, 13).

RUNX1 (AML1/PEBP2αB/CBFα2) is a key regulator of hematopoiesis in multiple cell types of the blood (14). The absence of RUNX1 results in embryonic lethality due to a complete lack of hematopoiesis (15, 16). RUNX1 is required for the expression of *Sfpi1* (PU.1), which in turn coordinates the development of myeloid cells, granulocytes, and lymphocytes (17). In T cell development, RUNX1 functions as both an activator and a repressor (18). The contributions of RUNX1 to B cell development have not been investigated extensively. RUNX1 is expressed in early B cell progenitors and in immature and mature B cells, where it increases cell survival (19, 20). The conditional ablation of *Runx1* genes in adult mice reduces numbers of common lymphoid progenitors and B cells (21). Expression of a fusion protein comprising the partner protein of RUNX1, CBFβ, and smooth muscle myosin heavy chain (SMMHC) decreased numbers of pro-B and pre-B cells and transcripts of the *Cd79a*, *Vpreb1*, and *Igll1* genes in mice (22). However, questions concerning the roles of RUNX1 in B cell development have not been addressed completely.

Previous work in our laboratory demonstrated DNA binding interactions between EBF1 and RUNX1, suggesting functional cooperation in vivo. EMSA and in vivo footprinting assays detected the binding of EBF1 and RUNX1 (with CBFβ) to tandem sites in the *Cd79a* promoter (23, 24). Coexpression of EBF1 and RUNX1 in terminally differentiated plasmacytoma cells reactivated *Cd79a* gene transcription in the context of hypermethylated DNA and inactive chromatin. EBF and RUNX1 function synergistically in B cells; however, little is understood concerning their coregulation of gene expression in these cells.

To determine the extent of gene regulation by EBF1 and RUNX1 in developing B cells, we turned to mice with reduced gene dosage of each factor. This step was necessitated by the extremely early arrest of B lymphopoiesis in *Ebf1*$^{-/-}$ mice and lack of hematopoiesis in *Runx1*$^{-/-}$ fetuses. To avoid these issues, we assessed the contributions of EBF1 and RUNX1 in the context of single and compound haploinsufficient mice. Such an approach reveals synergistic effects of reducing both genes simultaneously. Specific effects observed in compound, but not single, haploinsufficient mice, have provided evidence of cooperation between transcription factors and signaling proteins in multiple shared pathways (25, 26).

Unlike mice with homozygous knockouts of *Ebf1* or *Runx1* genes, *Ebf1*$^{+/-}$*Runx1*$^{+/-}$ (*ER*^{het}) mice are viable, and analysis of B cells over the full course of their development is possible. B cell numbers are reduced greatly in the spleens and bone marrow of *ER*^{het} mice. The maintenance of pro-B cells and relative absence of pre-B and immature and mature B cells in the bone marrow of compound haploinsufficient animals indicate that EBF1 and RUNX1 are required for developmental transitions following the expression of Ig μ chains. Reasons for this impeded development include the reduced and/or delayed expression of stage-specific genes in pro-B cells, including *Ikzf3* (Aiolos), *κ0* sterile transcripts, *Cd25* (CD25), and *Cd2* (CD2). We confirmed that these genes are downstream targets of EBF1 and RUNX1 by activating their endogenous counterparts in plasmacytoma cells. Together, these

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data confirm the importance of synergistic interactions between EBF1 and RUNX1 and define new roles of EBF1 in B cell lineage specification and progression.

Results

Production of B Cells Is Defective in ER^{het} Mice. To evaluate the impact of EBF1 and RUNX1 haploinsufficiencies on B cell development in vivo, we generated *WT* (*Wild Type*), R^{het} (*Ebf1*$^{+/+}$ *Runx1*$^{+/-}$), E^{het} (*Ebf1*$^{+/-}$*Runx1*$^{+/+}$), and ER^{het} mice. Flow cytometric analysis of B220$^+$ cells isolated from spleens of adult mice (4–6 weeks) revealed only a small difference between total numbers of B cells in *WT* relative to R^{het} spleens; however, splenic B cell numbers in E^{het} mice were reduced by 62% ($P = 0.0003$) relative to those in *WT* mice (Fig. 1*A*). We observed an even greater reduction (by 75%; $P = 1.4e\text{-}10$) in B cell numbers in spleens of ER^{het} mice. The difference between E^{het} and ER^{het} mice suggests that EBF1 and RUNX1 are both necessary to obtain normal numbers of peripheral B cells. In contrast, splenic T cell numbers were relatively unaffected by *Ebf1* or *Runx1* haploinsufficiencies (Fig. S1).

We hypothesized that the reduction of B cell numbers in the spleen was a consequence of defective B lymphopoiesis in the bone marrow. To assess this, we used flow cytometry to evaluate the populations of bone marrow cells expressing the definitive marker of committed B cells, CD19. The numbers of CD19$^+$ cells decreased to less than half between *WT* and ER^{het} mice (Fig.1*B*). Proportions of CD19$^+$ cells were similar in the bone marrow of *WT* and R^{het} mice (48% and 42%, respectively) (Fig.1*C*). In contrast, decreased proportions of CD19$^+$ cells were observed in bone marrow from E^{het} and ER^{het} mice (decreased from 48% to 27% and 29%, respectively) relative to that from *WT* mice ($P = 0.015$ and 0.001, respectively). An intermediate effect was observed in E^{het} mice. Moreover, the composition of the CD19$^+$ populations changed considerably between ER^{het} mice and the other genotypes. Coexpression of CD19 and CD117 (c-kit) indicates "committed" pro-B cells (27). ER^{het} mice exhibited 2- to 3-fold greater frequencies of CD19$^+$CD117$^+$ cells, although absolute numbers of these cells were relatively constant among the four genotypes (Fig.1*C* and Fig. S2*A*). The enhanced detection of CD117$^+$ cells is likely due to decreased representation of later stages of B cell development in ER^{het} bone marrow.

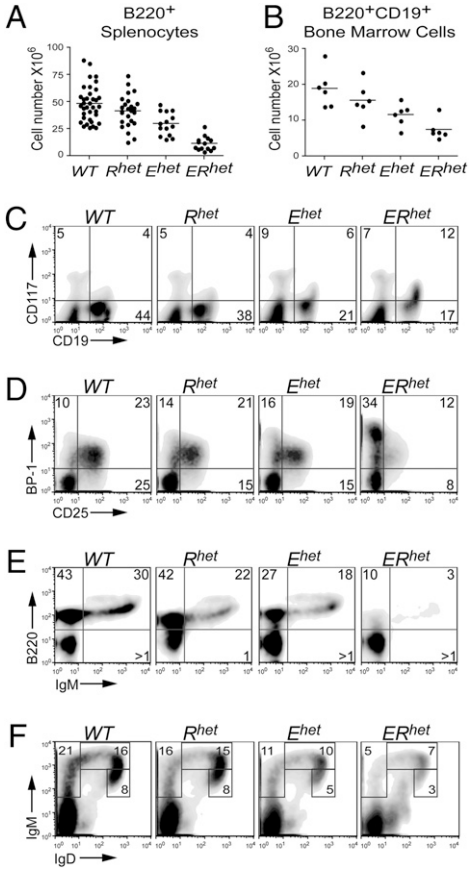

Fig. 1. Development of bone marrow B cells in *WT* and haploinsufficient mice. All mice were 4–6 weeks old ($n \geq 5$ per group). (*A*) Numbers of B220$^+$ splenocytes as determined by flow cytometry. (*B*) Numbers of B220$^+$CD19$^+$ bone marrow cells as determined by flow cytometry. (*C–F*) Representative flow cytometry assessing (*C*) CD117 (c-kit) and CD19 expression on IgM$^-$ bone marrow cells, (*D*) BP-1 and CD25 expression on CD19$^+$IgM$^-$ bone marrow cells, (*E*) B220 and IgM expression on CD43$^-$ bone marrow cells, and (*F*) IgM and IgD expression on CD19$^+$ bone marrow cells.

The overall reductions in the numbers of CD19$^+$ cells in the bone marrow of single and double haploinsufficient mice suggested an impediment in the generation of pre-B cells. To dissect the status of the pre-B cell compartment, we analyzed cell surface markers indicative of this bone marrow-derived population, BP-1 and CD25 (28–30). BP-1$^+$CD25$^-$CD19$^+$IgM$^-$ cells represent late pro-B cells, whereas BP-1$^+$CD25$^+$CD19$^+$IgM$^-$ cells represent the majority of pre-B cells. At a very late point in pre-B cell development, the cells become BP-1$^-$CD25$^+$CD19$^+$IgM$^-$ cells. We detected increased expression of BP-1 and proportions of BP-1$^+$CD25$^-$CD19$^+$IgM$^-$ cells in ER^{het} mice (Fig.1*D*); however, the numbers of these late pro-B cells were similar among all four genotypes (Fig. S2*B*). In contrast, both the proportions and the numbers of pre-B cells (BP-1$^+$CD25$^+$ and BP-1$^-$CD25$^+$) were reduced significantly (by 78 and 92%, respectively; $P = 0.0008$ and 0.0001) in ER^{het} mice. Intermediate effects were observed in E^{het} bone marrow, which also exhibited approximately half the number of pre-B cells observed in *WT* mice ($P = 0.03$ and 0.001). Significantly reduced numbers of BP-1$^-$CD25$^+$CD19$^+$ IgM$^-$ cells were detected as well in R^{het} bone marrow (by 62%; $P = 0.001$). Furthermore, we assessed the fraction D pre-B cell compartment as defined by Hardy et al. (30). As indicated in Fig.1*E*, we detected a substantial reduction in B220$^+$IgM$^-$CD43$^-$ bone marrow cells ($P = 0.04$). Together, these data suggest an inability of pro-B cells to transition efficiently to later stages of B cell development due to compound effects of R^{het} and E^{het} haploinsufficiencies.

We next examined immature and mature B cells in the bone marrow of mice with each of the four genotypes. We used flow cytometry to delineate IgM$^+$IgD$^-$, IgM^{hi}IgD$^+$, and IgM^{lo}IgD$^+$ B cells (Fig.1*F*). Reduced numbers of each of these populations were observed in mice with the mutant genotypes (Fig. S2*C*). The three IgM$^+$ B cell populations were reduced slightly in R^{het} bone marrow and dramatically in E^{het} (60, 54, and 57%, respectively; $P = 0.001$, 0.01, 0.001) and ER^{het} bone marrow (91, 93, and 88%, respectively; all $P < 0.0001$). We conclude that the production of mIg$^+$ B cells is defective in ER^{het} mice due to compound effects of *Ebf1* and *Runx1* haploinsufficiencies.

Haploinsufficiencies Alter Gene Expression in E^{het}, R^{het}, or ER^{het} Mice. Because EBF1 and RUNX1 are transcription factors, alterations in pre-B and mIg$^+$ cells in single and double haploinsufficient mice suggest perturbations of the expression of B cell stage-specific genes. We purified CD117$^+$CD19$^+$ pro-B or CD117$^-$CD19$^+$mIgM$^-$ late pro-B/pre-B cells and quantitated transcripts using quantitative real-time PCR (qRT-PCR). The assays focused on known targets of EBF1 and genes that reflect developmental transitions during B lymphopoiesis. Despite the presence of similar numbers of CD117$^+$CD19$^+$ cells in mice with each of the four genotypes, differences in gene expression were evident (Fig. 2*A*). The expression of known targets of EBF1 including *Cd79a*, *Cd79b*, the surrogate light (ψL) chain genes *Vpreb1* and *Igll1* (λ5), and *Recombination activating gene 1* (*Rag1*) was largely unaffected in CD117$^+$CD19$^+$ cells isolated from *WT*, R^{het}, or E^{het} mice. However, significant reductions of *Vpreb1* (36%; $P = 0.0048$) and *Igll1* (25%; $P = 0.0061$) transcripts were detected in ER^{het}

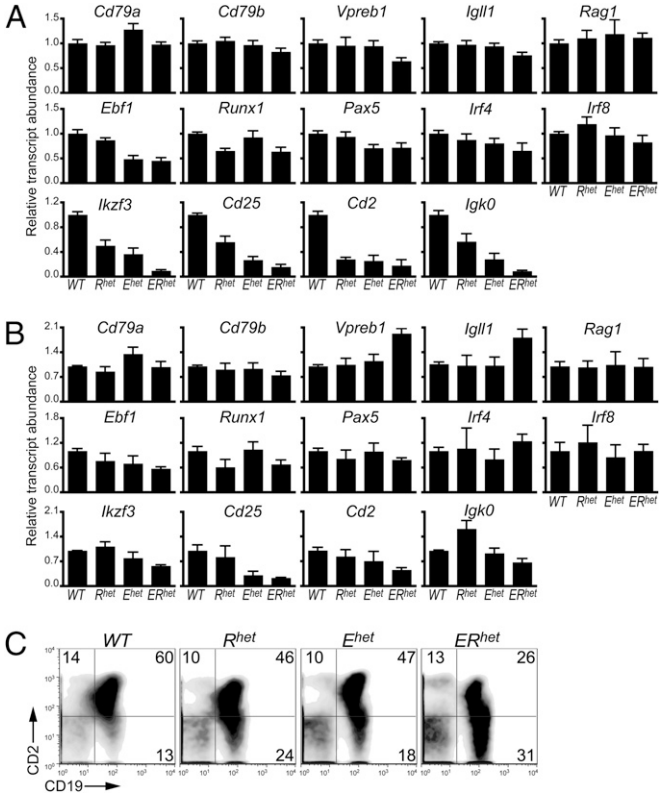

Fig. 2. Expression of stage-specific markers in CD117$^+$CD19$^+$mIgM$^-$ and CD117$^-$CD19$^+$mIgM$^-$ bone marrow cells of *WT* and haploinsufficient mice. (*A*) Quantitative PCR (qRT-PCR) of transcripts in CD117$^+$CD19$^+$mIgM$^-$ "committed" pro-B cells (n = 3–5 mice per group). All transcript levels were normalized to levels of β-*actin* transcripts. (*B*) qRT-PCR of transcripts in CD117$^-$CD19$^+$mIgM$^-$ late pro-B/total pre-B cells (n = 3–5 mice per group). All transcript levels were normalized to levels of *Hprt* transcripts. (*C*) Analysis of CD2 and CD19 expression on B220$^+$IgM$^-$ cells by flow cytometry.

mice relative to *WT* mice. As expected, expression of *Ebf1* and *Runx1* was reduced (52–55% and 35–36%, respectively; $P < 0.0034$) in the context of the mutant alleles. No additional decrease in transcripts of either gene was evident in CD117$^+$CD19$^+$ cells of ER^{het} mice. Reduced expression of *Pax5* transcripts (30%) was noted in CD117$^+$CD19$^+$ cells of E^{het} and ER^{het} mice. A similar decrease in *Irf4* transcripts (35%; $P = 0.061$) was noted in the cells from ER^{het} mice (changes in *Irf8* were not significant). Transcripts of *Ikzf3* (Aiolos), which is important for the development of pre-B cells (31, 32), were reduced by half in R^{het} and E^{het} mice and were nearly absent in CD117$^+$CD19$^+$ cells of ER^{het} mice ($P < 0.0001$). Other genes that encode markers of pre-B cells were expressed at low levels in CD117$^+$CD19$^+$ cells of each mutant genotype. Expression of *Cd25* was reduced in R^{het} (44%), E^{het} (73%), and ER^{het} (84%; $P < 0.0001$) CD117$^+$CD19$^+$ cells relative to *WT* CD117$^+$CD19$^+$ cells. Expression of *Cd2* genes was reducted in R^{het} (72%), E^{het} (75%), and ER^{het} (82%; $P = 0.0021$) CD117$^+$CD19$^+$ cells. *Igκ0* sterile transcripts, which precede κ *IgL* gene rearrangements, were reduced greatly in R^{het} (43%), E^{het} (72%), and ER^{het} (91%; $P < 0.0001$) CD117$^+$CD19$^+$ cells. These findings suggest that pre-B cell stage-specific genes, which are expressed significantly in committed pro-B cells, are reduced in ER^{het} mice.

We examined the same set of gene transcripts using cDNAs derived from CD117$^-$CD19$^+$mIgM$^-$ late pro-B/pre-B cells from mice with each of the four genotypes (Fig.2*B*). Several significant differences were noted in these cells in comparison with gene expression in CD117$^+$CD19$^+$ cells. First, levels of *Vpreb1* and *Igll1* transcripts were elevated (1.9- and 1.8-fold, respectively; P = 0.0043 and 0.025) in CD117$^-$CD19$^+$mIgM$^-$ cells of ER^{het} mice relative to *WT* mice. This likely reflects the skewing of these populations to early pre-B cells, which express pre-B cell receptors (pre-BCR). Interestingly, although *Ebf1*, *Runx1*, and other transcription factors show small differences in expression, these changes are muted in CD117$^-$CD19$^+$mIgM$^-$ cells relative to CD117$^+$CD19$^+$ cells. For example, differences in the expression of *Ikzf3* transcripts are reduced, but only by 43%, between pre-B cells of *WT* and ER^{het} mice. Levels of *Igκ0* sterile transcripts were affected less and even elevated (in R^{het} mice). However, *Cd25* transcripts were greatly reduced in E^{het} and ER^{het} (by 70 and 77%; $P = 0.028$ and 0.013) mice.

Our data suggest that, with a few notable exceptions, cells that make the transition from pro-B to pre-B cells have activated genes of the pre-B stage-specific program successfully. To confirm that the generation of late pre-B cells is impeded in the mutant mice, we examined the expression of CD2 on B220$^+$IgM$^-$ pre-B cells of mice with each of the four genotypes using flow cytometry (Fig.2*C*). CD2 is displayed on small resting pre-B cells in response to pre-BCR signaling (33, 34). In *WT* mice, CD19$^+$CD2$^+$ B cells constitute a discrete population including both late pre-B and mIgM$^+$ cells. In E^{het} and ER^{het} mice, CD2 expression is decreased on B220$^+$IgM$^-$ cells expressing slightly higher levels of CD19. In ER^{het} mice this population includes a high proportion of CD19$^+$ cells, which exhibit a continuous range of CD2 expression. These data suggest that the transition between pre-B and immature B cells is inefficient in E^{het} and ER^{het} bone marrow, possibly from inadequate pre-BCR signaling.

Decreased *Igλ* Gene Rearrangements in E^{het} and ER^{het} Mice. A hallmark of pre-B cell development is the activation of *IgL* gene rearrangements in response to pre-BCR signaling. Productive *IgL* gene rearrangements result in the replacement of ψ L chain proteins (VpreB1 and λ5) with κ or λ L chains, resulting in immature B cells expressing a mature BCR. To assess effects of *Ebf1* and *Runx1* haploinsufficiencies on the processes of *IgL* gene rearrangements, we isolated genomic DNA from purified CD117$^+$CD19$^+$ pro-B cells and used quantitative PCR assays to assess *Ig heavy* (*IgH*) chain gene rearrangements. We did not detect significant differences between frequencies and patterns of *IgH* rearrangements including proximal V_H7183 or distal V_HJ558 variable regions (Fig. S3). Next, we isolated genomic DNA from purified CD117$^-$CD19$^+$mIgM$^-$ pre-B cells for analysis of *IgL* chain gene rearrangements. *Igκ* rearrangements were decreased by 25% in E^{het} and ER^{het} mice, but not in R^{het} mice (Fig.3*A*). *Igλ* rearrangements were decreased more significantly, with reductions of 75% each in E^{het} and ER^{het} mice ($P = 0.0062$ and 0.0018). The overall decrease in *Igλ* rearrangements included reduced utilization of each of the three available *Jλ* loci in E^{het} mice (28.0, 18.0, and 31.6% of WT; $P = 0.001$, 0.011, and 0.059) and ER^{het} mice (27.0, 14.6, and 22.5% of *WT*; $P = 0.0002$, 0.0002, and 0.018) (Fig. 3*B*). We conclude that *IgL* chain, but not *IgH* chain, rearrangements are impaired in mice with a single functional *Ebf1* allele.

Early B Cell-Specific Genes Are Activated by EBF1 and RUNX1 in Terminally Differentiated Plasmacytoma Cells. We demonstrated previously that retroviral expression of EBF1 and RUNX1 in μM.2 plasmacytoma cells activates endogenous *Cd79a* and *Vpreb1* gene transcription (24, 35). To determine whether EBF1 and RUNX1 similarly regulate other genes that are decreased in ER^{het} mice, we infected μM.2 cells with retroviruses for expression of EBF1 or RUNX1, separately or simultaneously, and used qRT-PCR to measure the levels of specific transcripts at 48 h following infection (Fig. 4). Similar to previous results, *Cd79a* genes were activated only minimally by EBF1 or RUNX1 alone. However, in combination, they increased *Cd79a* transcripts by 49.7-fold ($P = 0.0042$). EBF1 and RUNX1 also strongly activated expression of *Vpreb1* and *Igll1* genes (596-fold and 405-fold, respectively; $P = 0.011$ and 0.052). As we have demonstrated previously for the *Cd79a* gene (24), this

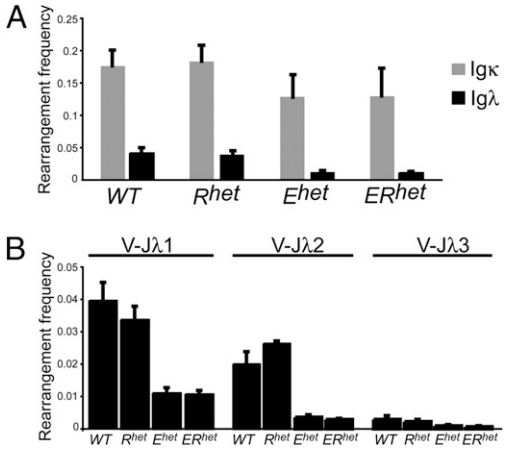

Fig. 3. Ig λ light chain gene rearrangements are reduced by *Ebf1* haploinsufficiency. (*A*) Frequencies of *Igκ* and *Igλ* gene rearrangements in pre-B cells of *WT* and haploinsufficient mice. (*B*) Reduced frequencies of *Igλ* gene rearrangements including *Jλ1*, *Jλ2*, and *Jλ3* in pre-B cells of E^{het} and ER^{het} mice (n = 3 mice per group).

robust activity likely reflects the high affinity of EBF1 and RUNX1 for the promoters of these genes. Thus, it was not surprising that haploinsufficiency of EBF1 and RUNX1 resulted in minor changes in transcript levels of these genes (Fig.2 *A* and *B*). When expressed separately, RUNX1 or EBF1 activated *Ikzf3* genes 2.8- or 4-fold, respectively, although synergistically increasing transcription of *Ikzf3* genes 6.9-fold (P = 0.0004). Robust induction of two other markers of pre-B cells, *Cd2* and *Cd25*, was dependent on cooperative effects of EBF1 and RUNX1 as well (6- and 66-fold, respectively; P = 0.0038 and 0.044). However, *Cd2* and *Cd25* transcripts were stimulated 2.5- and 15-fold, respectively, by EBF1 alone. Therefore, the combination of EBF1 and RUNX1 activates multiple silenced genes of the early B cell-specific program in terminally differentiated plasmacytoma cells. We conclude that this ability reflects the functional cooperation of EBF1 and RUNX1 in early B cell progenitors in the bone marrow.

Discussion

Here, we demonstrated the importance of regulatory gene dosage and functional cooperation between transcription factors in B lymphopoiesis. EBF1 drives expression of the B cell-specific transcriptional program in synergy with factors including PU.1, E2A, RUNX1, and Ikaros. The primary consequences of this process include the initiation of V(D)J recombination and assembly of the pre-BCR and BCR. Successful expression of these receptors allows cells to traverse checkpoints in developmental progression. Here, we demonstrated the importance of regulatory gene dosage and functional cooperation between transcription factors in B lymphopoiesis. B cell progenitors with lower than normal levels of EBF1 and RUNX1 are impeded during their transit through early B cell development, which results in greatly reduced pre-B and mIg^+ B cells in the bone marrow and mature B cells in the periphery.

In the original study of mice with targeted *Ebf1* alleles, *Ebf1* haploinsufficiency resulted in a 50% decrease in Hardy fractions B and C and a 10–30% reduction in B cells in the spleen (2). It was found subsequently that the effects of *Ebf1* haploinsufficiency are compounded by haploinsufficiency of *Tcfe2a* (encoding the E proteins E12 and E47) in $Ebf1^{+/-}Tcfe2a^{+/-}$ (ET^{het}) mice (25). Our studies revealed similarities and significant differences between ER^{het} and ET^{het} mice. In both models, B cell development is impeded by the combination of haploinsufficient loci, with decreased pre-B and mIg^+ cells in the bone marrow and spleen. However, unlike in ET^{het} mice, numbers of pro-B cells were not grossly affected in ER^{het} mice. In ET^{het} mice, late pre-B cell development was decreased, whereas $mIgM^+$ cells were nearly absent. Analysis of transcript levels in purified pro-B cells of ET^{het} mice demonstrated significant decreases in target genes including *Cd79a*, *Cd79b*, *Igll1*, *Vpreb1*, and *Rag2*. *Pax5* expression was decreased to nearly background levels. In contrast, there was little or no change in the expression of *Cd79a* and *Cd79b* and there were moderate changes in expression of *Igll1*, *Vpreb1*, and *Pax5* in pro-B cells of ER^{het} mice. Similar to that in ET^{het} mice, we observed potent effects of *Ebf1* haploinsufficiency on pre-B cells in ER^{het} mice. Although $BP\text{-}1^+CD25^-$ cells were not reduced in number in ER^{het} mice, they expressed greatly increased levels of BP-1. Numbers of $BP\text{-}1^+CD25^+$ and $BP\text{-}1^-CD25^+$ pre-B cells were decreased profoundly in E^{het} and ER^{het} mice. In part, reduction of these cells may be due to decreased expression of *Cd25*, which we identified as a transcriptional target of EBF1 and RUNX1. We also observed reduced numbers of $B220^+CD43^-IgM^-$ pre-B cells (Hardy fraction D). All mIg^+ stages of bone marrow B cell development were reduced as well. This deficiency likely contributes to the dearth of peripheral B cells in young ER^{het} mice.

We also identified changes in the expression of transcription factors and target genes that impact the development of pre-B cells in ER^{het} mice. *Irf4* transcripts were decreased modestly. Expression of *Ikzf3* (Aiolos) was delayed, as indicated by its reduced expression in ER^{het} $CD117^+CD19^+$ cells. Whereas *Ikzf3* expression initiates at the pro-B stage (32), its levels increase dramatically in pre-B cells, where it is important for silencing *Igll1* gene transcription. The delay in activating *Ikzf3* transcription could contribute to the increased expression of *Igll1* transcripts in pre-B cells of these mice. Transcripts of genes that are expressed at high levels in *WT* pre-B cells, including *Cd2*, were reduced in $CD117^+CD19^+$ cells, but increased in cells that successfully progressed to pre-B cells. Interestingly, graded expression of CD2 was observed on the surface of ER^{het} pre-B cells. Together, the data suggest that signals originating from the pre-BCR in ER^{het} mice are insufficient to drive the up-regulation of genes including *Ikzf3* and *Cd2* in pre-B cells, resulting in delayed or unstable activation of the pre-B cell program. In explanation of these effects, substantial up-regulation of *Ebf1* transcripts was detected in $B220^+CD43^+CD24^+BP\text{-}1^+$ cells (Hardy fraction C′) (30, 36). We propose that the low concentrations of EBF1 in E^{het} and ER^{het} mice slow the transition from early to late pre-B cells and hence to immature B cells. These data are consistent with stochastic mechanisms that function at the pre-B cell developmental boundary. Subsequently, cells that do succeed in activating the pre-B cell program progress to immature and mature B cells. Furthermore, our data highlight difficulties with rigidly associating a scheme of stage-specific markers with developmental subpopulations of cells and the fluid process of lymphopoiesis.

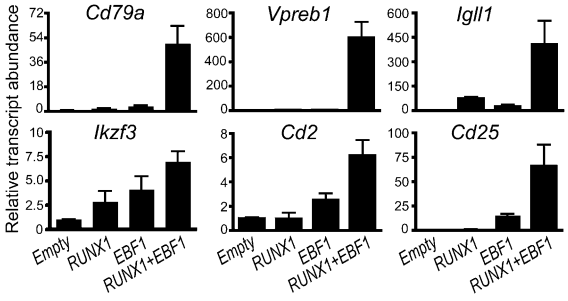

Fig. 4. Enforced expression of EBF1 and RUNX1 activates expression of early B cell-specific genes in plasmacytoma cells. μM.2 plasmacytoma cells were transduced with retroviruses for expression of EBF1 and/or RUNX1 as shown. Transduced cells were purified 48 h postinfection and analyzed using qRT-PCR. Data were normalized to control *Hprt* transcripts and represent three to five independent experiments.

Although significant differences in *IgH* or *Igκ* gene rearrangements were not detected, *Igλ* rearrangements were reduced significantly in *Ebf1* haploinsufficient pre-B cells. This did not appear to be due to decreased *Rag* (i.e., *Rag1*) gene expression in pre-B cells; however, we cannot rule out decreased V(D)J recombinase activity in a select population of B cells. The reduction in *Igλ* gene rearrangements may be a consequence of (*i*) decreased expression of EBF1 itself, (*ii*) decreased expression of other transcription factors that are regulated by EBF1, and/or (*iii*) defective pre-BCR signaling in E^{het} and ER^{het} pre-B cells. In regard to (*i*), ectopic expression of EBF1 in nonlymphoid cells preferentially induced *Igλ* rearrangements (37, 38). Together, these data suggest a role for EBF1 in the control of *Igλ* rearrangements.

Our data confirmed the importance of synergistic interactions between EBF1 and RUNX1 during early B cell development. We observed effects of *Ebf1* and *Runx1* haploinsufficiencies on previously unsuspected gene targets including *Cd2*, *Cd25*, and *Ikzf3*, which are expressed at low levels in $CD117^+CD19^+$ cells before their up-regulation in pre-B cells. Roles of EBF1 and RUNX1 in regulating these genes were confirmed by activation of their expression in μM.2 plasmacytoma cells. We demonstrated that EBF1 and RUNX1 activated *Cd79a*, *Igll1*, and *Cd25* transcription synergistically in μM.2 cells. Surprisingly, *Cd79a* expression was unaffected in ER^{het} mice. The lack of effects may be due to the high degree of DNA binding cooperativity between EBF1 and RUNX1 on *Cd79a* promoters, which compensates for their reduced dosage. We conclude that expression of EBF1 and RUNX1 is sufficient for the reprogramming of early B cell-specific genes in terminally differentiated plasmacytoma cells. However, details of how EBF1 and RUNX1 activate these genes are currently unknown and will be the subject of future investigations.

The effects of *Ebf1* and *Runx1* haploinsufficiencies have implications for human disease. Haploinsufficiencies of *Runx1* are associated with reduced hematopoietic stem cells in mice and familial thrombocytopenia and acute myelogenous leukemia in humans (39–41). Less is known concerning naturally occurring murine *Ebf1* haploinsufficiency; however, somatic mutations in *EBF1* genes are associated with human acute lymphoblastic leukemia (42). It will be interesting to determine whether these mutations result in phenotypes similar to those of E^{het} and R^{het} mice.

Materials and Methods

Mice. $Ebf1^{+/-}$ mice were obtained on a mixed 129Sv/C57BL/6J background from Y. Zhuang (Duke University). $Runx1^{+/-}$ mice were obtained on a mixed 129Sv/C57BL/6J background from Dan Littman (New York University). Mice were backcrossed onto a C57BL/6 background for >10 generations. Genotyping of *Ebf1* and *Runx1* haploinsufficent mice was performed by PCR using the following primers: (*Ebf1*) 5′-GGAGCCTCACCATTGCTGTAGAG-3′, 5′ATGGCGATGCCTGCTTGCCGAATA-3′, and 5′-AAAACGAGCGGAACCCTACTTG-3′ and (*Runx1*) 5′-TTAGCAGTAGATAGGTATGAGTCCC-3′, 5′-AAGGGGGCTCACTTACTTCG-3′, and 5′-TCGCAGCGCATCGCCTTCTA-3′. Tail DNA was amplified for 28 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. $Ebf1^{+/-}$ mice were bred with $Runx1^{+/-}$ mice to generate compound haploinsufficent mice. Mice were bred and housed in the Biological Resources Center at National Jewish Health. All experiments received prior approval from the National Jewish Health Institutional Animal Care and Use Committee.

Antibodies and Flow Cytometry. Cell staining was conducted using the following antibodies: CD45R (B220) (various fluorochromes) (Caltag Laboratories, eBiosciences, and BD Pharmingen); IgM-PE/Cy5 (Caltag Laboratories); CD117-PE-Cy5, CD25-Alexa Fluor 488, CD19-FITC/PE, CD2-FITC/PE, and CD117-FITC (eBioscience); CD19-APC, CD43-PE/APC, CD117-PE, IgM-FITC, and IgD-FITC (BD-Pharmingen); and IgM-Cy5 (Jackson Laboratories). Analytical flow cytometry was conducted using a CyAn cytometer. Data were analyzed using Summit (Dako Colorado) and FloJo software (Tree Star). Cell sorting was conducted using a MoFloXDP (Beckman Coulter) and analyzed using Summit software. For FACS analysis, bone marrow of adult mice (4–6 weeks) was harvested in Iscove's modified Dulbecco's medium (IMDM) (GIBCO/Invitrogen) supplemented with 1× Glutamax and 50 μg/mL gentamycin (Invitrogen) and 10% FBS (Biosource). Red blood cells were lysed in 1 mL of 144 mM NH_4Cl, 13.3 mM Tris-HCl (pH 7.2) buffer for 10 min and quenched with 10 mL complete IMDM. Cells were harvested, washed with FACS buffer (FB) [1× PBS (pH 7.6), 0.5% BSA, 0.02% NaN_3], and stained in FB containing antibodies and Fc block (anti-CD16) (BD-Pharmingen) at 20×10^6 cells/mL for 20 min at room temperature. Cells were washed three times with FB and analyzed. For cell sorting, bone marrow of adult mice (4–6 weeks) was harvested and treated as above; however, FB was replaced with MoFlo buffer [1× PBS (pH 7.6), 2% FBS]. Sorted cells were collected in a 50:50 mixture of complete IMDM and FBS.

Cell Transduction, RNA Isolation, and qRT-PCR. μM.2 plasmacytoma cells were grown and transduced as described previously (35). Sequences of primers used for qRT-PCR are in Table S1. Sorted cells were washed once with 1 mL of 1× PBS, 2 mM EDTA, 0.5% BSA. RNA was isolated using a PicoPure RNA Isolation Kit (Arcturus) according to the manufacturer's instructions. Preparation of cDNA used 2 μL of RNA eluate in the recommended reaction mixture for SuperScript II RT (Invitrogen). The mixture was incubated at 37 °C for 1 h and quenched with 1.4 mM EDTA/0.6 μg tRNA at 90 °C for 10 min. qRT-PCR used 12.5 μL SYBR Green Master Mix (Applied Biosystems), 50 nM primers, and 2 μL of cDNA per 25-μL sample, amplified and detected using an Applied Biosystems 7300 system. All samples were evaluated in triplicate. Data were analyzed for significance using Student's two-tailed *t* test.

Quantitative Analysis of *Ig* Gene Rearrangements. B cell populations were purified as above. Cells were snap frozen and lysed in 10 mM Tris-HCl, pH 8.3. Cell lysates were treated with 100 μg of proteinase K at 56 °C for 2 h. Proteinase K was inactivated at 95 °C for 10 min. Optical density of DNA samples was measured at 260 nm. Semiquantitative analysis of *IgH* gene rearrangements was performed using AmpliTaq Gold polymerase (Applied Biosystems) with primers listed in Table S1. DNA amplification was carried out in a final volume of 50 μL containing 15 mM Tris-HCl (pH 8.05), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.2 μM of each primer, 1.25 units of AmpliTaq polymerase, and 50 ng of template DNA. qPCR conditions were 10 min at 95 °C and 28 cycles of 30 sec at 94 °C, 30 sec at 60 °C, and 2 min at 72 °C. Ten-microliter aliquots of PCR were taken at 28, 32, and 38 cycles. PCR products were run on a 1.2% agarose gel and stained with EtBr. Quantitative analysis of κ, λ1, λ2, and λ3 rearrangements was performed by real-time PCR using a QuantiTect SYBR Green PCR Kit (Qiagen), using 0.2 μM of each primer and 10–50 ng of DNA template. Samples were amplified on an ABI7900 HT real-time PCR system (Applied Biosystems) and data were analyzed using SDS 2.1 software. *Igκ*, *λ1*, *λ2*, and *λ3* (*IgL*) rearrangement frequencies were calculated as $2^{\Delta Ct}$ with $\Delta Ct = Ct_{Actin} - Ct_{IgL}$.

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