Ebf1 and c-Myb Repress Rag Transcription Downstream of Stat5 during Early B Cell Development

Greg A. Timblin and Mark S. Schlissel

*J Immunol* 2013; 191:4676-4687; Prepublished online 25 September 2013; doi: 10.4049/jimmunol.1301675

http://www.jimmunol.org/content/191/9/4676

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/09/25/jimmunol.1301675.DC1

**References**

This article cites 58 articles, 22 of which you can access for free at:

http://www.jimmunol.org/content/191/9/4676.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2013 by The American Association of Immunologists, Inc. All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Ebf1 and c-Myb Repress Rag Transcription Downstream of Stat5 during Early B Cell Development

Greg A. Timblin* and Mark S. Schlissel*†

The temporal control of RAG (Rag) expression in developing lymphocytes prevents DNA breaks during periods of proliferation that could threaten genomic integrity. In developing B cells, the IL-7R and precursor B cell Ag receptor (pre-BCR) synergize to induce proliferation and the repression of Rag at the protein and mRNA levels for a brief period following successful Ig H chain gene rearrangement. Whereas the mechanism of RAG2 protein downregulation is well defined, little is known about the pathways and transcription factors that mediate transcriptional repression of Rag. Using Abelson murine leukemia virus–transformed B cells to model this stage of development, we identified early B cell factor 1 (Ebf1) as a strong repressor of Rag transcription. Short hairpin RNA–mediated knockdown of either Ebf1 or its downstream target c-Myb was sufficient to induce Rag transcription in these highly proliferative cells. Ebf1 and c-Myb antagonize Rag transcription by negatively regulating the binding of Foxo1 to the Rag locus. Ebf1 accomplishes this through both direct negative regulation of Foxo1 expression and direct positive regulation of Gfi1b expression. Ebf1 expression is driven by the IL-7R downstream effector Stat5, providing a link between the negative regulation of Rag transcription by IL-7 and a novel repressive pathway involving Ebf1 and c-Myb. The Journal of Immunology, 2013, 191: 4676–4687.

The generation of diverse B and T cell Ag receptor repertoires is dependent on the expression of the RAGs Rag1 and Rag2 (collectively known as Rag) during early lymphocyte development (1). RAG1 and RAG2 proteins form a complex that binds to conserved recombination signal sequences flanking a pair of Ag receptor gene segments and synchronously generates dsDNA breaks (DSBs) between each recombination signal sequence and its corresponding gene segment (2). Rag expression and DSB generation are restricted to the G0–G1 phases of cell cycle such that repair of DNA coding ends in the RAG-stabilized postcleavage complex is carried out by the nonhomologous end-joining pathway, resulting in assembly of the variable domain exons of Ag receptor genes (3). RAG-induced DSBs produced during S phase have the potential to be repaired by homologous recombination, a process that can lead to chromosomal translocations and transformation (4, 5). As lymphocytes go through periods of clonal expansion during their development, the balance between proliferation and differentiation, along with the expression of Rag, must be tightly regulated to maintain genomic integrity and ensure the production of diverse pools of B and T cells to mediate adaptive immune responses.

Following productive Ig H chain gene rearrangement in developing B cells in the bone marrow, expression of the precursor BCR (pre-BCR) activates signaling pathways that synergize with IL-7R signaling to direct two processes (6, 7). The first is clonal expansion of pre-BCR+ progenitors, a stage during which RAG protein and Rag mRNA levels are negatively regulated upon entry of these large, cycling pre-B cells into S phase (8, 9). The second process is differentiation to the small pre-B cell stage, which involves coordinated cell cycle exit, re-expression of Rag transcription downstream of the PI3K-Akt pathway downstream of these receptors has been implicated in the inactivation of Rag transcription via antagonism of Foxo1 transcription factors (12–14). Gfi1b and Stat5 have been implicated as direct negative regulators of Rag transcription (15, 16). Stat5 is activated by IL-7R signaling (17), consistent with the ability of IL-7 to repress Rag transcription (6, 12).

Abelson murine leukemia virus (AMuLV)–transformed B cell lines provide an in vitro model system to study the dynamics of Rag transcription during the developmental transition from the large to small pre-B cell stage. The constitutively active v-Abl kinase transforms B cell progenitors in a highly proliferative state in which Rag expression is low, mimicking the large, cycling pre-B cell stage of development. This developmental block can be released by inhibition of v-Abl with the small molecule kinase inhibitor STI-571 (STI) (18). STI treatment induces cell cycle arrest, upregulation of Rag transcription, and differentiation to a small pre-B cell-like state in which initiation of Ig κ L chain gene recombination occurs. In this study, we used the AMuLV system to identify novel pathways and factors responsible for the repression of Rag transcription. A gain-of-function screen identified unexpected roles for early B cell factor 1 (Ebf1) and c-Myb in the repression of Rag transcription. The expression of these factors is
driven by the IL-7R signaling effector Stat5, linking the negative regulation of Rag transcription by IL-7 to a novel repressive pathway involving Ebf1 and c-Myb.

Materials and Methods

Animal use statement

All experiments using C57/B6 mice were approved by the Animal Care and Use Committee at the University of California at Berkeley. The handling of animals was in accordance with protocol R253-0405.

Cell culture and chemicals

AMuLV-transformed B cells were cultured in RPMI 1640 (Life Technologies) supplemented with 5% v/v FCS (Gemini), 100 mg/ml penicillin and streptomycin (Life Technologies), and 55 mM sodium pyruvate (Life Technologies). All cells were grown at 37°C in 5% CO₂, STI-571 (Novartis) was used at a final concentration of 2.5 μM for 16 h for all experiments.

Expression plasmids

MSCV-based cDNA retroviral expression constructs were previously described (12). All cDNAs were PCR amplified with Platinum PfX DNA Polymerase (Invitrogen) and cloned into a multiple cloning site upstream of a FLAG sequence inserted into the multiple cloning site of CMSCV IRES hCD4. N-terminal 3×FLAG-tagged Ebf1 was created by amplifying the Ebf1 ORF lacking an ATG start codon and cloning it downstream of a 3×FLAG sequence inserted into the multiple cloning site of CMSCV IRES hCD4. CA-STAT5B cDNA (a gift of Drs. M. Clark and M. Mandel, University of Chicago, Chicago, IL) was excised from MIGR1 and cloned into CMSCV IRES hCD4.

MSCV-based short hairpin RNA (shRNA) retroviral expression constructs contain a hCD2 cell surface marker cDNA, followed by a miR-30 cassette, as previously described (19). Seed sequences for desired shRNA targets were identified with siRNA Wizard (InvivoGen), and shRNA oligonucleotides containing these seed sequences were designed using RNAi design tools. All cDNAs were PCR amplified with Platinum Pfx DNA Polymerase (Invitrogen) and cloned into a multiple cloning site upstream of a FLAG sequence for either human CD (hCD)4 or Thy1.1 cell surface proteins to mark infected cells. All cDNA open reading frames (ORFs) were sequenced to confirm the absence of mutations.

Murine c-Myb transcript variant 2 (NM_010848.3) ORF was PCR amplified from a primary B cell cDNA library, whereas murine Foxo1, Pax3, and Ebf1 ORFs were PCR amplified from existing plasmids and cloned into CMSCV IRES Thy1.1 or CMSCV IRES hCD4 retroviral vectors. N-terminal 3×FLAG-tagged Ebf1 was created by amplifying the Ebf1 ORF lacking an ATG start codon and cloning it downstream of a 3×FLAG sequence inserted into the multiple cloning site of CMSCV IRES hCD4. CA-STAT5B cDNA (a gift of Drs. M. Clark and M. Mandel, University of California, Chicago, IL) was excised from MIGR1 and cloned into CMSCV IRES hCD4.

Cell sorting, flow cytometry, and intracellular staining

Single-cell suspensions were prepared, and cells were labeled with fluorochrome-conjugated Abs using standard techniques. A FC500 (Beckman Coulter) or LSRII (BD Biosciences) flow cytometer was used for analysis, whereas a MoFlo or Influx cell sorter (DakoCytomation) was used for sorting. Data were analyzed with FlowJo software (Tree Star). Cell sorting, flow cytometry, and intracellular staining

Primary B cells were labeled with anti-IgM (B220), anti-IgG, anti-CD23 (RA3-6B2), anti-CD4 (1D3), and anti-CD19 (1D3) Abs. Anti-hCD2 (RPA-2.10), anti-hCD4 (RPA-74), and anti-Thy1.1 (OX-7) Abs were used to label cells transduced with retrovirus. Anti-B220, anti-CD43, anti-CD19, and anti-Thy1.1 Abs were obtained from BD Pharmingen. All others were obtained from eBioscience.

The Fix & Per Cell Permeabilization Kit (Invitrogen) was used for intracellular staining. Primary Ab was rabbit anti-EBF-1 (Millipore AB10523) or rabbit IgG control (GenScript). Secondary Ab was Alexa Fluor 647 F(ab’); fragment goat anti-rabbit. Gating strategy was as follows: pre-B cells (IgM⁺, B220⁺, CD43⁺), large cycling pre-B cells (IgM⁺, B220⁺, CD43⁺, forward scatter high), and small resting pre-B cells (IgM⁺, B220⁺, CD43⁺, forward scatter low).

Quantitative real-time PCR

Cells were collected by centrifugation and lysed in TRIzol, or sorted directly into TRIzol LS (both from Invitrogen). cDNA was prepared and reverse transcription was performed with Moloney murine leukemia virus–reverse transcriptase (Invitrogen) using random hexamer priming. Quantitative real-time PCR was carried out on an Applied Biosystems 7300 thermocycler using JumpStart Taq (Sigma-Aldrich) and EvoGreen (Biotium). PCR conditions were as follows: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 s; 60°C, 1 min. See Supplemental Table I for primer sequences. For gene expression analysis, transcript levels of all genes were normalized to Hprt. For chromatin immunoprecipitation (ChIP) quantitative real-time PCR (qPCR), data are presented as a percentage of ChIP input. Error bars on all plots represent the SD of triplicate qPCR assays.

Immunoblot

Cells were lysed in radioimmunoprecipitation assay buffer supplemented with Complete Protease Inhibitor Cocktail (Roche). Cell debris was cleared by centrifugation, and soluble protein was quantified with Bradford reagent (Bio-Rad). Laemmli SDS loading buffer was added to 20–40 μg protein per sample prior to separation on 8–10% SDS-PAGE gels. Following transfer to Immobilon-FL polyvinylidenefluoride membranes (Millipore) and blocking with 5% v/v milk/PBS, blots were probed with primary and secondary Abs and analyzed with the Odyssey Infrared Imaging System (LI-COR Biosciences). Primary Abs were used as follows: anti–EBF-1 (R&D Systems AF5156 and Millipore AB10523), anti-Myb (Millipore 05-175), anti-Pax5 (Santa Cruz sc-1974), anti-Stat5 (Santa Cruz sc-835), anti-Foxo1 (Cell Signaling L27), anti-lamin B1 (Abcam ab16048), anti-tubulin (Calbiochem CP006), and anti-FLAG M2 (Sigma-Aldrich). Infrared dye-conjugated secondary Abs were from Molecular Probes–Invitrogen. Quantification was performed using ImageJ.

ChIP

ChIP was performed, as previously described (20). Briefly, 80 million cells per experimental condition were harvested, fixed, lysed, and sonicated using a Branson 450 Digital Sonifier. Following centrifugation to remove insoluble material, chromatin was quantified and equal amounts were used in experimental and control immunoprecipitations. A total of 5 μg anti–FLAG M2 (Sigma-Aldrich), anti-Foxo1 (Abcam ab70382), anti-Pax5 (Santa Cruz sc-1974X), anti-Ebf1 (Sigma-Aldrich SAB2501166), or IgG control Abs (GeneScript) was conjugated to protein G Dynabeads (Invitrogen) and added to samples. Following overnight immunoprecipitation, beads were collected and washed three times with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.1], 150 mM NaCl), once with high salt buffer (0.5% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.1], 500 mM NaCl), once with LiCl buffer (250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris [pH 8.1]), and twice with TE buffer. DNA-protein complexes were eluted from beads with TE elution buffer (TE with 1% SDS), and cross-links were reversed overnight at 65°C. Following RNase A (Qiagen) and protease K (Invitrogen) treatment, chromatin was isolated using QIAquick columns (Qiagen) and subjected to qPCR analysis. See Supplemental Table I for primer sequences.

Results

Ebf1 overexpression negatively regulates Rag transcription

To screen for transcriptional regulators of Rag, we used retroviruses to individually overexpress a panel of transcription factors important for B cell development in a previously described AMuLV-transformed Rag1-GFP reporter B cell line (12). This line was derived from a heterozygous Rag1-GFP knock-in mouse that has a GFP cDNA in place of the coding exon at one Rag1 allele (21), allowing changes in Rag transcription to be monitored by flow cytometry. We assessed Rag1-GFP levels following overexpression

Downloaded from http://www.jimmunol.org/ by guest on July 25, 2017

4677

The Journal of Immunology
of the different factors in both untreated and STI-treated reporter cells.

To our surprise, early B cell factor 1 (Ebf1), a transcription factor implicated as a positive regulator of Rag transcription during early B cell development (22), repressed baseline Rag1-GFP levels when overexpressed in the reporter cell line as compared with empty vector control cells (Fig. 1A, top row). Moreover, when the Ebf1-overexpressing cells were treated with STI, Rag1-GFP induction was severely blunted compared with STI-treated control cells (Fig. 1A, bottom row). DNA binding by Ebf1 was required for Rag1-GFP repression as mutation of H157 in the Ebf1 zinc-finger motif abolished activity (23) (Fig. 1A, left). The repression of both Rag1 and Rag2 by Ebf1 was confirmed by qPCR (Fig. 1B), and the repressive effect of Ebf1 overexpression on Rag transcription was validated in independently generated Abelson cell lines (our unpublished observations). Interestingly, Ebf1 overexpression did not affect other aspects of STI-induced differentiation, such as the upregulation of Irf4 and Spi-B expression, or the induction of Igκ germine transcription (kGT) (Fig. 1C).

To test whether Ebf1 overexpression represses Rag transcription in primary cells, we transduced cultured pro-B cells isolated from heterozygous Rag1-GFP mice with Ebf1 or empty vector control retroviruses. In cultures with a high concentration of IL-7, flow cytometry analysis revealed that the Ebf1-overexpressing cells had a lower GFP mean fluorescence intensity (MFI) than empty vector control cells (Fig. 1D, left). When the IL-7 concentration in these cultures was lowered to induce Rag expression and differentiation to the pre-B cell stage, an increase in the GFP MFI was seen in the control cells, but not in the Ebf1-overexpressing cells (Fig. 1D, right), confirming that Ebf1 represses Rag induction upon IL-7 withdrawal. The repression of both Rag1 and Rag2 by Ebf1 in primary IL-7–cultured pro-B cells from wild-type C57/B6 mice was confirmed by qPCR (Fig. 1E). Together these data show that Ebf1 overexpression in Abelson cells and committed primary pro- and pre-B cells represses Rag transcription.

Ebf1 levels are inversely correlated with Rag expression at the large-to-small pre-B cell transition

Ebf1 mRNA levels are low in lymphoid progenitors, increase during progression through the pro-, pre-, and immature B cell stages, and decrease in mature and peripheral B cell subsets (24, 25). Our Ebf1 overexpression results prompted us to assess Ebf1

---

**FIGURE 1.** Ebf1 overexpression negatively regulates Rag transcription. (A) Flow cytometry analysis of GFP expression in an AMuLV-transformed B cell line generated from heterozygous Rag1-GFP knock-in mice, in which GFP reports transcription from the endogenous Rag locus. Cells were infected with empty retroviral vector, Ebf1 retrovirus, or Ebf1 H157A DNA-binding mutant retrovirus (23). Uninfected cells (shaded histogram) were distinguished from transduced cells (black line) by staining with anti-hCD4 (retroviral marker). Analysis was performed on untreated (UT, top row) or STI-treated (+STI, bottom row) cells. Numbers above gate indicate the percentage of GFP+ uninfected cells (Fig. 1A). The repression of both Rag1 and Rag2 by Ebf1 was confirmed by qPCR (Fig. 1B), and the repressive effect of Ebf1 overexpression on Rag transcription was validated in independently generated Abelson cell lines (our unpublished observations). Interestingly, Ebf1 overexpression did not affect other aspects of STI-induced differentiation, such as the upregulation of Irf4 and Spi-B expression, or the induction of Igκ germine transcription (kGT) (Fig. 1C).

To test whether Ebf1 overexpression represses Rag transcription in primary cells, we transduced cultured pro-B cells isolated from heterozygous Rag1-GFP mice with Ebf1 or empty vector control retroviruses. In cultures with a high concentration of IL-7, flow cytometry analysis revealed that the Ebf1-overexpressing cells had a lower GFP mean fluorescence intensity (MFI) than empty vector control cells (Fig. 1D, left). When the IL-7 concentration in these cultures was lowered to induce Rag expression and differentiation to the pre-B cell stage, an increase in the GFP MFI was seen in the control cells, but not in the Ebf1-overexpressing cells (Fig. 1D, right), confirming that Ebf1 represses Rag induction upon IL-7 withdrawal. The repression of both Rag1 and Rag2 by Ebf1 in primary IL-7–cultured pro-B cells from wild-type C57/B6 mice was confirmed by qPCR (Fig. 1E). Together these data show that Ebf1 overexpression in Abelson cells and committed primary pro- and pre-B cells represses Rag transcription.

Ebf1 levels are inversely correlated with Rag expression at the large-to-small pre-B cell transition

Ebf1 mRNA levels are low in lymphoid progenitors, increase during progression through the pro-, pre-, and immature B cell stages, and decrease in mature and peripheral B cell subsets (24, 25). Our Ebf1 overexpression results prompted us to assess Ebf1
mRNA and Ebf1 protein expression levels with respect to Rag expression in developing B cells. In agreement with microarray data that show Ebf1 transcript levels increasing from fraction C (large pre-B) to fraction D (small pre-B) (24), both STI treatment of Abelson cells and IL-7 withdrawal in primary cell cultures resulted in an induction of Ebf1 mRNA (Fig. 2B, 2D). However, we observed a decrease in Ebf1 protein levels upon STI treatment and IL-7 withdrawal in lysates prepared from these same cells (Fig. 2A, 2C), indicating that Ebf1 protein levels are inversely correlated with Rag expression in both Abelson cells and IL-7–cultured primary cells.

To further confirm the inverse relationship between Ebf1 and Rag expression, we performed intracellular staining for Ebf1 in primary B cells isolated from wild-type C57/B6 mouse bone marrow. Ebf1 expression in pro-B cells (IgM+ B220+, CD43+) was lower than in large cycling pre-B cells (IgM+, B220+, CD43+, forward scatter high) (Fig. 2E). And in agreement with our Western blot data, small resting pre-B cells (IgM+ forward scatter low) expressed less Ebf1 than large pre-B cells (Fig. 2E).

These data suggest that Ebf1 protein levels peak in large cycling pre-B cells in which Rag transcription is repressed, and that Ebf1 protein levels decrease upon differentiation to the small resting pre-B cell stage when Rag transcription is upregulated to initiate Ig L chain gene recombination. This inverse relationship between Ebf1 and Rag expression strongly correlates with our observation that Ebf1 overexpression negatively influences Rag transcription in Abelson cells and IL-7–cultured primary cells.

Ebf1 knockdown is sufficient to induce Rag transcription in Abelson cells

Upon STI-induced differentiation of Abelson cell lines, a multitude of changes in gene expression takes place, several of which are important for the induction of Rag transcription. If a decrease in Ebf1 protein levels is one of the changes essential for this induction, we reasoned that knockdown of Ebf1 might be sufficient to upregulate Rag transcription even in the absence of other STI-mediated effects. This was indeed the case as shRNA knockdown of Ebf1 was sufficient to induce Rag transcription in the Rag1-GFP reporter cell line, whereas a control luciferase shRNA had no effect on Rag levels (Fig. 3A). Reduction of Ebf1 mRNA levels by the Ebf1 shRNA was confirmed by qPCR (Fig. 3B). Thus, Ebf1 is a bona fide repressor of Rag transcription in Abelson cells.

The Ebf1 target gene c-Myb represses Rag transcription in Abelson cells

Conditional deletion in pro-B cells revealed that Ebf1 is critical for their proliferation and survival (26). In agreement with this, Ebf1 overexpression enhanced proliferation in our Rag1-GFP reporter cell line, whereas Ebf1 knockdown was deleterious to survival as Ebf1 shRNA-transduced cells could not be expanded in culture (Supplemental Fig. 1). The same study showed that the Ebf1 target gene c-Myb was important for cell survival, and that c-Myb overexpression rescued cell death in Ebf1-deficient AMuLV-transformed B cells. Consistent with c-Myb being downstream of Ebf1, qPCR analysis revealed that c-Myb mRNA levels decreased significantly upon Ebf1 knockdown (Fig. 4A). Although c-Myb has been previously described as a positive regulator of Rag transcription (27, 28), we found that c-Myb overexpression in the Rag1-GFP reporter cell line both decreased baseline Rag levels and severely blunted STI-induced Rag transcription (Fig. 4B).

To test whether c-Myb is a bona fide repressor of Rag transcription, we performed shRNA knockdown of c-Myb in the Rag1-GFP reporter line. As was the case for Ebf1 knockdown, c-Myb knockdown was sufficient to induce Rag transcription in the absence of STI treatment (Fig. 4C). Conditional deletion of c-Myb in lymphoid progenitors showed that this factor positively regulates Ebf1 expression during early B cell development (29). However, in our cell line, c-Myb knockdown does not affect Ebf1 transcript levels (Fig. 4D), suggesting that the observed increase in Rag transcription is due to c-Myb knockdown alone and not a concurrent decrease in Ebf1 expression. Thus, during later stages of B cell development represented by our Abelson cell line, c-Myb is downstream of Ebf1 in a pathway that represses Rag transcription.

Ebf1 and c-Myb independently repress Rag transcription

The observation that c-Myb is downstream of Ebf1 (Fig. 4A) suggested that upon Ebf1 knockdown, the increase in Rag transcription may be due to the subsequent decrease in c-Myb levels. To test whether a decrease in Ebf1 levels that occurs independent of a decrease in c-Myb levels would be sufficient to induce Rag transcription, we transduced cells overexpressing a c-Myb cDNA

---

**FIGURE 2.** Ebf1 protein levels are inversely correlated with Rag expression. (A and B) Immunoblot measuring Ebf1 protein levels (A) and qPCR measuring Ebf1 transcript levels (B) in AMuLV-transformed B cells in normal culture conditions or treated with STI. Tubulin immunoblot serves as a protein-loading control. Numbers below lanes indicate Ebf1/tubulin ratio for each sample. Data are representative of three independent experiments. (C and D) Immunoblot measuring Ebf1 protein levels (C) and qPCR measuring Ebf1 transcript levels (D) in CD19+IgM–Rag2−/−/c–/− primary B cells before and after IL-7 withdrawal. Tubulin immunoblot serves as a protein-loading control. Numbers below lanes indicate Ebf1/tubulin ratio for each sample. Data are representative of two independent experiments. (E) Flow cytometry analysis of intracellular Ebf1 expression in pro-B cells (1, dotted line), large cycling pre-B cells (2, dashed), and small resting pre-B cells (3, solid) isolated from wild-type C57/B6 mouse bone marrow. Shaded histogram represents total pre-B cell isotype control staining. Data are representative of two independent experiments.
with our Ebf1 shRNA or control luciferase shRNA. qPCR confirmed that the expression level of c-Myb mRNA was stable upon Ebf1 knockdown due to rescue by the exogenous c-Myb cDNA (Fig. 5A). However, Ebf1 knockdown still induced higher levels of Rag transcription in the absence of STI as compared with luciferase shRNA control cells (Fig. 5B, left). Moreover, whereas STI treatment was unable to fully upregulate Rag transcription in the luciferase shRNA control cells due to the repressive effects of c-Myb overexpression, STI-induced Rag transcription in the Ebf1 knockdown cells is robust (Fig. 5B, right). This result, along with the c-Myb knockdown result, shows that an independent decrease in either Ebf1 or c-Myb levels in our Rag1-GFP reporter Abelson cell line is sufficient to activate Rag transcription.

Ebf1 and c-Myb antagonize Foxo1 binding to the Rag locus upon STI-induced differentiation of Abelson cells

We hypothesized that Ebf1 and c-Myb might repress Rag transcription by antagonizing the activity of positive regulators of Rag. A recent study using Irf4−/−Irf8−/− pre-B cells implicated Foxo1 and Pax5 as critical factors for the upregulation of Rag transcription during the large to small pre-B cell transition (14). Individual overexpression of Foxo1 or Pax5 is sufficient to increase Rag1-GFP levels in the reporter Abelson cell line (Fig. 6A, Supplemental Fig. 2) (12), and knockdown of either factor severely blunts STI-induced Rag transcription (Fig. 6B, Supplemental Fig. 2) (12). Genome-wide ChIP-seq analysis in the Irf4−/−Irf8−/− pre-B cells...
identified Foxo1 and Pax5 binding sites in the Rag locus that are inductively bound by these factors upon IL-7 withdrawal and pre-B cell differentiation (14). These sites included previously identified elements such as the Ireg enhancer (30) and Rag2 promoter Pax5 binding site (28), along with novel promoter (P) and intergenic (I) binding sites (see Rag locus schematic in Fig. 6C). We performed Foxo1 and Pax5 ChIP in untreated and STI-treated Abelson cells and observed increased occupancy of these factors at these Rag locus sites following STI treatment (Fig. 6D, left, Supplemental Fig. 2). We also observed increased Foxo1 occupancy at sites in the Blnk and Syk loci, two other genes identified as direct Foxo1 targets during Ireg4−/−Ireg8−/− pre-B cell differentiation (14) (Fig. 6D, right). Together, these experiments implicate Foxo1 and Pax5 as direct transcriptional activators of Rag and other genes involved in pre-B cell differentiation in the AMuLV system following STI treatment.

We then tested whether Ebf1 and c-Myb overexpression affected the binding of Foxo1 or Pax5 to the Rag locus sites upon STI treatment. Whereas coimmunoprecipitation experiments revealed that both Ebf1 and c-Myb physically interact with Pax5 (our unpublished observations), Pax5 ChIP experiments showed that binding of this factor to the Rag locus upon STI treatment was not affected by Ebf1 or c-Myb overexpression (Supplemental Fig. 2). However, Foxo1 ChIP experiments revealed that overexpression of either Ebf1 or c-Myb clearly reduced the amount of Foxo1 bound to the Rag locus upon STI treatment (Fig. 6E, left). Ebf1 or c-Myb overexpression did not affect Foxo1 binding to the Blnk and Syk loci (Fig. 6E, right), suggesting these factors repress Rag induction, but not other aspects of pre-B cell differentiation. Thus, Ebf1 and c-Myb negatively regulate Rag transcription by antagonizing Foxo1 binding to the Rag locus.

**Ebf1 and c-Myb negatively regulate Foxo1 expression**

ImmGen microarray data show that Foxo1 mRNA levels are high in fractions B and C, decrease in fraction C′, and increase again in fraction D (24). In agreement with this, we found that Foxo1 transcript levels increase upon differentiation of primary B cell cultures and AMuLV cells following IL-7 withdrawal and STI treatment, respectively (Fig. 7A). Together these data suggest that factors actively repress Foxo1 transcription in large cycling pre-B cells. Reduced Foxo1 binding to the Rag locus in STI-treated Ebf1- and c-Myb-overexpressing cell lines prompted us to assess Foxo1 expression levels in these cells. Western blotting revealed lower levels of Foxo1 protein in cells overexpressing Ebf1 and c-Myb compared with control empty vector-transduced cells (Fig. 7B), suggesting that these factors act as negative regulators of Foxo1 expression. To further assess the relationship between Ebf1, c-Myb, and Foxo1, we assayed Foxo1 mRNA levels in cells transduced with shRNAs targeting Ebf1 and c-Myb. Indeed, both Ebf1 and c-Myb knockdown resulted in an increase in Foxo1 transcript levels compared with cells transduced with a control luciferase shRNA (Fig. 7C), supporting the notion that Ebf1 and c-Myb are negative regulators of Foxo1 transcription in Abelson cells.

**Ebf1 and c-Myb positively regulate Gfi1b expression**

Despite their negative influence on Foxo1 expression, Foxo1 binding to non-Rag locus targets upon STI-induced differentiation was unaffected by Ebf1 or c-Myb overexpression (Fig. 6E, right), suggesting Ebf1 and c-Myb influence chromatin accessibility at the Rag locus, but not other Foxo1 target genes. Gfi1b is both a direct negative regulator of Rag transcription and an indirect regulator of the Rag genes via its antagonism of Foxo1 expression (15). Gfi1b directly represses Rag transcription by binding near the Ireg2 and Ireg elements also bound by Foxo1 (Fig. 6) and recruiting chromatin modifiers that deposit H3K9me2, a chromatin mark associated with transcriptional repression (15). Given the effects of Ebf1 and c-Myb on both Foxo1 expression and Foxo1 binding to the Rag locus, we hypothesized that these factors may also regulate Gfi1b expression. Indeed, knockdown of both Ebf1 and c-Myb resulted in a strong decrease of Gfi1b mRNA levels (Fig. 8A), suggesting that Ebf1 and c-Myb repress Rag and Foxo1 in part by driving Gfi1b expression. Gfi1b has been previously identified as a direct target of Ebf1 in both pro-B and splenic B cells, with ChIP-seq revealing Ebf1 binding sites in the Gfi1b promoter (26, 31) (Fig. 8B). Conventional ChIP in cells overexpressing a 3×FLAG-tagged Ebf1 protein revealed strong binding of Ebf1 to this site (Fig. 8C), suggesting that Ebf1’s positive influence on Gfi1b expression is direct.
FIGURE 6. Ebf1 and c-Myb antagonize Foxo1 binding to the Rag locus during pre-B cell differentiation. (A) GFP expression in AMuLV-transformed Rag1-GFP reporter B cells transduced with Foxo1 cDNA retrovirus. Uninfected cells (shaded histogram) were distinguished cDNA-overexpressing cells (black line) by staining with anti-Thy1.1 (retroviral marker). Numbers above gate indicate the percentage of GFP⁺ uninfected cells (top) or cDNA-overexpressing cells (bottom). Data are representative of two independent experiments. (B) GFP expression in AMuLV-transformed Rag1-GFP reporter B cells transduced with Foxo1 shRNA and treated with STI. Uninfected cells (shaded histogram) were distinguished from shRNA-expressing cells (black line) by staining with anti-hCD2 (retroviral marker). Numbers above gate indicate the percentage of GFP⁺ uninfected cells (top) or shRNA-expressing cells (bottom). Data are representative of two independent experiments. (C) Schematic of the Rag locus showing approximate locations of sites bound by Foxo1 and Pax5 upon pre-B cell differentiation (14). “P” and “I” denote promoter and intergenic binding sites, respectively. (D) ChIP in AMuLV-transformed B cells with anti-Foxo1 Ab. Cells were cultured under normal conditions (white bars) or treated with STI (gray bars) prior to harvest. qPCR was performed with recovered chromatin to assess Foxo1 occupancy Rag locus sites depicted in (C), or at binding sites near other Foxo1 target genes (14). “Rag2PaxBS” serves as negative control, as Foxo1 does not bind this site. Data are representative of two independent experiments. (E) ChIP in AMuLV-transformed B cells with anti-Foxo1 or IgG control Ab. Empty vector-transduced, Ebf1-overexpressing, and c-Myb-overexpressing cells were treated with STI and harvested. qPCR was performed with recovered chromatin to assess Foxo1 occupancy at the binding sites studied in (D). Data are representative of two independent experiments.
Stat5 represses Rag through upregulation of Ebf1

The IL-7R signaling pathway has been implicated in both the control of Ebf1 expression (32–35) and the repression of Rag transcription (12, 16) during B cell development. Signaling through the IL-7R and transformation by v-Abl both result in the phosphorylation and activation of Stat5 (17, 36). Our IL-7 withdrawal and STI-treatment experiments showed that both IL-7R and v-Abl signaling positively regulate Ebf1 protein expression (Fig. 2). Thus, we hypothesized that Stat5 is the downstream effector in these signaling pathways that upregulates Ebf1 to repress Rag transcription.

As with Ebf1 and c-Myb, overexpression of a constitutively active form of Stat5 (CA-STAT5B) (37) in the Rag1- GFP reporter cell line repressed baseline Rag levels and severely blunted STI-induced Rag transcription (Fig. 9A). When we compared Ebf1 protein levels in sorted empty vector control and CA-STAT5B–transduced cells, Stat5 overexpression clearly upregulated Ebf1 (Fig. 9B, left lanes). Upon inhibition of v-Abl with STI, levels of phosphorylated endogenous Stat5a decrease (Fig. 9B, top 100-kDa band), and this correlates with a decrease in Ebf1 levels in empty vector control cells, as observed in Fig. 2A. However, in cells overexpressing CA-STAT5B, Ebf1 expression is rescued and remains high despite v-Abl inhibition and loss of active endogenous Stat5a (Fig. 9B, right lanes) compared with a negative control site in the Rag1 promoter. Data are representative of three independent experiments. (F) ChIP in AMuLV-transformed B cells with anti-Ebf1 Ab. Cells were cultured in normal conditions (white bars) or treated with STI (gray bars) prior to harvest. qPCR was performed on recovered chromatin to assess Ebf1 occupancy at sites in the Foxo1 locus described in (D). Data are representative of two independent experiments.
Increased Ebf1 dosage drives proliferation through activation of target genes involved in cell cycle progression such as cyclin D3 (Ccnd3) (26, 38) while simultaneously repressing Rag transcription via antagonism of Foxo1 and activation of Gfi1b. Thus, the promotion of proliferation by increased Ebf1 dosage is tightly linked with repression of the recombination machinery, ensuring genomic integrity is maintained. Upon the attenuation of IL-7R signaling and differentiation to the small pre-B cell stage, Ebf1 protein levels are reduced and Rag expression is upregulated to mediate Ig L chain gene rearrangement.

**Discussion**

Restricting expression of the recombinase machinery in developing lymphocytes to nondividing cells prevents DSBs during S phase that could be aberrantly repaired and lead to leukemic transformation. During B cell development, signals through the IL-7R and pre-BCR

**FIGURE 8.** Ebf1 and c-Myb positively regulate Gfi1b expression. (A) qPCR measuring Gfi1b transcript levels in sorted AMuLV-transformed B cells transduced with luciferase shRNA (LUC), and either Ebf1 (left) or c-Myb shRNA (right). Data are representative of three independent experiments. (B) Published Ebf1 ChIP-seq data (26, 31) visualized in the UCSC Genome Browser at the Gfi1b locus. An Ebf1 binding site (5’-CCCNNGGG-3’) identified within the Gfi1b promoter peak is shown below, along with its location relative to the Gfi1b transcriptional start site. (C) ChIP in AMuLV-transformed B cells overexpressing a 3×FLAG-tagged Ebf1 protein with anti-FLAG or IgG control Ab. qPCR was performed on recovered chromatin to assess Ebf1 occupancy at the Gfi1b promoter site described in (B) compared with a negative control site in the Rag1 promoter. Data are representative of four independent experiments.
drive proliferation and the repression of Rag expression via targeted degradation of RAG2 protein and transcriptional repression of the Rag genes. We have identified a novel pathway that adds an additional layer of complexity to how this transcriptional repression is accomplished. Although this pathway involves upstream components in IL-7 and Stat5 that have been previously implicated in Rag repression, the downstream effectors identified (Ebf1 and c-Myb) paradoxically have been previously described as activators of the Rag locus during early B cell development.

Ebf1 was identified as a B lineage-specific factor that bound and activated the mb-1 promoter (39, 40), and Ebf1-deficient mice revealed its essential role in early B cell development (41). Ebf1 promotes specification to the B lineage in multipotent progenitors via simultaneous activation of B lineage genes and repression of genes involved in the development of other lineages (42, 43). Predicted Ebf1 targets during the earliest stages of development include Rag1, Rag2, and Foxo1, as Ebf1-deficient common lymphoid progenitors display reduced expression of these genes (22). This raises the question of how Ebf1 is converted to a repres sor of Foxo1 transcription during later stages of B cell development. Our experiments suggest that Ebf1 dosage may play a role, as unexpectedly we found increased levels of Ebf1 in both AMuLV-transformed cells and IL-7–cultured primary B cells resulting in decreased Rag expression (Fig. 1). Whereas Ebf1 transcript levels peak in small pre-B cells (24), our data show that Ebf1 protein levels are highest in proliferating pro-B and early pre-B cells where Rag expression is repressed (Fig. 2). IL-7R expression levels vary within the pro-B cell compartment (16), and pre-BCR expression enhances IL-7R responsiveness (44, 45). We hypothesize that brief periods of strong IL-7R signaling in proliferating pro-B and large cycling pre-B cells could cause a spike in Ebf1 dosage that contributes to Rag transcriptional repression. Ebf1 dosage has been shown to be important for transcriptional repression of NK cell–specific genes by Ebf1 (46). Perhaps high levels of Ebf1 protein induced by strong IL-7R signaling allow for the formation of repressive complexes at binding sites in the Foxo1 locus that are not formed under conditions of low Ebf1 expression during earlier stages of development. Stat5 has been shown to simultaneously activate and repress genes in B cells by assembling into distinct dimeric and tetrameric complexes (47). It would be interesting to test biochemically whether Ebf1 can form dose-dependent higher-order complexes on Foxo1 locus binding sites, and whether such complexes are competent for corepressor recruitment.

Alternatively, late pro-B and early pre-B cells may express an Ebf1 corepressor that is not present at appreciable levels during earlier stages of B cell development to allow for stage-specific Foxo1 repression. Interestingly, expression levels of the H3K27 methyltransferase Ezh2, which is recruited to the Ig κ locus by tetrameric Stat5 complexes to repress premature recombination in cycling pre-B cells (47), increase throughout early B cell development and peak in fraction C′ when Rag is repressed (24). As Ebf1 has been shown to target the repressive H3K27me3 chromatin mark on non-B lineage genes it actively represses in pro-B cells (48, 49), it would be interesting to test whether a stage-specific interaction between Ebf1 and Ezh2 is responsible for Foxo1 and Rag repression in late pro-B and early pre-B cells.

Our experiments also revealed that Ebf1 protein levels decrease upon differentiation to the small pre-B stage and re-expression of Rag1 and Rag2 (Fig. 2). This occurs despite an increase in Ebf1 transcript levels upon differentiation, suggesting Ebf1 protein is actively targeted for degradation at this developmental transition. Interestingly, analysis of GFP expression in mice expressing an Ebf1(1–148 aa)-GFP fusion protein revealed high GFP levels in pro-B cells, but greatly reduced GFP expression in pre-B cells (25). Whereas the authors attribute this to nonspecific destabilization of the Ebf1-GFP fusion protein, it is consistent with the decrease in Ebf1 protein levels we observed during pre-B cell differentiation that appears necessary for full re-expression of the Rag genes. Identifying the factors and pathways responsible for this posttranslational control of Ebf1 will be of considerable interest, as disruption of this process could prevent recombination re-expression and impair Ig L chain gene rearrangement in resting pre-B cells.

Previous reports by our group and others implicated c-Myb as a positive regulator of Rag promoter activity (27, 28). Whereas these studies relied heavily on reporter constructs containing cloned Rag locus elements transfected into both B and non-B lineage cells, our analysis of c-Myb’s effects on transcription from the endogenous Rag locus in an AMuLV-transformed B cell line clearly shows this factor represses Rag expression in this context. Analysis of mice in which c-Myb is conditionally deleted in early B cell progenitors, and of mice expressing a hypomorphic c-Myb allele, revealed defects in expression of IL-7R and of other early B lymphoid genes (29, 50). Whereas Rag expression was not affected in the B lineage compartments of these mice, it appeared that, similar to Ebf1, c-Myb plays a role in B lineage specification via the activation of a large set of genes important for early B cell development. Thus, how c-Myb is co-opted along with Ebf1 as a repressor of the Rag genes during a later stage of B cell development will be an important question to answer. Unlike Ebf1, c-Myb protein levels are unchanged following STI treatment of AMuLV cells (our unpublished observations), suggesting that posttranslational modifications or the availability of novel corepressors control c-Myb activity at this developmental transition. Identifying the factors controlling c-Myb activity, along with the location of functional c-Myb binding sites in the Rag, Foxo1, and Gfi1b loci in B cells, will be essential for understanding the mechanism by which c-Myb represses Rag transcription.

Foxo1 activity is necessary for the two waves of Rag expression that mediate Ig H chain and L chain gene rearrangement in pro-B and pre-B cells, respectively (51). Studies suggest that posttranslational mechanisms negatively regulate Foxo1 activity in large pre-B cells, and that, upon differentiation, this regulation is relieved to allow Foxo1 to activate Rag transcription in small pre-B cells (12, 14). However, microarray analysis with sorted primary cells shows a clear decrease in Foxo1 transcription levels, as pro-B cells (fractions B/C) differentiate to large cycling pre-B cells (fraction C′), followed by an increase in Foxo1 mRNA in small pre-B cells (fraction D) (24). When we quantified Foxo1 transcripts in both Abelson-transformed and primary cultured pro-B cells, we observed a significant increase in Foxo1 mRNA levels upon STI- and IL-7 withdrawal-induced differentiation (Fig. 7A), indicating that Foxo1 is also regulated at the transcription level during this developmental transition. Our experiments suggest that the repression of Foxo1 transcription by Ebf1 and c-Myb is an important additional level of control of Foxo1 activity (and thus Rag expression) in proliferating late pro-B and early pre-B cells.

IL-7R signaling and Stat5 have been implicated in the ordered rearrangement of the Ig loci via activation of chromatin in the H chain locus (52, 53) and repression of chromatin in the L chain locus (47, 54) in pro-B cells. Recent studies have shown that, in addition to controlling recombination accessibility, IL-7R signaling and Stat5 regulate recombination at the level of transcription of the Rag genes (6, 12, 14, 16). Our findings provide additional mechanistic insight into how this negative regulation by Stat5 is accomplished. While potentially acting as a direct negative regulator of Rag transcription (16), we showed that Stat5 also acti-
vates an Ebf1-dependent pathway that leads to enhanced proliferation and the transcriptional repression of FoxO1. Thus, in addition to being controlled posttranslationally by the PI3K-Akt pathway, FoxO1 is also regulated at the transcriptional level by the IL-7R/Stat5 pathway to prevent Rag expression during periods of cellular proliferation.

The transcriptional repressor Gfi1b is an additional downstream factor in the repressive Ebf1-dependent pathway (Fig. 8). Although its ability to negatively regulate Rag transcription both directly and through repression of FoxO1 has been described (15), the pathways controlling Gfi1b expression and activity during early B cell development are ill defined. Gfi1b mRNA levels are inversely correlated with Rag transcription and peak in large cycling pre-B cells (15). This is consistent with a model in which enhanced IL-7R signaling and Stat5 activity at this stage upregulate Ebf1 and c-Myb, which in turn drive increased levels of Gfi1b transcription that lead to Rag repression. But whereas our experiments shed light on how Gfi1b expression might be regulated during this specific stage of B cell development, it has been reported that Ebf1 is a negative regulator of Gfi1b expression in common lymphoid progenitors (43). So again, the paradox of how Ebf1 switches between transcriptional activator and repressor must be solved to better understand how this factor directs gene expression during distinct developmental stages.

Stat5 is constitutively activated in AMuLV-transformed cells (36) and in human hematopoietic malignancies, including BCR-ABL+ acute lymphoblastic leukemia, as Stat5-deficient hematopoietic progenitors are impaired for pro-B cell differentiation. Thus, Ebf1 and c-Myb represent at least two different pathways that converge at Stat5 activity during early B cell development are correlated with increased Gfi1b mRNA levels (10). Moreover, the repression of FoxO1.


