

E2A Promotes the Survival of Precursor and Mature B Lymphocytes¹

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The basic helix-loop-helix transcription factor E2A is an essential regulator of B lymphocyte lineage commitment and is required to activate the expression of numerous B lineage-specific genes. Studies involving ectopic expression of Id proteins, which inhibit E2A as well as other basic helix-loop-helix proteins such as HEB, suggest additional roles of E2A at later stages of B cell development. We use E2A-deficient and E2A and HEB double-deficient pre-B cell lines to directly assess the function of E2A and HEB in B cell development after lineage commitment. We show that, in contrast to the established role of E2A in lineage commitment, elimination of E2A and HEB in pre-B cell lines has only a modest negative impact on B lineage gene expression. However, E2A single and E2A and HEB double-deficient but not HEB single-deficient cell lines show dramatically enhanced apoptosis upon growth arrest. To address the possible role of E2A in the regulation of B cell survival *in vivo*, we crossed IFN-inducible Cre-transgenic mice to E2A conditional mice. Cre-mediated E2A deletion resulted in a block in bone marrow B cell development and a significant reduction in the proportion and total number of splenic B cells in these mice. We show that Cre-mediated deletion of E2A in adoptively transferred mature B cells results in the rapid depletion of the transferred population within 24 h of Cre induction. These results reveal that E2A is not required to maintain B cell fate but is essential in promoting pre-B and B cell survival. *The Journal of Immunology*, 2006, 177: 2495–2504.

B lymphocyte development in mice is one of the best characterized models to study mammalian cell lineage commitment and differentiation (for reviews, see Refs. 1 and 2). Commitment of lymphoid progenitors to the B cell lineage in the adult bone marrow is guided by the coordinated activities of transcription factors and cytokine signals. The transcription factors Pu.1, E2A, early B cell factor (EBF),³ and Pax-5 play key roles in regulating the transition from lymphoid precursor to lineage-committed B cell progenitor (3–7). Progression through B cell development is characterized by the sequential somatic recombination of the Ig H (IgH) and L chain (IgL) Ag receptor genes which ultimately leads to the expression of a functional BCR on the surface of mature B lymphocytes. B lineage-specific gene expression is regulated in part by the combined activities of E2A, EBF, and Pax-5. In addition, it has been shown that E2A and Pax-5 may also play a role in the suppression of non-B lineage gene expression (8, 9).

The E2A gene encodes the basic helix-loop-helix (bHLH) transcription factors E12 and E47 and is absolutely required for commitment of lymphoid progenitors to the B cell lineage (4, 5, 10). In addition, the bHLH transcription factors HEB and E2-2 have also been implicated in the regulation of B cell development (11). E12, E47, HEB, and E2-2 are classified in a transcription factor family

known as E proteins based on structural and functional similarities. Mice transheterozygous for *E2A/HEB*, *E2A/E2-2*, or *HEB/E2-2* exhibit reductions in progenitor B cell number that are greater than a mouse heterozygous for any single one of these genes (11). Therefore, the combined expression of these three E proteins is required for an optimal level of B cell development. E2A has also been directly implicated in the transcriptional regulation of many B lineage-specific genes and has been shown to be essential for Ig H and L chain recombination (5, 12–14). Recent studies using E2A-deficient hemopoietic progenitors and pre-B cell lines demonstrate that E2A promotes, as well as suppresses, expression of a broad array of transcripts in B cells (8, 15). E2A is required to initiate expression of many B lineage-specific genes such as *EBF*, *mb-1*, and *B29*. However, analysis of *E2A*^{-/-} pre-B cells revealed that E2A is not required to maintain the expression of these genes. These results raise the possibility that other E proteins, such as HEB, compensate for the loss of E2A and maintain B cell-specific gene expression.

E proteins have also been implicated in the regulation of lymphocyte survival. Multiple studies have demonstrated that increased expression of Id proteins, which are specific E protein inhibitors, correlates with enhanced apoptosis in developing T and B cells (16–18). Specifically, in B cells, induction of Id3 expression in progenitor B cells has been shown to induce apoptosis through a caspase-2-dependent mechanism (19). Although these studies suggest that E proteins play a role in promoting lymphocyte survival, they do not identify the specific E protein that may be mediating these effects. Additionally, Id proteins have been shown to directly inhibit the activity of members of the ternary complex factor subfamily of ETS transcription factors as well as members of the Pax transcription factor family (20, 21). Thus, it is possible that the observed phenotype in Id overexpression studies is a result of the combined inhibition of multiple transcription factors. Id3 expression also severely perturbs progenitor B cell proliferation raising the possibility that apoptosis may be a secondary consequence of catastrophic growth arrest.

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³ Abbreviations used in this paper: EBF, early B cell factor; bHLH, basic helix-loop-helix; pIpC, polyinosinic-polycytidylic acid; Ab-MuLV, Abelson murine leukemia virus; for, forward; rev, reverse; 7AAD, 7-actinomycin-D; qRT-PCR, quantitative RT-PCR; qPCR, quantitative PCR; C_μ, μ H chain constant exon.

We created pre-B cell lines deficient in E2A, HEB, or both E2A and HEB to evaluate the contribution of these E proteins to B cell gene expression, proliferation, and survival. In addition, we created mice in which E2A deletion could be induced to directly examine the consequence of E2A deletion beyond B lineage commitment. We found B lineage identity is maintained in $E2A^{-/-}HEB^{-/-}$ pre-B cell lines and that E2A deficiency correlated with enhanced apoptosis when growth arrest was induced by treatment with STI-571 (Gleevec). We also show that disruption of E2A in mature B cells results in rapid cell loss. Together, these data directly implicate E2A in the regulation of progenitor and mature B lymphocyte survival.

Materials and Methods

Mice and cell lines

$E2A^{flx}Mx-Cre^{tg}$ mice were created by crossing mice carrying the type I IFN-inducible Cre transgene (Mx-Cre) (22) with E2A conditional mice (23). Mx-Cre expression was induced by i.p. injection of 400 μ g of polyinosinic-polycytidylic acid (pIpC) (gamma-irradiated; Sigma-Aldrich) dissolved in 1 \times PBS. Wild-type CD45.1⁺CD45.2⁺ donor cells used in adoptive transfer experiments were obtained from the F₁ progeny of CD45.1 and CD45.2 congenic strains on a B6 background. E2A/HEB double conditional pre-B cell lines ($E2A^{flx}HEB^{flx}$) were derived from Abelson murine leukemia virus (Ab-MuLV) transformed bone marrow pro-B cells of an $E2A^{flx}HEB^{flx}$ mouse (J. Wojciehowski and Y. Zhuang, unpublished data). Cre recombinase was expressed in the cell lines by transduction with MIGR1-Cre retrovirus. Floxed and deleted E2A alleles were detected by genomic PCR with the following primers: E2A^{flx} for (CTGCACTC CGAATTGTGCCTG), PGKneo for (GCCCATTCGACCACCAAGCG), and YZ198 (GATCCTCGTCTCATTGGTACTG). Floxed and deleted HEB alleles were detected by genomic PCR with the following primers: JW1 (CTGGACAGAAAGTTCAGCACTAGTAC) and JW2 (CATTC CTATACATCAGCTTCTTGGACG). All pre-B cell lines were maintained at 37°C in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (HyClone), 100 U/ml penicillin (Invitrogen Life Technologies), 100 μ g/ml streptomycin (Invitrogen Life Technologies), and 50 μ M 2-ME (J. T. Baker).

STI-571 preparation

STI-571 (Gleevec; Novartis) was prepared as a 10-mM stock solution dissolved in H₂O plus 10 mM HCl, sterile filtered, and stored at -20°C until use.

Quantitative real-time PCR gene expression analysis

Total RNA was prepared using TRIzol (Invitrogen Life Technologies) followed by isopropanol precipitation. For all samples, 2 μ g of purified total RNA was treated with RNase free DNase I (Sigma-Aldrich) and random-primed cDNA was made using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Quantitative RT-PCR was performed with a Roche LightCycler and the FastStart DNA master SYBR green I kit (Roche) as per the manufacturer's instructions. All of the samples were normalized to the expression of GAPDH. The primers for RT-PCR are as follows: EBF, EBF no. 2 forward (for) (CATGTCCTGGC AGTCTCTGA) and EBF no. 2 reverse (rev) (CAACTCACTCCA GACCAGCA); Pax-5, Pax-5 for (CCGCCAAAGGATAGTGGAACTTG) and Pax-5 rev (CACAGTGTCAATTGTACAGACTCGC); E2-2, E2-2 for (GGTCCGGTCCCACTTC) and E2-2 rev (CGTCACTCTTCT GCTCTGG); IgH I μ , Imu for (GAGAGCCCCCTGTCTGATAAGAATC) and Imu rev (CGGTTTTGGAGTGAAGTTCGTG); mb-1 (I α), mb-1 for (CCTCTCCTCTTCTTGTGCATACG) and mb-1 rev (CCCCTGTGT TCTTGTACTTCCGG); B29 (I β), B29 for (TGTTCTGTCTGCT GCTCTTCTC) and B29 rev (TCGGTGACATTATGGTTGGCG); λ 5, λ 5 for (CAGATCATCCACGGGGAGC) and λ 5 rev (TGAGTGACAGG GACCCATC); VpreB, VpreB no. 2 for (CGTCTGTCTGCTCATGCT) and VpreB no. 2 rev (ACGGCACAGTAATACACAGCC); GAPDH, GAPDH for (CCTGGAGAAACCTGCCAAGTATG) and GAPDH rev (AGAGTGGGAGTTGCTGTTGAAGTC).

Quantitative PCR analysis of E2A deletion

A standard curve of deleted E2A alleles was created by mixing predetermined numbers of $E2A^{-/-}HEB^{-/-}$ (del) and $E2A^{flx}HEB^{flx}$ (floxed) pre-B cells together. A total of 1 \times 10⁵ pre-B cells were mixed in the following ratios: 90% del: 10% floxed; 75% del: 25% floxed; 50% del: 50% floxed; 25% del: 75%

floxed; 10% del: 90% floxed. Cells were lysed in Triton lysis buffer (Tris-EDTA (pH 7.5) + 0.2% Triton X-100 + 0.2 mg/ml proteinase K) by incubation at 55°C for 30 min followed by 95°C for 10 min then quickly cooled and stored at 4°C. Quantitative PCR was performed on these samples to generate a standard curve of E2A deletion. Quantitative PCR was performed on a Roche LightCycler using the FastStart DNA master SYBR green I kit (Roche) as per the manufacturer's instructions. Each sample was first normalized by quantitative PCR amplification of the CD14 genomic locus and then the quantitative value of E2A deletion for each sample was plotted. A linear trend line was fitted to the data points using Microsoft Excel. The equation of the trend line was used to calculate the percent of E2A deletion in unknown samples which had been normalized to CD14. The primers E2A^{flx} for and YZ198 are used to amplify the deleted E2A allele and CD14 is amplified with CD14 for (GCTCAAACCTTCAGAACTCTAC CGAC) and CD14 rev (AGTCAGTTCGTGGAGCCGGAAATC).

Adoptive B cell transfer experiments

Single-cell suspensions of donor (age: 2–3 mo) splenocytes were prepared in FACS buffer (1 \times PBS (pH 7.4) + 5% bovine calf serum). RBC were lysed using 0.16 M NH₄Cl and total splenocytes were labeled on ice with CD43 (BD Pharmingen), CD93 (eBioscience), CD4 (BD Pharmingen), CD8 β (BD Pharmingen), Mac-1 (Caltag Laboratories), Ter¹¹⁹ (BD Pharmingen), and pan-NK (BD Pharmingen) PE-conjugated Abs and Gr-1 (Caltag Laboratories) Cy5-PE (Cy5PE) conjugated Ab in FACS buffer. Immature, transitional and non-B splenocytes were depleted by AutoMACS (Miltenyi Biotec) using anti-PE Ab labeled microbeads (Miltenyi Biotec) as per the manufacturer's instructions. Enriched B cells were washed and suspended in 1 \times PBS (pH 7.4) and injected via the tail vein into sublethally irradiated (300 rad) recipients (B6 CD45.1 congenic mice, age 2–3 mo). All animal procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Cell cycle analysis and annexin V staining

For cell cycle analysis, pre-B cells were fixed in ice-cold ethanol and stained with propidium iodide in the presence of RNase A (Sigma-Aldrich). For annexin V staining, cells were first stained with appropriate Abs, washed in 1 \times annexin V binding buffer (BD Pharmingen) and then labeled with Annexin V^{PE} conjugate (BD Pharmingen) at room temperature in 1 \times annexin V binding buffer as per the manufacturer's instructions.

Flow cytometry

Bone marrow was prepared in FACS buffer and stained with CD43 PE, B220 allophycocyanin-conjugated Abs (BD Pharmingen), and 7-actinomycin-D (7AAD; BD Pharmingen). Splenocytes were prepared in FACS buffer and stained with IgM FITC, CD19 PE, IgD biotin, and B220 allophycocyanin-conjugated Abs followed by streptavidin-Cy5 PE staining. Mice analyzed were 2–3 mo old. All staining was done on ice. Samples were run on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Results

The loss of E2A and HEB perturbs but does not abolish B lineage-specific gene expression in pre-B cells

We have previously established E2A-deficient Ab-MuLV transformed progenitor B cell lines to study E2A regulation of pre-B lymphocyte gene expression and differentiation (13, 15). $E2A^{-/-}$ pre-B cells exhibit a block in Ig L chain recombination but retain expression of many B lineage-specific genes including known E2A targets such as *EBF*, *Ig H chain*, and *mb-1* (13, 15). These findings raise the possibility that other E proteins may be able to compensate for the loss of E2A. To test this hypothesis, we created Ab-MuLV transformed pre-B cell lines that are deficient in both E2A and HEB. Conditional E2A ($E2A^A$), previously referred to as $E2A^{loxP}$, mice were crossed to mice carrying a conditional HEB allele (HEB^f) to produce $E2A^{flx}HEB^{flx}$ offspring. The conditional E2A and HEB alleles were created by flanking the exons that encode the E47 and E12 bHLH domains of E2A and the bHLH domain of HEB with loxP recombination sites (Ref. 23; J. Wojciehowski and Y. Zhuang, unpublished data). Whole bone marrow from an adult $E2A^{flx}HEB^{flx}$ mouse was transformed with Ab-MuLV and the resulting stable pre-B cell line was infected with a retrovirus expressing Cre recombinase to simultaneously delete

E2A and HEB (Fig. 1A). Clonal cell lines were derived by limiting dilution plating of retrovirally infected cells and genomic PCR screening was performed to confirm the deletion of E2A and HEB. Both E2A and HEB alleles were deleted in the majority of Cre-transduced clones screened (Fig. 1B).

Once $E2A^{-/-}HEB^{-/-}$ clones were established, we examined the expression of known E2A target genes by quantitative RT-PCR

(qRT-PCR). Gene expression in $E2A^{-/-}HEB^{-/-}$ clones was compared with that of $E2A^{-/-}$ clones and the parental $E2A^{+/+}HEB^{+/+}$ cell line. E2A is required to properly induce EBF expression upon commitment of lymphoid progenitors to the B cell lineage (12, 24). Therefore, we measured the expression of EBF and Pax-5 in $E2A^{+/+}HEB^{+/+}$, $E2A^{-/-}$, and $E2A^{-/-}HEB^{-/-}$ pre-B cells. We have previously shown that EBF and Pax-5 expression is unchanged in

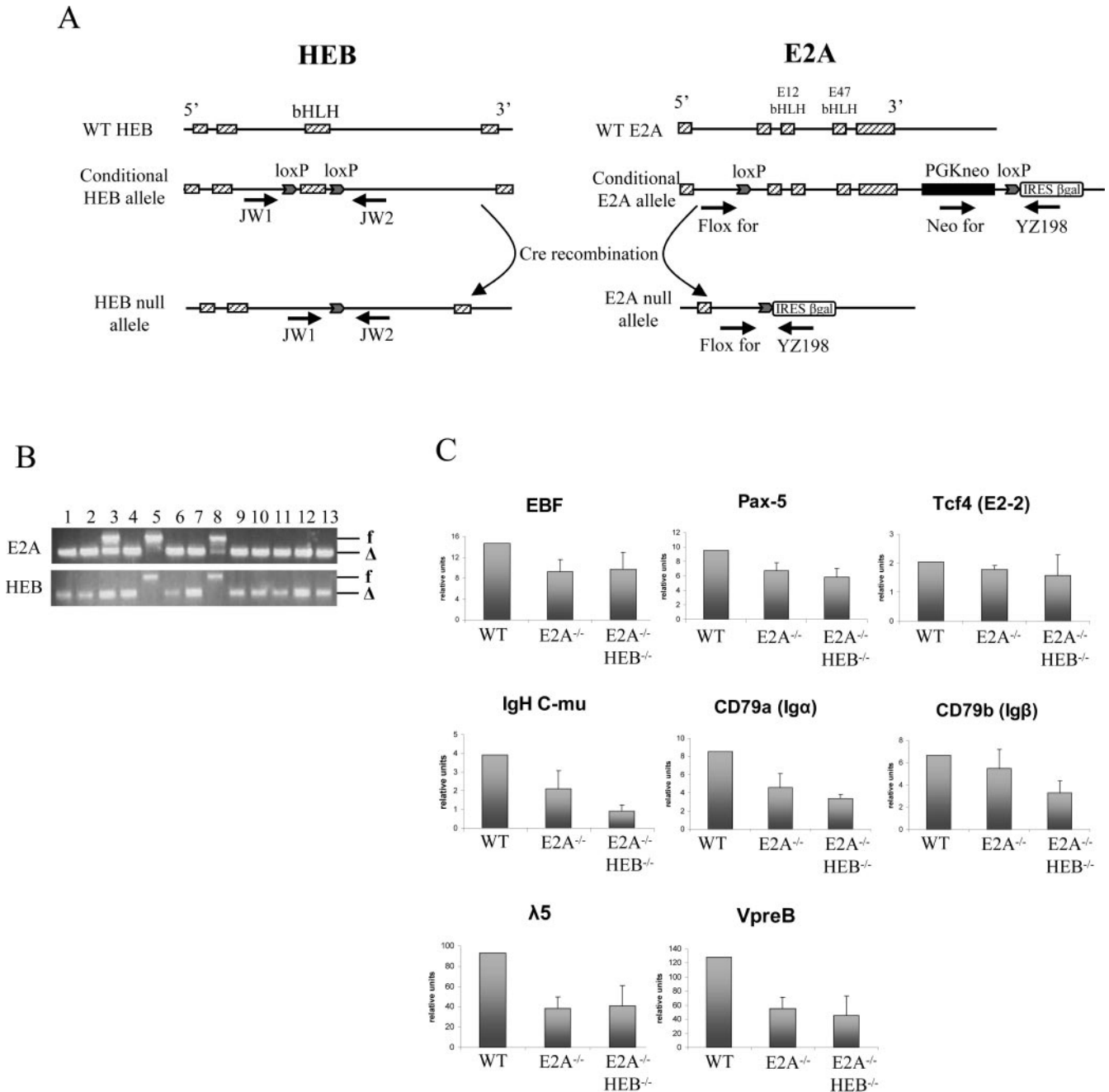


FIGURE 1. Gene expression in $E2A^{-/-}$, $HEB^{-/-}$, and $E2A^{-/-}HEB^{-/-}$ pre-B cell lines. **A**, Diagram of HEB and E2A floxed and deleted alleles. The HEB floxed allele contains loxP sites flanking exon 18 of the *HEB* gene, which encodes the bHLH domain. The E2A conditional allele contains loxP sites flanking exons 17–20, which include the E47 and E12 bHLH encoding exons. Cre-mediated recombination results in deletion of these exons creating null *HEB* and *E2A* alleles. Floxed and null alleles are detected using the primers JW1 + JW2 or E2Aflox for + PGKneo for + YZ198 for HEB or E2A, respectively. **B**, PCR genotyping of E2A and HEB deletion in Cre-transduced pre-B cells. Clonal pre-B cell lines (numbered 1–13) were derived by limited dilution plating of retrovirally transduced cells. PCR were performed to detect floxed (f) and deleted (Δ) HEB and E2A alleles using primers described in **A**. **C**, Analysis of B lineage specific gene expression in $E2A^{-/-}$ and $E2A^{-/-}HEB^{-/-}$ pre-B cells. The expression of the E-protein E2-2 and multiple B lineage restricted genes was assessed by quantitative RT-PCR in $E2A^{+/+}HEB^{+/+}$ (WT), $E2A^{-/-}$, and $E2A^{-/-}HEB^{-/-}$ cell lines. Gene expression levels in $E2A^{-/-}$ and $E2A^{-/-}HEB^{-/-}$ cells are represented as the average expression, relative to wild-type bone marrow, across three independent clones of each genotype with SD between clones indicated. For each transcript, relative expression in whole bone marrow was set as 1 and quantitative PCR values were calculated against a standard curve derived from 5-fold serial dilutions of bone marrow cDNA. All samples were normalized to the housekeeping gene GAPDH.

$E2A^{-/-}$ pre-B cells as determined by semiquantitative RT-PCR analysis (15). Quantitative RT-PCR analysis revealed a small decrease in the expression of both EBF and Pax-5 in $E2A^{-/-}$ and $E2A^{-/-}HEB^{-/-}$ pre-B cells (Fig. 1C). These data demonstrate that the expression of EBF and Pax-5 can be maintained despite the loss of both E2A and HEB.

E2A is known to regulate the expression of Ig H chain (IgH) (25). Therefore, we examined IgH expression by measuring transcription occurring through the μ H chain constant exon ($C\mu$). $C\mu$ expression was reduced 2-fold in $E2A^{-/-}$ cells and 4-fold in $E2A/HEB$ double-deficient pre-B cells relative to wild type indicating that IgH expression is influenced by E protein gene dose (Fig. 1C). We also examined expression of known E2A targets $CD79a$ (Ig α), $CD79b$ (Ig β), $\lambda 5$, and $VpreB$. Expression of Ig α and Ig β was decreased 2-fold in $E2A^{-/-}HEB^{-/-}$ pre-B cells relative to the control pre-B cell line. The expression of surrogate L chain genes $\lambda 5$ and $VpreB$ was reduced 2-fold in both $E2A^{-/-}$ and $E2A^{-/-}HEB^{-/-}$ pre-B cell lines when compared with $E2A^{fl/fl}HEB^{fl/fl}$ pre-B cells. We confirmed that expression of the E protein E2-2 remained unchanged by $E2A$ and HEB gene status thus ruling out the possibility that increased E2-2 expression was compensating for the deficiency in $E2A$ and HEB expression. These data indicate that B lineage-specific gene expression is maintained in the absence of both $E2A$ and HEB and show that HEB is involved in regulating the expression of a subset of $E2A$ target genes in B cells.

E2A-deficient pre-B cells undergo rapid cell death upon induction of growth arrest and differentiation

Previous studies have shown that overexpression of Id3 proteins, which block E protein activity, can inhibit progenitor B cell proliferation and induce cell death (19). Because Id proteins are non-specific inhibitors of E protein activity, it is not clear whether Id-mediated growth inhibition and cell death is due to the direct inhibition of $E2A$ or due to the inhibition of another target. Therefore, we investigated whether $E2A^{-/-}$ and/or $HEB^{-/-}$ pre-B cells exhibit altered proliferation.

We examined total cellular DNA content to determine the cell cycle kinetic profiles of the $E2A^{fl/fl}$ and $E2A^{-/-}$ transformed pre-B cell lines that we previously established (15). We observed that slightly more of the $E2A^{fl/fl}$ pre-B cells were actively cycling (57% cycling cells) versus $E2A^{-/-}$ pre-B cells (46% cycling cells) (Fig. 2A). Next, we examined the cell cycle kinetics of pre-B cell lines deficient in both $E2A$ and HEB . We measured total cellular DNA content of the parental $E2A^{fl/fl}HEB^{fl/fl}$ cell line, a Cre-transduced clone which had lost HEB but retained both floxed copies of $E2A$ ($E2A^{fl/fl}HEB^{-/-}$) and three $E2A^{-/-}HEB^{-/-}$ clones. Cell cycle analysis of three independent $E2A^{-/-}HEB^{-/-}$ clones revealed variable differences in the proportion of cycling cells compared with $E2A^{fl/fl}HEB^{fl/fl}$ or $E2A^{fl/fl}HEB^{-/-}$ cells (Fig. 2B). We observed that in $E2A^{-/-}HEB^{-/-}$ clones 7, 9, and 11, ~37, 45, and 47% of the cells were actively cycling, respectively. Approximately 51% of the $E2A^{fl/fl}HEB^{fl/fl}$ or $E2A^{fl/fl}HEB^{-/-}$ cells were actively cycling. Analysis of population growth kinetics confirmed that $E2A^{-/-}HEB^{-/-}$ clones 7 and 9 proliferate slower than either of the $E2A^{fl/fl}HEB^{fl/fl}$ or $E2A^{fl/fl}HEB^{-/-}$ cell lines (data not shown).

Abelson virus-transformed pre-B cells can be induced to undergo growth arrest and differentiation by inhibiting the activity of the v-Abl oncogene with the small molecule inhibitor STI-571 (26). Therefore, we asked whether E proteins are required for cell survival of pre-B cell lines that are reverted to an untransformed state with STI-571. We treated $E2A^{fl/fl}HEB^{fl/fl}$, $E2A^{fl/fl}HEB^{-/-}$, $E2A^{-/-}$ or $E2A^{-/-}HEB^{-/-}$ pre-B cell lines with 1 μ M STI-571 for 12 h and monitored cell viability by annexin V and 7AAD staining. We observed a large proportion of apoptotic cells

(7AAD⁻annexin V⁺) in $E2A^{-/-}$ and $E2A^{-/-}HEB^{-/-}$ pre-B cell cultures but not in $E2A^{fl/fl}HEB^{fl/fl}$ or $E2A^{fl/fl}HEB^{-/-}$ cultures after 12 h of STI-571 treatment (Fig. 2C). The $E2A^{fl/fl}HEB^{-/-}$ pre-B cell line showed only a small increase in 7AAD⁻annexin V⁺ cells after 12 h of STI-571 treatment. The increase in annexin V⁺ cells observed in $E2A^{-/-}$ and $E2A^{-/-}HEB^{-/-}$ clones after STI-571 treatment coincided with a rapid decline in the number of viable cells over the 12 h of treatment (Fig. 2D). STI-571-treated $E2A^{fl/fl}HEB^{fl/fl}$ and $E2A^{fl/fl}HEB^{-/-}$ pre-B cells showed little decline in cell viability over this same time period. Clones deficient in both $E2A$ and HEB did not exhibit a further decrease in cell viability when compared with $E2A$ single-deficient clones, indicating that the loss of $E2A$ but not HEB is sufficient to sensitize pre-B cell lines to apoptosis. These data indicate that $E2A$ protects pre-B cell lines from apoptotic cell death when the cells are induced to undergo growth arrest and differentiation. These data raise the possibility that $E2A$ may play a broader role in promoting B cell survival.

Conditional disruption of E2A in vivo perturbs B lymphocyte development in the bone marrow

Results from $E2A$ -deficient pre-B cell lines indicate that $E2A$ regulates the expression of multiple B lineage-specific genes and may play an important role in promoting B lymphocyte survival. Because $E2A$ knockout mice lack B cells, we used a conditional gene disruption approach to investigate the in vivo function of $E2A$ in developing and mature B cells. We crossed type I IFN inducible Cre (Mx-Cre) transgenic mice with our $E2A$ conditional mice ($E2A^{\Delta}$) to generate $E2A^{fl/fl}Mx-Cre^{tg}$ mice (22). $E2A$ deletion was induced by injecting these mice with pIpC. $E2A^{fl/fl}$ or $E2A^{fl/fl}Mx-Cre^{tg}$ mice were given three i.p. injections of 400 μ g of pIpC every other day and sacrificed 2 days after the last injection. Analysis of pIpC-treated Cre⁺ mice revealed a near complete loss of pro-B cells (B220⁺CD43^{high}) and a significant reduction in pre-B cells (B220⁺CD43^{low}) in the bone marrow (Fig. 3A). Cre⁻ mice treated with pIpC also had significantly smaller proportion of pre-B cells than untreated controls. This side effect of pIpC treatment is thought to be due to the ability of type I IFN to antagonize IL-7-dependent growth and development of progenitor B lymphocytes (27). We also found that the proportion of pre-B cells in untreated Cre⁺ mice was reduced as well, possibly as a consequence of leaky Cre expression.

To determine the efficiency of $E2A$ deletion, we developed a quantitative PCR (qPCR) based method. We adopted this method because it can be used to assay small populations of cells and it is faster than traditional Southern analysis. The qPCR assay was validated using genomic DNA obtained from populations of $E2A^{fl/fl}HEB^{fl/fl}$ and $E2A^{-/-}HEB^{-/-}$ pre-B cells that were mixed together in known ratios (Fig. 3B). qPCR analysis revealed that ~60% of the $E2A$ alleles had been deleted in the remaining bone marrow cells of pIpC-treated Cre⁺ mice (Fig. 3C). However, because B cell development depends on $E2A$, it is likely that this value underestimates the actual level of Cre-mediated $E2A$ deletion in the bone marrow. A small percentage (<10%) of deleted $E2A$ alleles was also detected in the untreated Cre⁺ mice indicating that there is leaky expression of the Cre transgene. No $E2A$ deletion was detected in the untreated or pIpC-treated $E2A^{fl/fl}Cre^{-}$ mice by qPCR. These results indicate that remaining bone marrow B cells still retain at least one copy of the floxed $E2A$ allele after pIpC treatment. The significant reduction of the pro-B cell population observed in pIpC-treated $E2A^{fl/fl}Mx-Cre^{tg}$ mice could be due to the partial or complete loss of $E2A$.

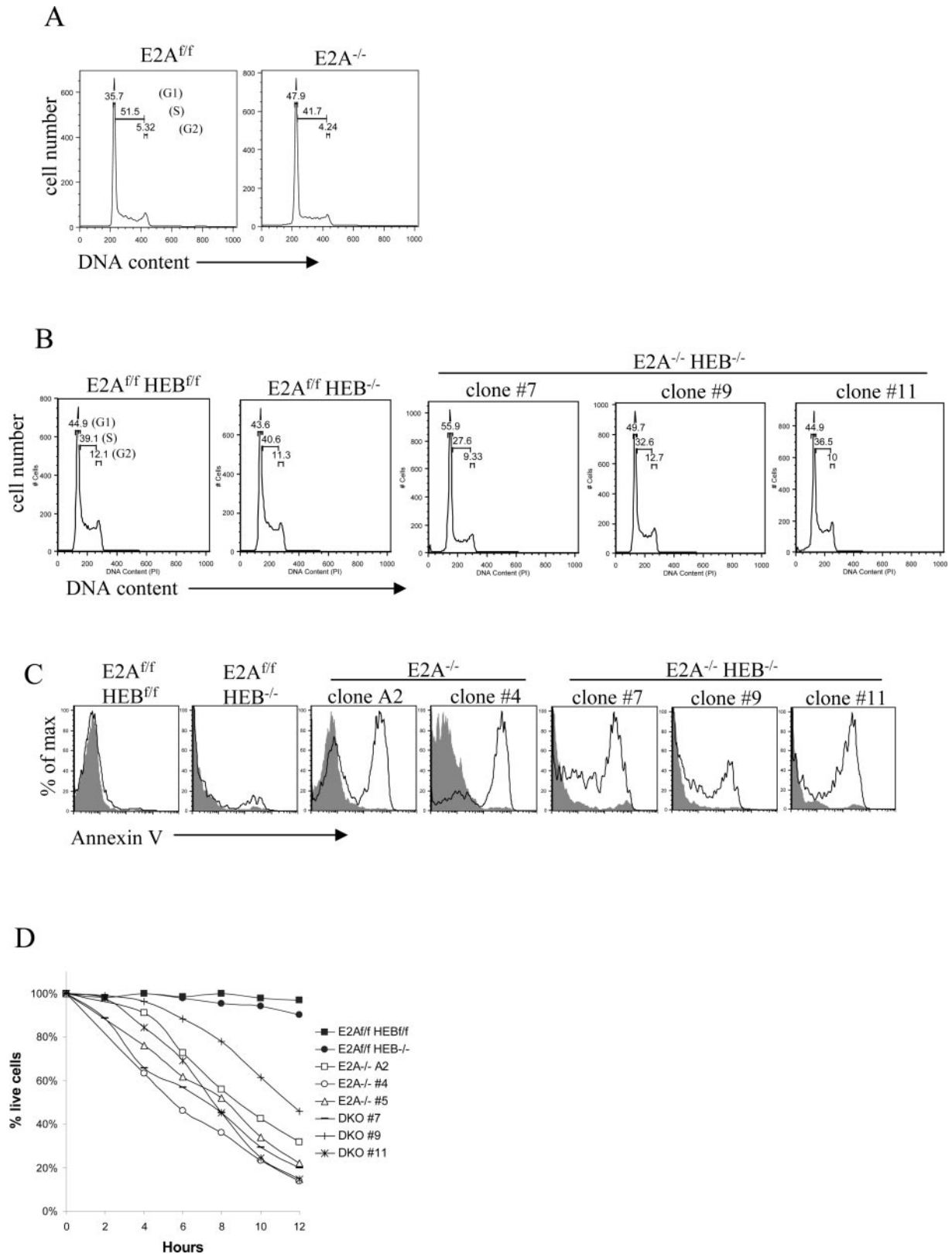


FIGURE 2. E2A-deficient cells undergo rapid apoptosis upon the induction of growth arrest and differentiation. **A**, Cell cycle kinetics of *E2A^{-/-}* and *E2A^{f/f}* pre-B cell lines. Cre-retrovirus-transduced *E2A^{-/-}* pre-B cells or antisense-Cre-transduced *E2A^{f/f}* pre-B cells were fixed in ethanol and stained with propidium iodide. Total DNA content was measured by FACS. The gates for G₁, S, and G₂ cells were determined using FlowJo software. **B**, Cell cycle kinetics of *E2A^{-/-}HEB^{-/-}* pre-B cell populations. Fixed cells were stained with propidium iodide and DNA content was measured by FACS. Cell cycle gates were determined with FlowJo software. **C**, *E2A^{-/-}* and *E2A^{-/-}HEB^{-/-}* pre-B cell lines exhibit enhanced apoptosis after STI-571 treatment. Cells were treated with 1 μM STI-571 and the proportion of apoptotic cells (7AAD⁻annexin V⁺) was evaluated immediately after treatment (solid gray) and 12 h after treatment (line) by FACS. The histograms presented are pregated on 7AAD-negative cells. **D**, *E2A^{-/-}* and *E2A^{-/-}HEB^{-/-}* pre-B cells undergo rapid cell death after STI-571 treatment. *E2A^{f/f}HEB^{f/f}* (WT) cells, an *E2A^{f/f}HEB^{-/-}* clone, three *E2A^{-/-}* clones and three *E2A/HEB* double knockout clones (DKO nos. 7, 9, 11) were treated with 1 μM STI-571 and the percent of live cells (annexin V⁻7AAD⁻) cells was determined at various times after treatment by FACS.

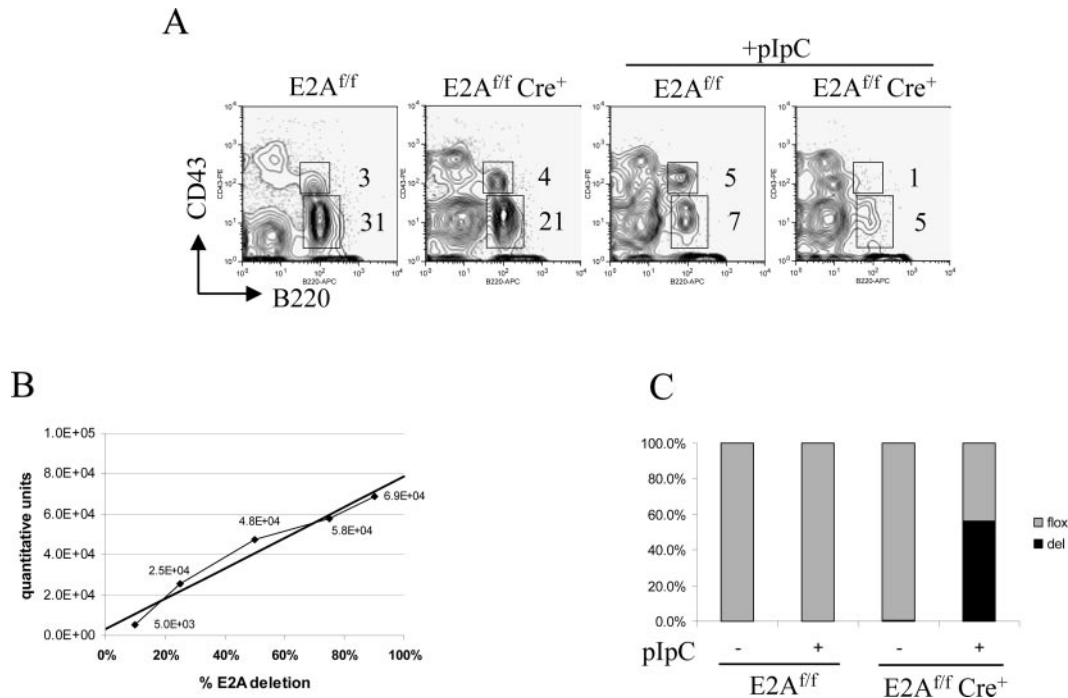


FIGURE 3. Mx-Cre-mediated E2A deletion blocks B cell development in bone marrow. *A*, Representative FACS plots of bone marrow B cell precursor populations from untreated or pIpC-treated $E2A^{f/f}$ or $E2A^{f/f}Mx-Cre^{tg}$ mice. FACS plots are size gated on lymphocytes and the frequencies of pro-B ($B220^+ CD43^{high}$) and pre-B ($B220^+ CD43^{low}$) cells are presented. Plots presented are representative of three independent experiments. *B*, Quantitative PCR analysis of E2A deletion. $E2A^{f/f}HEB^{f/f}$ and $E2A^{-/-}HEB^{-/-}$ pre-B cells were mixed together in known ratios, genomic DNA was prepared, and a standard curve of E2A deletion was generated by real-time PCR. Details of the standard curve generation and qPCR are provided in *Materials and Methods*. Quantitative PCR amplification of the CD14 genomic locus was used to normalize each of the samples. *C*, Quantitative PCR analysis of E2A deletion in bone marrow. E2A deletion was detected in whole bone marrow by quantitative real-time PCR. Samples were normalized to CD14 and the percent of E2A deletion was determined by plotting the normalized quantitative PCR value of E2A deletion on the standard curve in *B*. Bar graphs are presented as the proportion of deleted $E2A$ allele (del) and floxed $E2A$ allele (flux) of 100%. E2A deletion corresponds to the mice analyzed in *A*.

The conditional deletion of E2A results in the loss of splenic B lymphocytes

The $E2A^{f/f}Mx-Cre^{tg}$ mouse is a particularly useful model to examine the effect of $E2A$ gene disruption on mature B lymphocytes. Cre^+ and Cre^- mice were given three injections of pIpC every other day and splenic B cells were examined 2 days after the final injection. We found that the total number and percent of splenic B cells was significantly reduced in pIpC-treated Mx-Cre⁺ mice when compared with pIpC-treated Cre^- mice (Fig. 4A). The mean total number of $B220^+ CD19^+ IgM^+$ B cells was reduced from $1.0 \times 10^7 \pm 1.8 \times 10^6$ in Cre^- mice ($n = 3$) to $3.8 \times 10^6 \pm 1.2 \times 10^6$ in Mx-Cre⁺ mice ($n = 4$) and the mean proportion of splenic B cells was $56 \pm 5.7\%$ ($n = 3$) and $37 \pm 2.9\%$ ($n = 8$) in Cre^- and Mx-Cre⁺ mice, respectively. Because E2A regulates the expression of the Ig genes as well as $Ig\alpha$ and $Ig\beta$ which are key signaling molecules required for BCR expression, we asked whether E2A disruption could influence the expression level of surface IgM and IgD on splenic B cells. However, we found that the expression of IgM and IgD does not differ significantly between pIpC-treated Cre^- and Cre^+ mice (Fig. 4B). Quantitative analysis of E2A deletion in pIpC-treated mice revealed that the floxed $E2A$ allele was significantly enriched (60% floxed) in the $CD19^+$ -enriched fraction of $E2A^{f/f}Mx-Cre^{tg}$ splenocytes versus the $CD19^-$ splenocyte fraction (20% floxed) (Fig. 4C). We performed an additional experiment to determine whether enrichment of the floxed $E2A$ allele in pIpC-treated $CD19^+$ $E2A^{f/f}Mx-Cre^{tg}$ splenocytes could be due to inefficient E2A deletion in this population. $E2A^{f/f}Mx-Cre^{tg}$ and $E2A^{f/f}Mx-Cre^{tg}$ mice were given two pIpC treatments (on days 0 and 2) and sacrificed for analysis 48 h

after the last treatment. The relative proportion of wild-type/floxed $E2A$ to deleted $E2A$ in whole spleen and $CD19^+$ enriched splenocytes was determined by Southern analysis. We found that enrichment of the floxed allele in $E2A^{f/f} Mx-Cre^{tg}$ mice is not due to inefficient E2A deletion in splenic B cells because $CD19^+$ enriched splenocytes from the pIpC-treated $E2A^{f/f}Mx-Cre^{tg}$ mouse show approximately equal representation of wild-type and deleted $E2A$ alleles. (Fig. 4D). We also observed that whole spleen from the $E2A^{f/f}Mx-Cre^{tg}$ mouse exhibited ~50% floxed and 50% deleted $E2A$ while only the floxed $E2A$ allele was detectable in the $CD19^+$ -enriched splenocyte population. Thus, we consistently observe an overrepresentation of floxed allele in the $CD19^+$ splenocyte fraction of $E2A^{f/f}Mx-Cre^{tg}$ mice treated with pIpC. It is unlikely that the loss of E2A results in a down-regulation of CD19 surface expression on mature B cells because CD19 expression is normal on $E2A^{-/-}$ pre-B cell lines (15). These results indicate that E2A-deficient B cells are lost from the spleens of pIpC-treated $E2A^{f/f}Mx-Cre^{tg}$ mice.

Adoptively transferred $E2A^{f/f}Mx-Cre^{tg}$ mature B lymphocytes are lost upon induction of E2A deletion

The underrepresentation of E2A-deficient splenic B cells observed may result from a block in B cell development in the bone marrow or may be directly due to the deletion of $E2A$ in mature B cells. To distinguish these possibilities, we isolated mature B cells from $E2A^{+/f}$, $E2A^{f/f}Mx-Cre^{tg}$, or $E2A^{f/f}Mx-Cre^{tg}$ mice and transferred these cells to sublethally irradiated wild-type recipients. Recipient mice then received a total of three pIpC injections given every other day and were sacrificed 2 days after the final treatment (Fig.

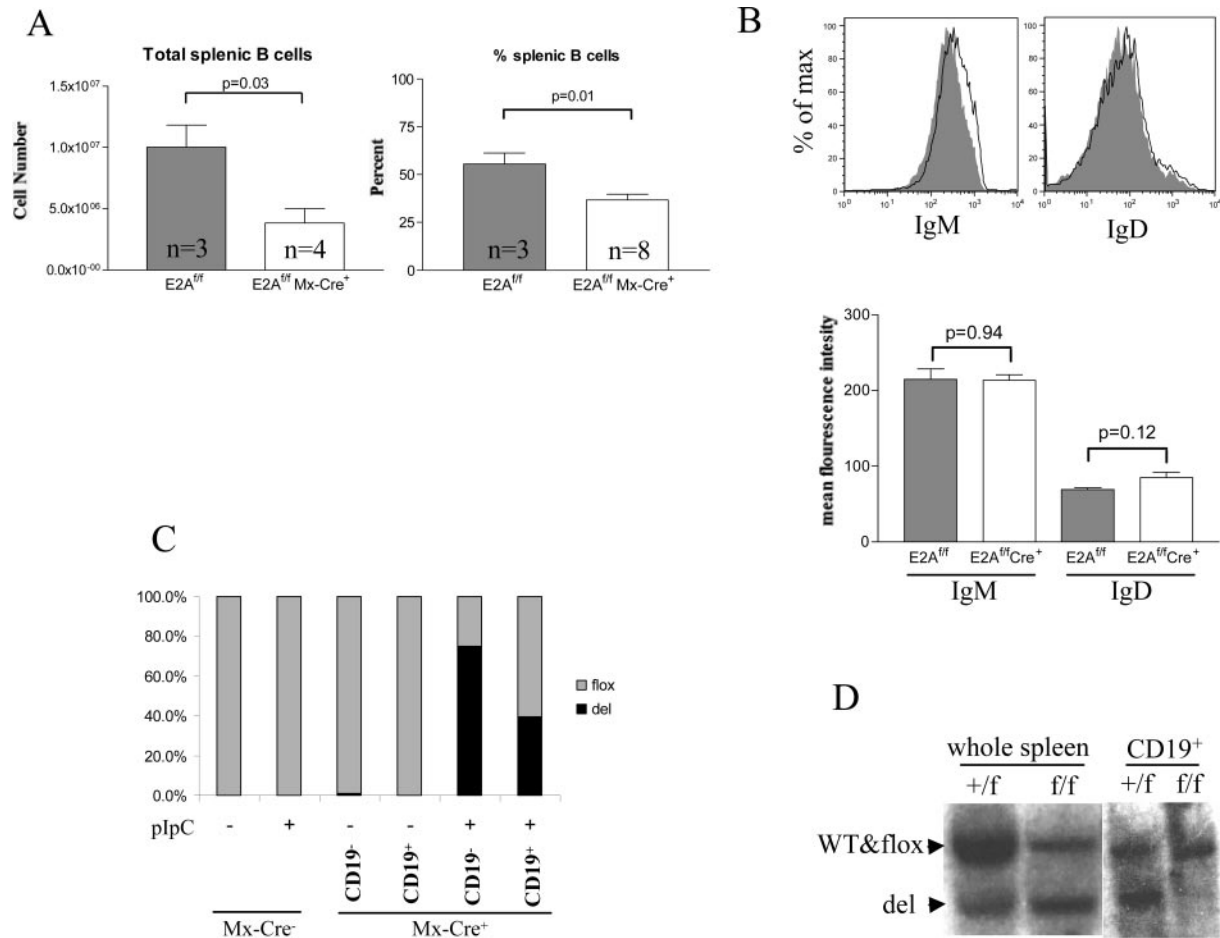


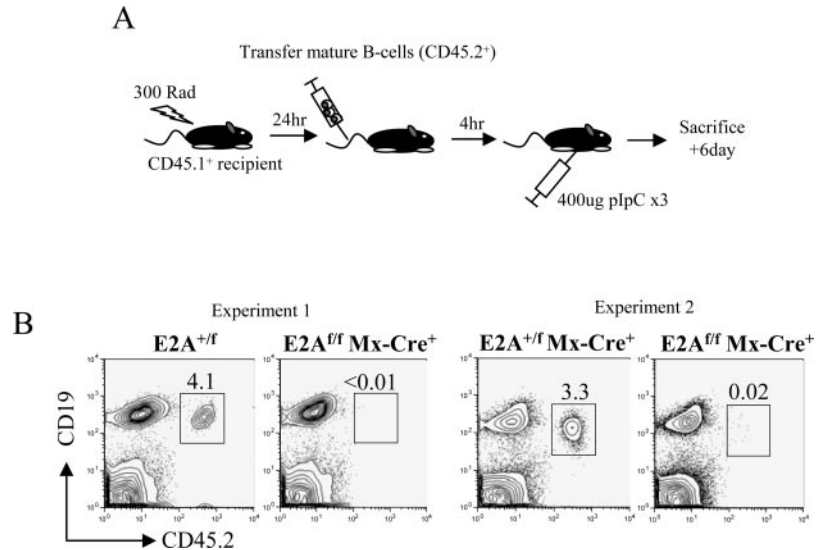
FIGURE 4. Conditional E2A deletion results in the reduction of B cells in the spleen. *A*, $E2A^{fl/fl}$ or $E2A^{fl/fl}Mx-Cre^{tg}$ mice were treated with pIpC and the mean total number and mean percent of $B220^+CD19^+$ splenic B cells was plotted with SEM indicated. *B*, Conditional deletion of E2A does not alter surface expression of IgM and IgD on splenic B cells. Histograms compare IgM and IgD expression on $B220^+$ splenocytes from pIpC-treated $E2A^{fl/fl}$ (solid gray) or $E2A^{fl/fl}Mx-Cre^{tg}$ (line) mice. The mean fluorescence intensity of IgM and IgD expression on $B220^+$ splenocytes from pIpC-treated $E2A^{fl/fl}$ ($n = 3$) and $E2A^{fl/fl}Mx-Cre^{tg}$ ($n = 4$) mice was plotted. All samples shown were prepared on $B220^+$ lymphocytes. *C*, Quantitative PCR analysis of E2A deletion in the spleen. Quantitative PCR was used to detect E2A deletion in whole spleen of $E2A^{fl/fl}$ mice treated with or without pIpC. Splenocytes from $E2A^{fl/fl}Mx-Cre^{tg}$ mice treated with or without pIpC were separated into $CD19^+$ and $CD19^-$ fractions by Ab-conjugated magnetic bead enrichment and quantitative PCR was used to detect E2A deletion in each fraction. Each sample was normalized to CD14 and the percent of E2A deletion was calculated using the standard curve generated in Fig. 1*B*. Bar graphs are presented as the proportion of floxed E2A allele (flox) and deleted E2A allele (del) of 100%. The pIpC-treated Cre^- and Cre^+ genotypes correspond to the histograms in *B*. *D*, Southern blot analysis of E2A gene status in splenocytes from pIpC-treated $E2A^{fl/fl}Mx-Cre^{tg}$ and $E2A^{fl/fl}Mx-Cre^{tg}$ mice. Genomic DNA from total splenocytes and CD19-enriched splenocytes was digested with *Bam*HI and probed with PCR-amplified E2A genomic probe. Bands corresponding to the wild-type/floxed E2A allele (WT/flox) and deleted E2A allele (del) are indicated.

5A). Approximately 2×10^6 mature B cells of each genotype were transferred. Transferred B cells were distinguished from host cells by CD45.2 surface expression. $E2A^{+/+}$ and $E2A^{+/+}Mx-Cre^{tg}$ B cells were readily detectable in spleens of the recipient mice after pIpC treatment, however, very few $E2A^{fl/fl}Mx-Cre^{tg}$ splenic B cells could be detected after the pIpC treatments (Fig. 5*B*). These results indicate that the deletion of E2A in mature B cells results in the loss of these cells in vivo.

The nearly complete lack of $E2A^{fl/fl}Mx-Cre^{tg}$ B cells found in recipient spleens after the three pIpC treatments suggests that the deletion of both copies of E2A results in the rapid loss of mature B cells. To better understand the kinetics by which transferred $E2A^{fl/fl}Mx-Cre^{tg}$ B cell are lost, we mixed purified mature B cells from wild-type mice with an equal number of B cells from $E2A^{fl/fl}$ or $E2A^{fl/fl}Mx-Cre^{tg}$ mice and transferred these mixed populations to sublethally irradiated wild-type recipients. Differential surface expression of the markers CD45.1 and CD45.2 was used to distinguish donor wild-type ($CD45.1^+CD45.2^+$), donor $E2A^{fl/fl}$ or $E2A^{fl/fl}Mx-Cre^{tg}$ ($CD45.1^-CD45.2^+$), and recipient ($CD45.1^+CD45.2^-$)

B cells. A total of 2×10^6 wild-type + 2×10^6 $E2A^{fl/fl}$ or $E2A^{fl/fl}Mx-Cre^{tg}$ B cells were transferred to each recipient and 400 μ g of pIpC was administered to the recipient mice 4 h after cell transfer (Fig. 6*A*). Recipient mice were sacrificed at 4 and 24 h after the pIpC treatment and the ratio of donor-derived wild-type to $E2A^{fl/fl}$ or $E2A^{fl/fl}Mx-Cre^{tg}$ splenic B cells was determined by FACS. We found that the proportion of both Cre^- and Cre^+ B cells to wild-type B cells had decreased 4 h after pIpC treatment (Fig. 6, *B* and *C*). These results suggest that both $E2A^{fl/fl}$ and $E2A^{fl/fl}Mx-Cre^{tg}$ B cells engraft less efficiently than wild-type cells. By 24 h post-pIpC treatment, the ratio of donor $E2A^{fl/fl}Mx-Cre^{tg}$ to wild-type B cells decreased an additional 30%, whereas the ratio of $E2A^{fl/fl}$ to wild-type B cells increased by $\sim 20\%$. We examined the change in the ratio of transferred $E2A^{fl/fl}Mx-Cre^{tg}$ to wild-type B cells over time in two independent experiments and observed an equivalent decline in the proportion of Cre^+ B cells between 4 and 24 h after pIpC treatment (Fig. 6*D*). These data indicate that a single pIpC treatment is sufficient to eliminate a significant proportion of the transferred $E2A^{fl/fl}Mx-Cre^{tg}$ B cells within 24 h of Cre induction.

FIGURE 5. Adoptively transferred $E2A^{fl}/Mx-Cre^{tg}$ mature B cells are lost after multiple rounds of Cre induction. **A**, Donor-derived $E2A^{+/+}$, $E2A^{+/+}Mx-Cre^{tg}$, or $E2A^{fl}/Mx-Cre^{tg}$ ($CD45.2^{+}$) mature splenic B cells were transferred to sublethally irradiated wild-type $CD45.1^{+}$ recipients. Each recipient received three injections of pIpC every other day and splenocytes were analyzed 2 days after the last pIpC treatment for the presence of donor-derived B cells. **B**, Detection of donor-derived mature B cells in the spleens of recipient mice by FACS. Total splenocytes from pIpC-treated recipients were analyzed by FACS to detect transferred B cells. The frequency of $CD19^{+}CD45.2^{+}$ donor-derived B cells is indicated. Results of two representative experiments are shown. The plots presented were size gated on lymphocytes.



We were able to detect both floxed and deleted $E2A$ alleles in splenic genomic DNA from recipients that received $E2A^{fl}/Mx-Cre^{tg}$ B cells at 24 h, indicating that a single round of Cre induction does not completely delete all floxed $E2A$ alleles (data not shown). These data support the conclusion that mature B cells are lost upon deletion of $E2A$.

Discussion

$E2A$ -deficient pre-B cell lines express many B lineage-specific genes, including known $E2A$ targets, raising the possibility that other E proteins can regulate B lineage-specific gene expression in the absence of $E2A$. As expected, disruption of $E2A$ alone reduced the transcript levels of known $E2A$ targets. However, the deletion of both $E2A$ and HEB in pre-B cells negatively impacted the expression of only a subset of $E2A$ target genes. Of the genes we assayed, IgH $C\mu$ expression was most dependent on expression of $E2A$ and HEB . These data indicate IgH expression is particularly sensitive to E protein gene dose and suggest that the expression level of IgH may be regulated by the overall level of E protein activity within the B cell. Interestingly, the disruption of both $E2A$ and HEB had only a minimal effect on the expression of EBF. $E2A$ directly associates with the 5' region of the EBF locus and is required to induce EBF expression at the earliest stages of B cell development (12, 14). Based on these results it seems plausible that $E2A$ may play a role in regulating the expression level of EBF in B cells. However, we find that EBF expression is maintained at near normal levels in $E2A^{-/-}HEB^{-/-}$ pre-B cells, suggesting that at later stages of B cell development, EBF expression is regulated independently of E protein activity. These data suggest that EBF expression switches from an $E2A$ -dependent mechanism to a mechanism less dependent on E proteins once lymphoid precursors commit to the B cell lineage. It is possible that this switch in control of EBF expression may serve as one method to enforce commitment to the B cell lineage. Our results also demonstrate that while disruption of $E2A$ and HEB negatively impacts the expression of multiple B lineage-specific genes, overall B cell lineage identity is retained, presumably due to $E2A$ - and HEB -independent maintenance of EBF and Pax-5 expression.

$E2A$ has been shown to both promote or inhibit cell proliferation under different experimental conditions (28–30). Studies using EBF-reconstituted $E2A^{-/-}$ progenitor B cells revealed a role for E proteins in the optimal expression of the pro-proliferative genes $N-myc$ and $c-myc$ and in the promotion of IL-7-dependent

proliferation (24). However, we observe only a minor defect in proliferation of $E2A^{-/-}$ or $E2A^{-/-}HEB^{-/-}$ pre-B cell lines suggesting that there may be other mechanisms that drive proliferation of these cells independently of $E2A$ and HEB . It has been shown that v-Abl can promote $c-myc$ expression, thus v-Abl signaling may be sufficient to maintain $c-myc$ in the absence of $E2A$ and HEB to promote proliferation (31). Alternately, it is possible that the regulation of $N-myc$ and $c-myc$ expression in our pre-B cell lines is mediated through a mechanism that is independent of $E2A$ and HEB . Future studies should help to resolve these questions.

Our studies in $E2A$ -deficient pre-B cell lines and in $E2A$ -conditional mice strongly suggest that $E2A$ regulates an important intrinsic survival pathway in B cells. We found that the loss of $E2A$ correlates with dramatically enhanced cell death in pre-B cell lines. Interestingly, this phenotype was only observed when we treated $E2A^{-/-}$ or $E2A^{-/-}HEB^{-/-}$ pre-B cell lines with STI-571. These data suggest that v-Abl provides survival signals which can complement $E2A$ deficiency. Mature B cell survival is dependent in part on signaling through a functional BCR on the cell surface. Ablation of the Ig H chain or the BCR signaling molecule $Ig\alpha$ on mature B cells has been shown to block this necessary BCR survival signal ultimately resulting in cell death (32, 33). Because $E2A$ regulates the expression of multiple genes which are required to produce the functional BCR, we investigated whether insufficient BCR expression could explain the reduction of B cells observed in $E2A^{fl}/Mx-Cre^{tg}$ mice after Cre induction. However, we did not observe a down-regulation of surface IgM or IgD on $B220^{+}$ splenic B cells in pIpC-treated $E2A^{fl}/Mx-Cre^{tg}$ mice. Adoptive transfer experiments revealed that $E2A^{fl}/Mx-Cre^{tg}$ B cells were lost within 24 h after Cre induction. These data show that B cells die very rapidly upon $E2A$ deletion. It is possible that BCR expression is quickly down-regulated upon $E2A$ deletion and we fail to observe a population of B cells with low BCR expression because these cells do not survive long enough to establish a significant, detectable population.

However, we do not believe that ablation of BCR expression is the primary cause of B cell death in our experimental system for the following reasons. First, qRT-PCR analysis of our E-protein-deficient pre-B cell lines suggests that the expression of $Ig\alpha$, $Ig\beta$, or IgH is not absolutely dependent on $E2A$ and may be regulated by HEB and/or $E2-2$. Second, $IgM^{low}B220^{+}$ B cells can be detected in the spleen of mice after Mx-Cre mediated disruption of Ig

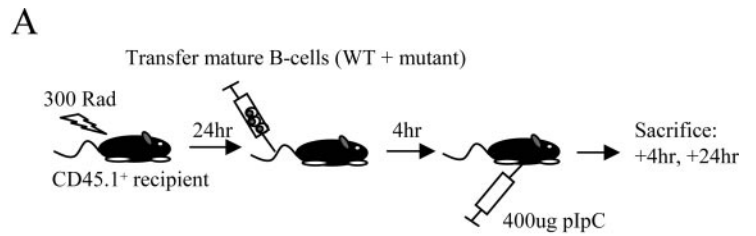
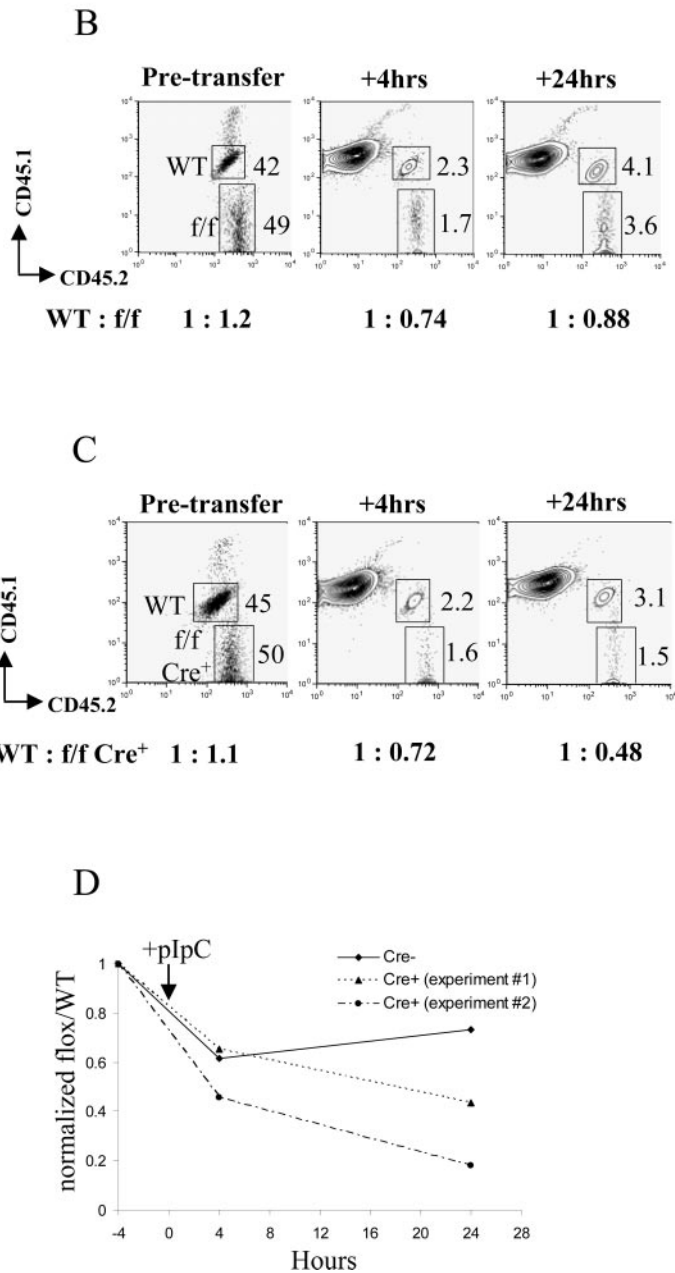


FIGURE 6. A single round of Cre induction results in the rapid loss of *E2A^{flox}Mx-Cre^{tg}* B cells after adoptive transfer. *A*, Diagram of the competitive adoptive transfer experiment. An equal number of wild-type (WT) (CD45.1⁺CD45.2⁺) plus *E2A^{flox}* (CD45.1⁻CD45.2⁺) or *E2A^{flox}Mx-Cre^{tg}* (CD45.1⁻CD45.2⁺) mature B cells were mixed and transferred into sublethally irradiated wild-type hosts (CD45.1⁺CD45.2⁻). After transfer, the hosts were given a single injection of pIpC and sacrificed at various time points thereafter. Donor-derived B cells were identified in the spleens of recipients by differential expression of surface Ags CD45.1 and CD45.2. *B*, Competitive adoptive transfer of WT + *E2A^{flox}* B cells into sublethally irradiated mice. Host and donor-derived splenic B cells were resolved by surface expression of CD45.1 and CD45.2 at each time point after pIpC treatment. The FACS plots presented were size gated on lymphocytes and then on B220⁺ CD19⁺ cells. The frequency of the donor derived populations and the ratio of WT to *E2A^{flox}* donor B cells is indicated for each time point. Time points indicated are hours after pIpC treatment. *C*, Competitive adoptive transfer of WT + *E2A^{flox}Mx-Cre^{tg}* B cells into sublethally irradiated wild-type recipients. FACS plots were size gated on lymphocytes and then on B220⁺ CD19⁺ cells. The frequency of donor-derived populations and the ratios of WT to *E2A^{flox}Mx-Cre^{tg}* B cells at each time point are indicated. Time points indicated are hours after pIpC treatment. *D*, Analysis of the change in proportion of floxed to wild-type transferred B cells over time. The initial ratio of floxed to wild-type mature B cells at the time of cell transfer ($t = -4$ h) was set as 1 and subsequent time points taken after cell transfer were normalized accordingly and graphed as a function of time with $t = 0$ h set as the time of pIpC injection. Cre⁻ and Cre⁺ (experiment no. 1) correspond to the experiments shown in *B* and *C*, respectively.



H chain on mature B cells (33). These data suggest that the pre-existing BCR on the cell surface is gradually down-regulated when the synthesis of new receptors is blocked. In our studies, we do not observe an analogous IgM^{ow}B220⁺ B cell upon E2A disruption indicating that E2A-deficient B cells die before a reduction in BCR expression can be observed. Finally, E2A protects transformed pre-B cells from apoptosis in a manner which is not dependent on BCR expression as these cells are at a developmental stage in

which they have not fully rearranged their Ig genes. These lines of evidence strongly suggest that E2A promotes B cell survival through a novel mechanism which is independent of its role in regulating B cell Ag receptor expression.

We also observed that *E2A^{flox}* B cells failed to engraft as efficiently as wild-type B cells in a competitive adoptive transfer. The *E2A^f* allele is hypomorphic due to the presence of the PGKneo cassette 3' of the *E2A* gene. Therefore, *E2A^{flox}* mice exhibit a

phenotype that is intermediate between wild-type and $E2A^{+/-}$. It is possible that decreased E2A expression may contribute to differences in initial B cell engraftment. Additionally, differences in the genetic backgrounds of $E2A^{fl/fl}$ donor mice, which are mixed between B6, 129, and SJL, and wild-type donor mice, which are B6 CD45 congenic, may also contribute to the observed differences in B cell engraftment efficiency.

The specific mechanism through which E2A may regulate B cell survival is currently unclear. It has been shown that Id3 overexpression in progenitor B cells leads to growth arrest and death via a caspase-2-dependent pathway (19). It was demonstrated that Id3-mediated cell death occurred in a p53-independent manner and could not be rescued by a Bcl-2 transgene. Currently, none of the genes that are known to be directly regulated by E2A can fully account for the apparent antiapoptotic activity of E2A in our pre-B cell lines. However, $E2A^{-/-}$ pre-B cell lines will serve as a useful tool to dissect the molecular pathways through which E2A regulates B cell survival.

The finding that E2A is required for the survival of mature B cells is notable. We propose that E2A promotes B cell survival by a common mechanism in progenitor and mature B cells. The level of E2A protein declines significantly as developing B lymphocytes mature and enter the resting peripheral B cell pool but increases rapidly upon B cell activation (34–36). It is well-established that pIpC can transiently activate resting B cells in T cell-independent manner; therefore, it is possible E2A may promote the survival of recently activated B cells. Thus, it will be particularly interesting to determine whether E2A is involved in promoting the survival of resting versus activated peripheral B cells.

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Disclosures

The authors have no financial conflict of interest.

References

- Smith, E., and M. Sigvardsson. 2004. The roles of transcription factors in B lymphocyte commitment, development, and transformation. *J. Leukocyte Biol.* 75: 973–981.
- Singh, H., K. L. Medina, and J. M. Pongubala. 2005. Contingent gene regulatory networks and B cell fate specification. *Proc. Natl. Acad. Sci. USA* 102: 4949–4953.
- Medina, K. L., J. M. Pongubala, K. L. Reddy, D. W. Lancki, R. Dekoter, M. Kieslinger, R. Grosschedl, and H. Singh. 2004. Assembling a gene regulatory network for specification of the B cell fate. *Dev. Cell* 7: 607–617.
- Zhuang, Y., P. Soriano, and H. Weintraub. 1994. The helix-loop-helix gene *E2A* is required for B cell formation. *Cell* 79: 875–884.
- Bain, G., E. C. Maandag, D. J. Izon, D. Amsen, A. M. Kruisbeek, B. C. Weintraub, I. Krop, M. S. Schlissel, A. J. Feeney, M. van Roon, et al. 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* 79: 885–892.
- Urbanek, P., Z. Q. Wang, I. Fetka, E. F. Wagner, and M. Busslinger. 1994. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* 79: 901–912.
- O'Riordan, M., and R. Grosschedl. 1999. Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity* 11: 21–31.
- Ikawa, T., H. Kawamoto, L. Y. Wright, and C. Murre. 2004. Long-term cultured E2A-deficient hematopoietic progenitor cells are pluripotent. *Immunity* 20: 349–360.
- Mikkola, I., B. Heavey, M. Horcher, and M. Busslinger. 2002. Reversion of B cell commitment upon loss of Pax5 expression. *Science* 297: 110–113.
- Bain, G., E. C. Robanus Maandag, H. P. te Riele, A. J. Feeney, A. Sheehy, M. Schlissel, S. A. Shinton, R. R. Hardy, and C. Murre. 1997. Both E12 and E47 allow commitment to the B cell lineage. *Immunity* 6: 145–154.
- Zhuang, Y., P. Cheng, and H. Weintraub. 1996. B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. *Mol. Cell. Biol.* 16: 2898–2905.
- Kee, B. L., and C. Murre. 1998. Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J. Exp. Med.* 188: 699–713.
- Lazorchak, A. S., M. S. Schlissel, and Y. Zhuang. 2006. E2A and IRF-4/Pip promote chromatin modification and transcription of the immunoglobulin κ locus in pre-B cells. *Mol. Cell. Biol.* 26: 810–821.
- Greenbaum, S., and Y. Zhuang. 2002. Identification of E2A target genes in B lymphocyte development by using a gene tagging-based chromatin immunoprecipitation system. *Proc. Natl. Acad. Sci. USA* 99: 15030–15035.
- Greenbaum, S., A. S. Lazorchak, and Y. Zhuang. 2004. Differential functions for the transcription factor E2A in positive and negative gene regulation in pre-B lymphocytes. *J. Biol. Chem.* 279: 45028–45035.
- Kim, D., X. C. Peng, and X. H. Sun. 1999. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Mol. Cell. Biol.* 19: 8240–8253.
- Kee, B. L., R. R. Rivera, and C. Murre. 2001. Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF- β . *Nat. Immunol.* 2: 242–247.
- Qi, Z., and X. H. Sun. 2004. Hyperresponse to T-cell receptor signaling and apoptosis of Id1 transgenic thymocytes. *Mol. Cell. Biol.* 24: 7313–7323.
- Kee, B. L. 2005. Id3 induces growth arrest and caspase-2-dependent apoptosis in B lymphocyte progenitors. *J. Immunol.* 175: 4518–4527.
- Yates, P. R., G. T. Atherton, R. W. Deed, J. D. Norton, and A. D. Sharrocks. 1999. Id helix-loop-helix proteins inhibit nucleoprotein complex formation by the TCF ETS-domain transcription factors. *EMBO J.* 18: 968–976.
- Roberts, E. C., R. W. Deed, T. Inoue, J. D. Norton, and A. D. Sharrocks. 2001. Id helix-loop-helix proteins antagonize pax transcription factor activity by inhibiting DNA binding. *Mol. Cell. Biol.* 21: 524–533.
- Kuhn, R., F. Schwenk, M. Aguet, and K. Rajewsky. 1995. Inducible gene targeting in mice. *Science* 269: 1427–1429.
- Pan, L., J. Hanrahan, J. Li, L. P. Hale, and Y. Zhuang. 2002. An analysis of T cell intrinsic roles of E2A by conditional gene disruption in the thymus. *J. Immunol.* 168: 3923–3932.
- Seet, C. S., R. L. Brumbaugh, and B. L. Kee. 2004. Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. *J. Exp. Med.* 199: 1689–1700.
- Choi, J. K., C. P. Shen, H. S. Radomska, L. A. Eckhardt, and T. Kadesch. 1996. E47 activates the Ig-heavy chain and TdT loci in non-B cells. *EMBO J.* 15: 5014–5021.
- Muljo, S. A., and M. S. Schlissel. 2003. A small molecule Abl kinase inhibitor induces differentiation of Abelson virus-transformed pre-B cell lines. *Nat. Immunol.* 4: 31–37.
- Wang, J., Q. Lin, H. Langston, and M. D. Cooper. 1995. Resident bone marrow macrophages produce type 1 interferons that can selectively inhibit interleukin-7-driven growth of B lineage cells. *Immunity* 3: 475–484.
- Prabhu, S., A. Ignatova, S. T. Park, and X. H. Sun. 1997. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. *Mol. Cell. Biol.* 17: 5888–5896.
- Park, S. T., G. P. Nolan, and X. H. Sun. 1999. Growth inhibition and apoptosis due to restoration of E2A activity in T cell acute lymphoblastic leukemia cells. *J. Exp. Med.* 189: 501–508.
- Engel, I., and C. Murre. 1999. Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. *Proc. Natl. Acad. Sci. USA* 96: 996–1001.
- Noronha, E. J., K. H. Sterling, and K. L. Calame. 2003. Increased expression of Bcl-x_L and c-Myc is associated with transformation by Abelson murine leukemia virus. *J. Biol. Chem.* 278: 50915–50922.
- Kraus, M., M. B. Alimzhanov, N. Rajewsky, and K. Rajewsky. 2004. Survival of resting mature B lymphocytes depends on BCR signaling via the Iga/β heterodimer. *Cell* 117: 787–800.
- Lam, K. P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90: 1073–1083.
- Zhuang, Y., A. Jackson, L. Pan, K. Shen, and M. Dai. 2004. Regulation of E2A gene expression in B-lymphocyte development. *Mol. Immunol.* 40: 1165–1177.
- Herblot, S., P. D. Aplan, and T. Hoang. 2002. Gradient of E2A activity in B-cell development. *Mol. Cell. Biol.* 22: 886–900.
- Quong, M. W., D. P. Harris, S. L. Swain, and C. Murre. 1999. E2A activity is induced during B-cell activation to promote immunoglobulin class switch recombination. *EMBO J.* 18: 6307–6318.