

B-cell development fails in the absence of the *Pbx1* proto-oncogene

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***Pbx1*, a homeodomain transcription factor that was originally identified as the product of a proto-oncogene in acute pre-B-cell leukemia, is a global regulator of embryonic development. However, embryonic lethality in its absence has prevented an assessment of its role in B-cell development. Here, using *Rag1*-deficient blastocyst complementation assays, we demonstrate that *Pbx1* null embryonic stem (ES) cells fail to generate common lymphoid progenitors (CLPs) resulting in**

a complete lack of B and NK cells, and a partial impairment of T-cell development in chimeric mice. A critical role for *Pbx1* was confirmed by rescue of B-cell development from CLPs following restoration of its expression in *Pbx1*-deficient ES cells. In adoptive transfer experiments, B-cell development from *Pbx1*-deficient fetal liver cells was also severely compromised, but not erased, since transient B lymphopoiesis was detected in *Rag*-deficient recipients. Conditional inactivation

of *Pbx1* in pro-B (CD19⁺) cells and thereafter revealed that *Pbx1* is not necessary for B-cell development to proceed from the pro-B-cell stage. Thus, *Pbx1* critically functions at a stage between hematopoietic stem cell development and B-cell commitment and, therefore, is one of the earliest-acting transcription factors that regulate de novo B-lineage lymphopoiesis. (Blood. 2007;109:4191-4199)

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Introduction

Hematopoiesis is a dynamic life-long process that is initiated and maintained by hematopoietic stem cells (HSCs), which differentiate through a series of committed progenitors to generate mature blood elements of diverse hematopoietic lineages. The molecular programs that are required for generation of specific progenitors and that orchestrate their critical transitions to more developmentally restricted progeny remain poorly defined. Some of the candidate factors that serve these master regulatory roles are transcription factors that are activated by chromosomal translocations in acute leukemia, which result in dramatic perturbations of normal hematopoiesis. In support of this, the translocated genes *AML1* (*Runx1/CBF*), *LMO2* (*Rbtm2, Tig2*), *SCL/TAL1*, *TANI/Notch1*, and *MLL1* have all been shown to be critical for embryonic hematopoiesis,^{1,2} their loss blocking or significantly perturbing hematopoiesis through a variety of pathways.

Pbx1 is also a proto-oncogene with a role in hematopoiesis. It was originally discovered at the site of chromosomal translocations in pre-B-cell acute leukemia^{3,4} and codes for a TALE (3-amino acid loop extension) class homeodomain transcription factor, which is a component of hetero-oligomeric protein complexes that regulate developmental gene expression. Lack of *Pbx1* results in embryonic lethality at E15 and is associated with multiple patterning malformations, including homeotic transformations, and hypoplasia or aplasia of most internal organs.⁵⁻⁸ In addition, reduced numbers and impaired functions of committed hematopoietic progenitors in the fetal liver result in inadequate maintenance of definitive hematopoiesis and severe anemia.⁹ Finally, several of the embryonic defects partially phenocopy those associated with loss of various Hox and orphan homeodomain proteins, indicating an in

vivo role for *Pbx1* in multiple transcriptional programs as a DNA-binding cofactor for a large subset of homeodomain transcription factors, some of which are implicated in the regulation of hematopoietic progenitor expansion.

In leukemia, *PBX1* mutations are restricted to a subset with pre-B-cell features and result in in-frame fusions with the *E2A* gene,^{3,4} which codes for critical regulators of B-lineage development.^{10,11} These features suggest that *Pbx1* may be required for normal B-lineage lymphopoiesis. Because *Pbx1* null embryos die at E15, we have used *Rag1*-deficient chimera analyses and cell transfer studies to evaluate this possibility. We demonstrate here that *Pbx1* null embryonic stem (ES) cells fail to generate common lymphoid progenitors (CLPs), resulting in the complete lack of B and natural killer (NK) cells in the chimeric mice and a partial impairment of T-cell development. B-cell development from null fetal liver cells in adoptive *Rag*-deficient recipients is similarly compromised, although B and T cells are partially reconstituted in a subset of mice. Because conditional inactivation of *Pbx1* at the pro-B-cell stage and thereafter does not prevent B-cell development, our studies indicate that *Pbx1* is required very early in B-cell commitment and acts at a stage between the development of HSCs and the origin of CLPs.

Materials and methods

Animals

Four- to 8-week-old C57BL/6 *Rag1*^{-/-} mice (Jackson Laboratories, Bar Harbor, ME), C57BL/6 *Rag2*^{-/-} mice (Taconic, Hudson, NY), and

Submitted October 25, 2006; accepted January 15, 2007. Prepublished online as *Blood* First Edition Paper, January 23, 2007; DOI 10.1182/blood-2006-10-054213.

The online version of the article contains a data supplement.

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C57BL/6 (Stanford Animal Facility) were used for experiments. All animals were maintained according to Public Health Service policy for Humane Care and Use of Laboratory Animals.

Antibodies

The following fluorochrome-conjugated antibodies were produced in our laboratories: M1/70 (anti-Mac-1/CD11b), 8C5 (anti-Gr-1), 6B2 (anti-B220), 145-2C11 (anti-CD3), GK1.5 (anti-CD4), 53-6.7 (anti-CD8), A7R34 (anti-IL-7R/CD127), 2B8 (anti-c-Kit/CD117), 3C11 (anti-c-Kit/CD117), E13-161-7 (anti-Sca-1), 331 (anti-IgM), 11-26 (anti-IgD), 6C31BP1 (anti-BP-1), and B3-B4 (CD23) (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Monoclonal antibodies against Ly9.1 (CD229), B220, CD19, CD43, CD24, CD5, CD21, IgM (Igh-6a), IgM (Igh-6b), TCR β , CD4, CD8, NK1.1, CD11b, CD11c, and Gr-1 and isotype controls were purchased from BD Pharmingen (San Diego, CA). For visualization of biotinylated antibodies, streptavidin-conjugated FITC, PE, Cy5.5-PE, and Alexa 594 were used (BD Pharmingen). The lineage cocktail included unconjugated rat antibodies specific for CD3 (KT31.1), CD4 (GK1.5), CD8 (53-6.7) B220 (6B2), Mac-1 (M1/70), Gr-1 (bC5), and TER119, which were visualized with Cy5-PE-conjugated goat anti-rabbit IgG polyclonal antibodies (Caltag, Burlingame, CA).

Targeting vector construction and generation of *Pbx1*^{-/-} ES cells

The establishment of *Pbx1*^{+/-} ES cell lines and mice is described elsewhere.⁸ For the generation of *Pbx1*^{-/-} ES cell lines, the remaining wild-type allele was floxed by transfection of *Pbx1*^{+/-} ES cells with the targeting construct illustrated in Figure S1. Correct gene targeting in 6 of 400 clones was confirmed by Southern blot analysis (3' external probe on *Ssp1*-digested DNA). The floxed exon 3 was removed by Cre recombination. Floxed (6567 bp) and Cre-deleted (3268 bp) *Pbx1* alleles were distinguished by long-distance polymerase chain reaction (PCR) analysis (primers: 5'-TGCTACTTCCATTGTGTCACGTCCTGCACGA-3' and 5'CCATCAGAAGCGGTGCTAGAGCTTGCGG-3'). Homozygous mutation of *Pbx1* was confirmed by Southern blot analysis. Two homozygous *Pbx1* mutant ES cell lines (A11 and D2) were used for *Rag1*-deficient blastocyst complementation assays and yielded similar results.

Restoration of *Pbx1* expression in *Pbx1*^{-/-} ES cells

Pbx1b cDNA was introduced into *Pbx1*^{-/-} ES cells (clones A11 and D2) using a lentiviral vector to restore *Pbx1* expression (Figure S2). Lentivirus-expressing *Pbx1b* under control of the *PGK* promoter was produced using a conditional packaging system¹² and 293FT cells (Invitrogen, Carlsbad, CA) as previously described¹³ and concentrated 100-fold. Single-cell suspensions of 1×10^5 ES cells were infected overnight with the lentivirus in a volume of 500 μ L.¹⁴

Rag1-deficient blastocyst complementation assay

Rag1^{-/-} blastocysts (E3.5) were microinjected with either of 2 *Pbx1*^{-/-} ES cell clones, the parental *Pbx1*^{+/-} ES cells, or the original *Pbx1*^{+/+} ES cells using standard procedures, and then transferred into pseudopregnant recipients. Peripheral blood from the chimeric offspring was analyzed for reconstitution of mature lymphocytes by flow cytometry. Fluorescence-activated cell sorting (FACS)-purified Ly9.1⁺ cells were analyzed for the presence of wild-type and mutant *Pbx1* alleles by PCR.

Fetal liver reconstitution of lymphoid system in *Rag*-deficient mice

Single-cell suspensions of 1×10^6 whole fetal liver mononuclear cells harvested from E14.5 *Pbx1*^{-/-} donor embryos⁸ were injected intravenously into the lethally irradiated (850 cGy, using a 200 kV x-ray source) 8-week-old male *Rag2*^{-/-} recipient mice. Peripheral blood from the chimeric mice was analyzed for B and T cells, 6, 12, and 18 weeks after transplantation.

Generation of *Pbx1*^{fl/fl}-*CD19Cre* mice

Pbx1^{+/-} mice were bred with *CD19Cre* knock-in mice expressing Cre recombinase under control of the endogenous *CD19* promoter¹⁵ or with

mice containing a floxed *Pbx1* gene to obtain *Pbx1*^{+/-}-*CD19Cre* and *Pbx1*^{fl/fl}-offspring, respectively, which were then interbred to obtain *Pbx1*^{fl/fl}-*CD19Cre* mice and control littermates. *Pbx1* and *Cre* genotypes were determined by PCR.

Southern blot analysis

Genotype analysis was performed on DNA extracted from targeted ES cells or FACS-sorted B (CD19⁺) cells. Following digestion with *SspI*, DNAs were subjected to Southern blot analysis using the *Pbx1* 3' external probe.⁸

Immunocytochemistry

Cytospin preparations of 1×10^4 flow-sorted CLPs were fixed in cold acetone, blocked, and permeabilized (10% normal goat serum, 0.5% Triton-X 100) at room temperature, and incubated overnight with mouse anti-Pbx1b antibody at 4°C. For visualization, Texas red-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used. The cells were mounted in a medium containing DAPI (Vector Laboratories, Burlingame, CA). Spleens were processed for cryosections and stained with Texas red-conjugated anti-B220 antibody. Microscopic images were obtained using an Eclipse E800M microscope (Nikon, Melville, NY) with a SPOT RT digital camera and acquisition software (Diagnostic Instruments, Sterling Heights, MI) with a final magnification of 600 (objective, 20 \times /0.45 numerical aperture). Images were processed using Photoshop 7 (Adobe Systems, San Jose, CA).

Western blot analysis

Double FACS-sorted pro-B (B220⁺CD43⁺), pre-B (B220⁺CD43⁻), and mature B cells (B220⁺IgM⁺) were lysed, electrophoresed, and subjected to immunoblotting using monoclonal antibodies specific for Pbx1a, Pbx1b, or β -actin as described previously.¹⁶ The presence of Pbx1 isoforms was also analyzed in *Pbx1*^{+/+}, *Pbx1*^{+/-}, *Pbx1*^{-/-}, and *Pbx1*^{-/-} (rescued) ES cells and *Pbx1*^{+/+} and *Pbx1*^{-/-} mouse embryonic fibroblasts (MEFs).

High-dimensional 11-color FACS analysis

Peripheral blood (7-10 drops) was collected in 500 μ L 10 mM EDTA in phosphate-buffered saline (PBS) and incubated with 1 mL 2% dextran in PBS, for 2 hours at 37°C. The supernatant was then transferred to a fresh tube for red cell lysis. Bone marrow cells were harvested by flushing femurs and tibias with PBS. Spleen, thymus, and lymph nodes were harvested in PBS and pressed through nylon mesh. To obtain peritoneal cells, PBS was injected into the peritoneal cavity of a recently killed mouse, and the fluid was withdrawn. The single-cell preparations were incubated with 150 mM ammonium chloride for 5 minutes for red cell lysis, washed, and resuspended in PBS containing 2% fetal calf serum. Cells were stained with cocktails of fluorochrome-conjugated antibodies.¹⁷ Propidium iodide was added to all samples to exclude dead cells. High-dimensional FACS data were collected on a modified triple-laser FACS instrument. FlowJo (Tree Star, San Carlos, CA) software was used for subsequent analysis.

Real-time PCR analysis

RNA was prepared from double FACS-sorted cell populations using TRIzol reagent (Invitrogen), treated with DNase, and converted to single-strand cDNA using the SuperScript II kit (Invitrogen). cDNA equivalent to 200 cells/reaction were mixed with FastStart Master SYBR Green polymerase mix (Roche, Indianapolis, IN) and primers (Pbx1a: ACAATAAAGCAGGC-CCAAGTT and ATACATGACTTGCCCAACAGC, Pbx1b: GAGGTTG-CAGGATGCTACTAC and ACTGCCAGGGCCTTCTGTA, Pbx1: AG-GACATCGGGGACATTTTAC and CATTAAACAAGGCAGGCTTCA), and real-time PCR was performed using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Results of triplicate analyses were normalized based on expression of 18S RNA.

Assessment of immune response

Fetal liver chimeras were immunized by intraperitoneal injection of 100 μ g DNP-conjugated keyhole limpet hemocyanin (DNP-KLH) with complete

Freund adjuvant. Preimmune and postimmune (14 days) sera were collected, and the titers of DNP-specific IgM and IgG measured by enzyme-linked immunosorbent assay (ELISA) using DNP-BSA as capture antigen.

Results

Pbx1 is expressed at the earliest stages of B-cell development

Two alternative isoforms of Pbx1, designated Pbx1a (47 kDa) and Pbx1b (38 kDa), arise from differential splicing of the *Pbx1* transcript. Pbx1b, but not Pbx1a, is present in the mesenchyme throughout the mid-gestation embryo¹⁸ and at E11.5 in the aorta-gonad-mesonephros (AGM), the primary site for intraembryonic hematopoiesis. Notably, Pbx1b is present in committed myeloid progenitors and HSCs of the fetal liver at E14.5 as well as postnatally in the adult bone marrow.⁹

We investigated Pbx1 expression during B-cell development starting with CLPs, isolated by flow cytometry from adult bone marrow as Lin⁻CD127⁺c-Kit^{int}Sca-1^{int} cells.¹⁹ Immunocytochemical analysis showed that Pbx1b was present in CLPs (Figure 1A). Western blot analysis of FACS-purified B-cell progenitors from the adult bone marrow revealed Pbx1b in pro-B (B220⁺CD43⁺), pre-B (B220⁺CD43⁻), and mature B (B220⁺IgM⁺) cells (Figure 1B). Quantitative reverse transcription PCR (RT-PCR) analysis of purified progenitors and B-cell precursors showed that *Pbx1* expression levels generally decreased as cells progressed toward

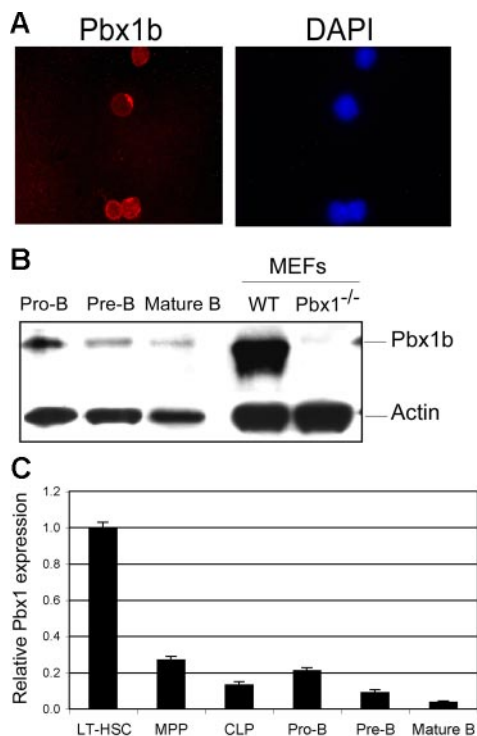


Figure 1. Pbx1 is present throughout B-cell development. (A) FACS-purified CLPs (Lin⁻CD127^{int}c-kit^{int}Sca-1⁺) were analyzed by immunofluorescence using a Pbx1b-specific monoclonal antibody (left panel). Red staining (Texas red) that was confined to the nucleus (blue staining right panel, DAPI) indicates the presence of Pbx1b. (B) Western blot analysis of double-sorted B-cell populations shows presence of Pbx1b protein in pro-B (B220⁺CD43⁺), pre-B (B220⁺CD43⁻), and mature B (B220⁺IgM⁺) cells that progressively decreases with increasing maturation. *Pbx1*^{+/+} and *Pbx1*^{-/-} MEFs were used as positive and negative controls, respectively. Faint cross-reactive band in last lane is not representative of residual Pbx1b protein. (C) *Pbx1* transcripts were assessed in purified progenitors and B-cell populations by quantitative RT-PCR analysis. Relative levels of expression are compared to long-term HSCs. Error bars represent SEMs for triplicate analyses.

maturity (Figure 1C). Pbx1a, in contrast, was not detected in any of these populations. Thus, all cells with B-lineage differentiation potential (HSCs, CLPs, and pro-B, pre-B, and mature B cells) selectively express Pbx1b, which is significantly down-regulated with maturation.

Failure of in situ B-cell development in *Pbx1*^{-/-} *Rag1*^{-/-} blastocyst-complemented chimeric mice

The embryonic lethality of *Pbx1*^{-/-} embryos at E15 prior to establishment of a lymphoid system prevented an analysis of their in situ lymphoid development. Therefore, the role of *Pbx1* in successive stages of the B-cell developmental pathway was examined in chimeric mice using the *Rag1*-deficient blastocyst complementation assay.^{20,21} Initially, *Pbx1*^{-/-} ES cell lines were generated by gene targeting (Figure S1 presents targeting strategy), and the lack of Pbx1 protein in appropriately targeted ES clones was confirmed by Western blot analysis (Figure 2A). Somatic chimeras were then generated by injecting *Pbx1*^{-/-} (or control) ES cells into *Rag1*-deficient (*Rag1*^{-/-}) blastocysts. Analysis of the offspring mice revealed that *Pbx1*^{+/+} and *Pbx1*^{+/-} ES cells contributed to high levels (85%-95%) of coat chimerism in most of the progeny, whereas only low levels of chimerism (10%-15%) were observed in a minority of mice resulting from *Pbx1*^{-/-} ES cell blastocyst injections, likely reflecting the essential role of *Pbx1* in embryogenesis. Nevertheless, a subset of *Pbx1*^{-/-} *Rag1*^{-/-} coat color chimeras (20%) contained *Pbx1* null cells in the peripheral blood as detected by PCR analysis (Figure 2B).

Flow cytometric analysis showed that chimeric mice generated from blastocysts injected with *Pbx1*^{+/+} or *Pbx1*^{+/-} ES cells contained mature B (IgM⁺B220⁺) and T (TCRαβ⁺) lymphocytes in the peripheral blood. Since *Rag1*^{-/-} mice do not produce mature lymphocytes (Figure 2C) due to a deficiency in V(D)J recombination,²² circulating B and T cells in these somatic chimeras must have exclusively derived from the microinjected ES cells indicating that they complemented the *Rag1* deficiency in lymphopoiesis. The chimeras generated from *Pbx1*^{-/-} ES cells displayed a complete absence of B cells (IgM⁺ or IgM⁺B220⁺) in the peripheral blood (Figure 2C) demonstrating that *Pbx1* was required for B-lineage lymphopoiesis. In contrast to the lack of circulating B cells, TCRαβ⁺ cells comprised of mature CD4 and CD8 single-positive T cells (Figure 2C) were present in the blood of *Pbx1*^{-/-} *Rag1*^{-/-} chimeric mice suggesting a differential requirement of *Pbx1* for B- versus T-lineage development.

The absence of B cells in the peripheral blood prompted an analysis for abnormalities in the B-cell compartments of secondary lymphoid organs in *Pbx1*^{-/-} *Rag1*^{-/-} chimeric mice. Expression of the Ly9.1 alloantigen was used to distinguish cells derived from recipient blastocyst (C57BL/6 background) versus microinjected ES cells (129 background). First described as an alloantigenic marker of lymphocyte differentiation,^{23,24} Ly9.1 is a surface glycoprotein (Lgp-100, CD229) that is expressed on most thymocytes, peripheral T and B cells, bone marrow lymphoid cells, and hematopoietic progenitors. In chimeras generated from *Pbx1*^{-/-} ES cells, no B220⁺ cells were detected in the Ly9.1⁺ fraction of the spleen and lymph node (Figure 3A), demonstrating the absence of *Pbx1*^{-/-} conventional (B2) B lymphocytes in these secondary lymphoid locations. Analysis of the peritoneal cells revealed an absence of detectable B2 as well as B1 cells (Figure 3A). Thus, the peripheral lymphoid tissues were completely devoid of mature B220⁺ B cells representative of either the B1 or B2 lineages.

The bone marrow was investigated to localize the stage where B-cell development was blocked by *Pbx1* deficiency. B-cell

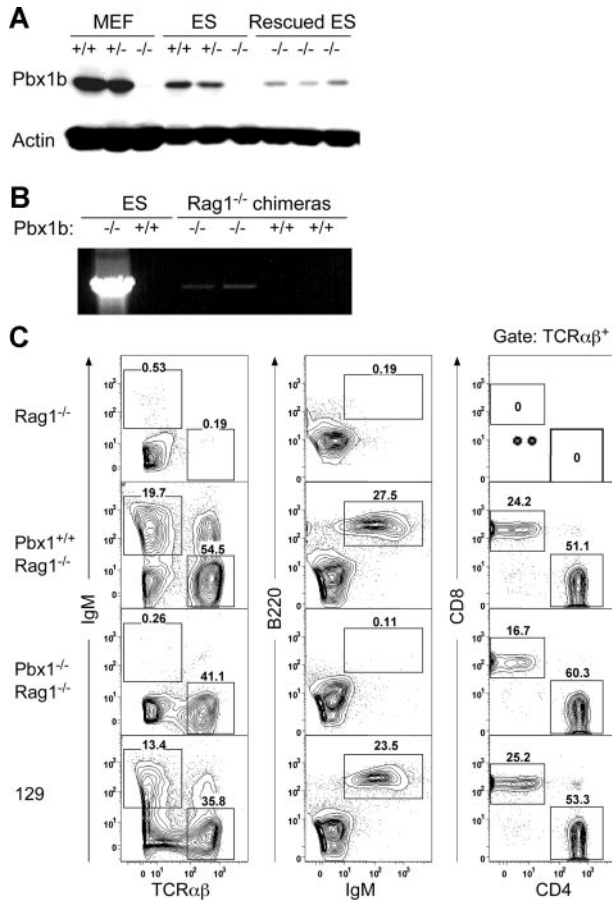


Figure 2. Absence of B lymphocytes in the peripheral blood of *Pbx1*^{-/-} *Rag1*^{-/-} blastocyst-complemented mice. (A) Western blot analysis of ES cells and MEFs (genotypes indicated at the top of the panel) demonstrates the absence of Pbx1b protein in homozygous null cells and the presence of exogenous Pbx1b in *Pbx1*^{-/-} ES cells that were transfected with a lentiviral vector expressing *Pbx1b* cDNA under the control of the *PGK* promoter. Actin was used as control for protein loading. (B) PCR analysis confirmed the presence of *Pbx1*^{-/-} ES cell-derived cells in the blood of *Pbx1*^{-/-} *Rag1*^{-/-} chimeric mice. (C) FACS analysis using B and T cell-specific fluorochrome-conjugated antibodies was performed on cells from the peripheral blood mononuclear cells (PBMCs) of chimeric and control mice. No IgM⁺ or IgM⁺B220⁺ B cells were detected in the *Pbx1*^{-/-} *Rag1*^{-/-} adult chimeric mice (third row panels). Conversely, TCRαβ⁺ CD4 and CD8 single-positive cells were detected in the blood of *Pbx1*^{-/-} *Rag1*^{-/-} mice. The ratio of CD4⁺CD8⁻ to CD4⁻CD8⁺ cells was not significantly altered. *Pbx1*^{+/+} *Rag1*^{-/-} chimeric control mice had normal numbers of B and T cells in their PBMCs (second row panels). Analyses from age-matched *Rag1*^{-/-} and 129 mice are also shown.

progenitor analysis (Hardy profile)²⁵ revealed that Hardy fractions A through F representing all stages of B-cell development from pro-B through mature B cells were completely missing in the Ly9.1⁺ bone marrow fraction of *Pbx1*^{-/-} *Rag1*^{-/-} chimeric mice (Figure 3B). This contrasted with the normal course of B-lymphocyte development that was present in the bone marrows of chimeric mice generated from *Pbx1*^{+/+} ES cells. These observations indicate that in situ B-lineage lymphopoiesis fails in *Rag1*^{-/-} chimeras in the absence of *Pbx1* and is blocked at an early stage of development prior to Hardy fraction A.

Development of CLPs is dependent on *Pbx1*

B cells differentiate from CLPs.¹⁹ Because *Pbx1*^{-/-} ES cells did not contribute to the earliest stages of B-cell development in the bone marrow, the chimeric mice were analyzed by FACS for potential alterations of CLPs. Despite significant chimerism in secondary lymphoid tissues and the bone marrow of *Pbx1*^{-/-}

Rag1^{-/-} chimeric mice, no Ly9.1⁺ CLPs were detected in their bone marrows (Figure 3C). By comparison, a substantial proportion (up to 39% of total) of CLPs in the bone marrows of *Pbx1*^{+/+} *Rag1*^{-/-} mice was ES cell-derived (Ly9.1⁺). We further analyzed upstream of CLPs for the presence of HSCs. *Pbx1*^{-/-} *Rag1*^{-/-} chimeras produced donor-derived (Ly9.1⁺) HSCs; however, the percentage was lower compared to the control chimeras (Figure 3D). Therefore, development of CLPs in *Rag1*^{-/-} chimeras requires *Pbx1*, and the failure of B-lineage lymphopoiesis in the absence of *Pbx1* is likely due to a lack of upstream CLP.

T-lineage developmental potential is partially preserved in the absence of *Pbx1*

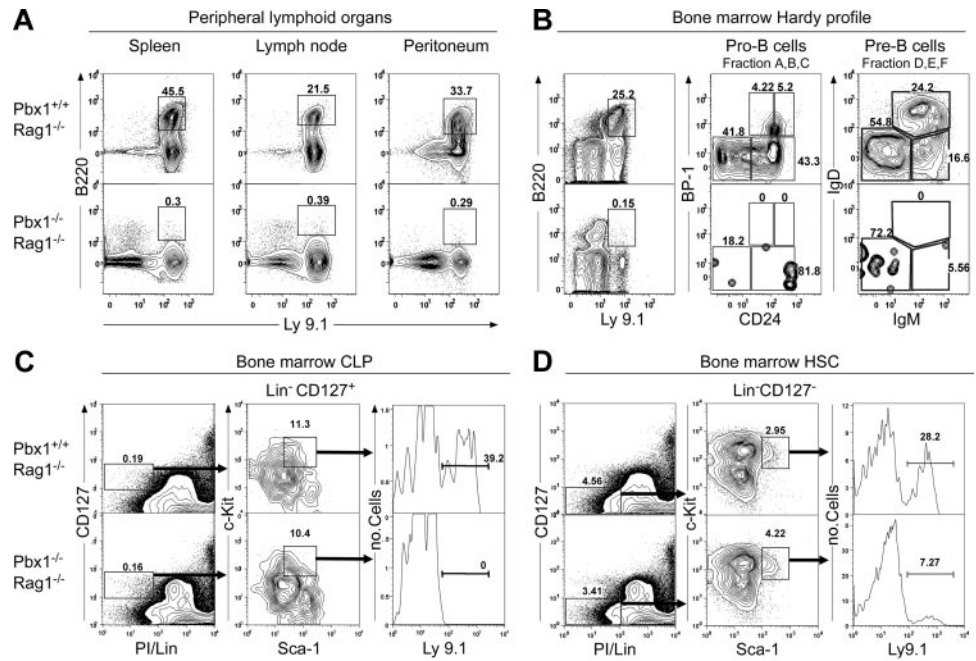
CLPs have been reported to differentiate into lymphocytes of the T-, B-, and NK-cell lineages.¹⁹ Consistent with the absence of CLPs and B lymphocytes, very few *Pbx1*^{-/-} ES cell-derived NK1.1⁺ cells were detected in the *Pbx1*^{-/-} *Rag1*^{-/-} chimeric mice (Figure 4A). Nonetheless, despite the absence of detectable CLPs in *Pbx1*^{-/-} *Rag1*^{-/-} chimeric mice, substantial numbers of TCRαβ⁺ T cells were present in the peripheral blood (Figure 2C) and secondary lymphoid tissues (data not shown) at 5 to 20 weeks of age. The few NK1.1⁺ cells detected in the Ly9.1⁺ fraction of these mice were exclusively NKT cells (NK1.1⁺TCRαβ⁺; Figure 4A). Furthermore, the ratios of CD4⁺ to CD8⁺ cells appeared to be normal in *Pbx1*^{-/-} *Rag1*^{-/-} chimeric mice, similar to those in the control *Pbx1*^{+/+} ES-complemented mice.

Transplantation experiments were performed to further interrogate the lymphoid differentiation potential of *Pbx1*-deficient bone marrow progenitors. Lin⁻ cells (Ly9.1⁺) were purified by flow cytometry from chimeric mice and transplanted into lethally irradiated C57BL/6 (Ly9.2) recipient mice along with a rescue dose of *Rag1*-deficient bone marrow cells. *Pbx1*^{-/-} cells engrafted very poorly as evidenced by the low percentage of Ly9.1⁺ cells in the recipient bone marrow when compared to the high levels of Ly9.1⁺ cells present in the control mice given transplants with equal numbers of *Pbx1*^{+/+} cells (Figure 4B). *Pbx1*^{-/-} cells did not contribute to development of B cells; however, a low level of TCRαβ⁺ T cells was detected in the peripheral blood of the transplant recipients (Figure 4B). Similar reduced levels of chimerism and lack of B cells or their early progenitors were observed in all lymphoid organs and the bone marrow of the recipients of transplants with *Pbx1*^{-/-} cells (data not shown). Therefore, *Pbx1* deficiency severely compromises hematopoietic differentiation potential along the B- and NK-cell lineages initiating from CLPs, but permits at least partial long-term T-lineage lymphopoiesis.

B-cell development is restored by constitutive expression of *Pbx1b*

Rescue of the *Pbx1* lymphoid deficiency in chimeric mice was attempted to confirm the specific role of *Pbx1b* in CLPs and B-lineage development. *Pbx1*^{-/-} ES cells were transduced with a lentiviral vector constitutively expressing *Pbx1b* cDNA under control of the *PGK* promoter (Figure S2). Clones (*Pbx1*^{-/-R}) with the highest level of re-expressed Pbx1b protein by Western blot analysis (Figure 2A) were selected for injection into *Rag1*^{-/-} blastocysts. FACS analysis of the resulting chimeric mice showed the presence of IgM⁺ and B220⁺ cells in the peripheral blood at levels comparable to the control *Pbx1*^{+/+} *Rag1*^{-/-} chimeric mice (Figure 5A), indicating successful rescue of the B-cell deficiency observed in *Pbx1*^{-/-} *Rag1*^{-/-} chimeras. Donor ES cell-derived B220⁺ cells were also present in the spleen, lymph nodes, and peritoneal cavity, and complete restoration of the B-cell differentiation pathway from Hardy fractions A through F was observed

Figure 3. Absence of B cells in the secondary lymphoid tissues and impaired B-cell development in the bone marrow of *Pbx1*^{-/-}*Rag1*^{-/-} chimeric mice. (A) FACS analysis was performed on cells harvested from the spleen, lymph node, and peritoneal cavity of chimeric mice. Staining with anti-B220 (B cells) and anti-Ly9.1 (donor) antibodies showed that all sites in *Pbx1*^{-/-}*Rag1*^{-/-} mice were devoid of donor-derived B cells (Ly9.1⁺B220⁺). (B) Hardy profile analysis²⁵ was performed on bone marrow cells of chimeric mice. Donor-derived (Ly9.1⁺) cells from fractions A through C (B220⁺CD43⁺) and D through F (B220⁺CD43⁻) were completely absent in the bone marrow of the *Pbx1*^{-/-}*Rag1*^{-/-} mice. (C) FACS analysis of bone marrow cells for CLPs¹⁹ revealed the absence of Ly9.1⁺ CLPs in *Pbx1*^{-/-}*Rag1*^{-/-} chimeras. (D) FACS analysis of bone marrow cells for HSCd revealed the presence of Ly9.1⁺ HSCs in *Pbx1*^{-/-}*Rag1*^{-/-} chimeras.



in the bone marrow (not shown). Similarly, reintroduction of *Pbx1b* restored the loss of CLPs in the bone marrow (Figure 5B). These data demonstrate phenotypic rescue of adult B lymphopoiesis and further confirm a critical role for *Pbx1b* in specification of the B-lymphoid lineage.

Transient B-cell development occurs when *Pbx1*-deficient fetal liver cells are transferred to *Rag*-deficient mice

Pbx1^{-/-} embryos die by E15 with a significant defect in myeloid/erythroid development.⁹ The fetal liver is small (about 20% of normal size) but yields cells that support long-term (> 9 months) reconstitution and persistent erythroid development. To assess their B-cell developmental potential, fetal liver mononuclear cells from E14.5 *Pbx1*^{-/-} and control (*Pbx1*^{+/+}) embryos were transplanted into lethally irradiated *Rag*-deficient adult mice and monitored for reconstitution of lymphocytes by analyzing peripheral blood at 4, 8, and 12 weeks after transfer. B cells (B220⁺CD19⁺) in control recipients increased steadily with time, whereas B cells in *Pbx1*^{-/-} fetal liver recipients, although initially detectable, did not increase,

resulting in a significant difference between B-cell numbers in the peripheral blood in control and *Pbx1*^{-/-} recipients at 8 and 12 weeks after transfer (*P* < .001 and *P* < .001, respectively; Figure 6A). Examination of the T-cell compartment in recipients of fetal liver from *Pbx1*^{-/-} embryos indicated that T-cell development was significantly decreased but that mature CD4⁺ and CD8⁺ T cells were detectable in these mice (Figure 6B-C) and followed similar kinetics of reconstitution as B cells. *Pbx1*^{-/-} fetal liver recipients displayed disorganized lymphoid follicles (Figure S3) and very few immature B cells (B220⁺IgM^{high}IgD^{low}) in the spleen, but nevertheless mounted comparable antigen-specific IgM (not shown) and IgG (Figure 6D) antibody responses against a T-dependent antigen (KLH-DNP). Thus, the fetal liver transfer experiments suggest that there was a transient wave of lymphocyte development in the *Pbx1*^{-/-} fetal liver chimeras in contrast to the continued lymphocyte development that occurred in wild-type chimeras. However, B cells in the absence of *Pbx1* mount comparable immune responses.

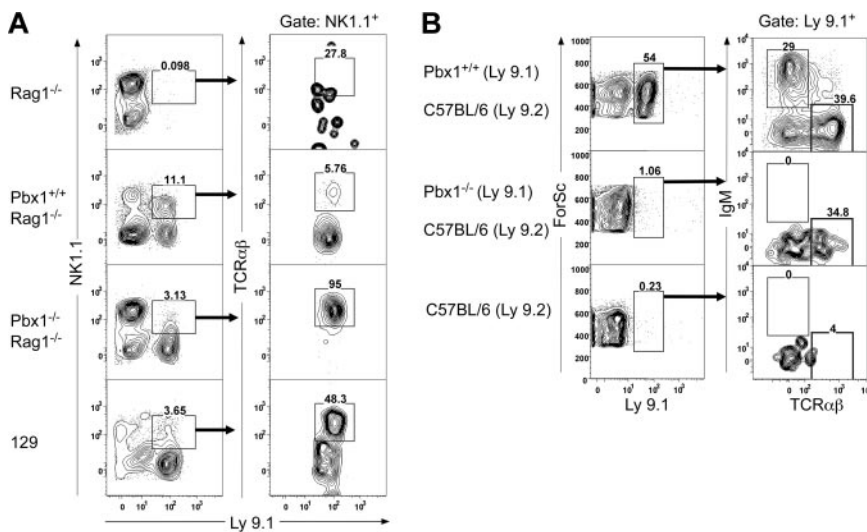


Figure 4. Poor lymphoid reconstitution in transplant recipients of *Pbx1*^{-/-}*Rag1*^{-/-} bone marrow cells. (A) FACS analysis using NK and T cell-specific fluorochrome-conjugated antibodies was performed on cells from the peripheral blood of chimeric and control mice.²⁶ Very few Ly9.1⁺NK1.1⁺ cells were detected in the *Pbx1*^{-/-}*Rag1*^{-/-} adult chimeric mice. All NK1.1⁺ cells in *Pbx1*^{-/-}*Rag1*^{-/-} chimeric mice were TCRαβ⁺ (NKT cells) in contrast to *Pbx1*^{+/+}*Rag1*^{-/-} mice. Analyses of *Rag1*^{-/-} and 129 mice are shown for comparison. (B) Equal numbers of donor-derived (Ly9.1⁺) *Pbx1*^{-/-} or *Pbx1*^{+/+} cells were FACS purified from *Rag1*^{-/-} blastocyst-complemented mice and transplanted into lethally irradiated C57BL/6 recipients along with 2 × 10⁶ *Rag1*^{-/-} bone marrow cells. No donor-derived B cells were detected in the peripheral blood of recipients receiving *Pbx1*^{-/-}*Rag1*^{-/-} bone marrow in contrast to recipients of control *Pbx1*^{+/+}*Rag1*^{-/-} bone marrow. Reconstitution of TCRαβ cells was present, but poor, in recipients receiving *Pbx1*^{-/-}*Rag1*^{-/-} bone marrow.

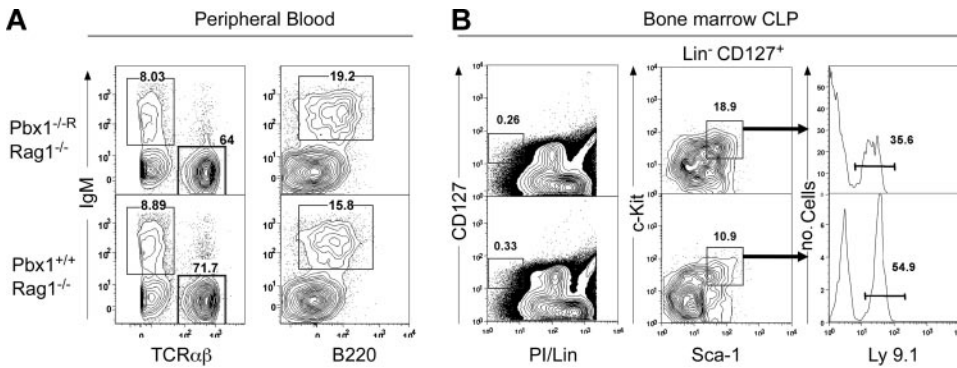


Figure 5. Reintroduction of *Pbx1b* in *Pbx1*^{-/-} ES cells restores B-cell and CLP development in *Rag1*-deficient blastocyst-complemented mice. (A) FACS analysis shows the presence of IgM⁺B220⁺ B cells and normal numbers of TCRαβ⁺ cells in the peripheral blood of mice chimeric for *Pbx1b*-rescued ES cells at levels comparable with *Pbx1*^{+/+} *Rag1*^{-/-} control mice. (B) FACS analysis shows that donor-derived (Ly9.1⁺) CLPs are present in *Pbx1*^{-/-R} *Rag1*^{-/-} chimeras.

B-cell development occurs in the absence of *Pbx1* from the pro-B cell (CD19⁺) stage onward

The foregoing results obtained from blastocyst complementation assays and fetal liver transplantation experiments demonstrated that *Pbx1b* is required at the earliest stages of B-cell development. To assess its potential continued requirement at later stages, a conditional knockout approach was used to specifically inactivate the *Pbx1* gene at the pro-B-cell stage of differentiation. Mice containing a floxed *Pbx1* gene were bred with *Pbx1*^{+/-} mice and then with mice that express the Cre recombinase under control of the *CD19* locus¹⁵ to obtain *Pbx1*^{f/f} *CD19Cre* mice. Southern blot analysis confirmed specific and complete deletion of *Pbx1* in CD19⁺ B-cell populations in the spleen and bone marrow of *Pbx1*^{f/f} *CD19Cre* mice (Figure 7A). FACS analysis demonstrated that mice lacking *Pbx1* at the pro-B stage (CD19⁺, Hardy fraction B) had normal percentages of B220⁺IgM⁺ cells in the peripheral blood (Figure 7B) and secondary lymphoid organs (Figure 7C). In the bone marrow, normal numbers and ratios of cells in Hardy fractions A through F indicated that B-cell differentiation proceeded in mice lacking *Pbx1* from CD19⁺ pro-B cells onward

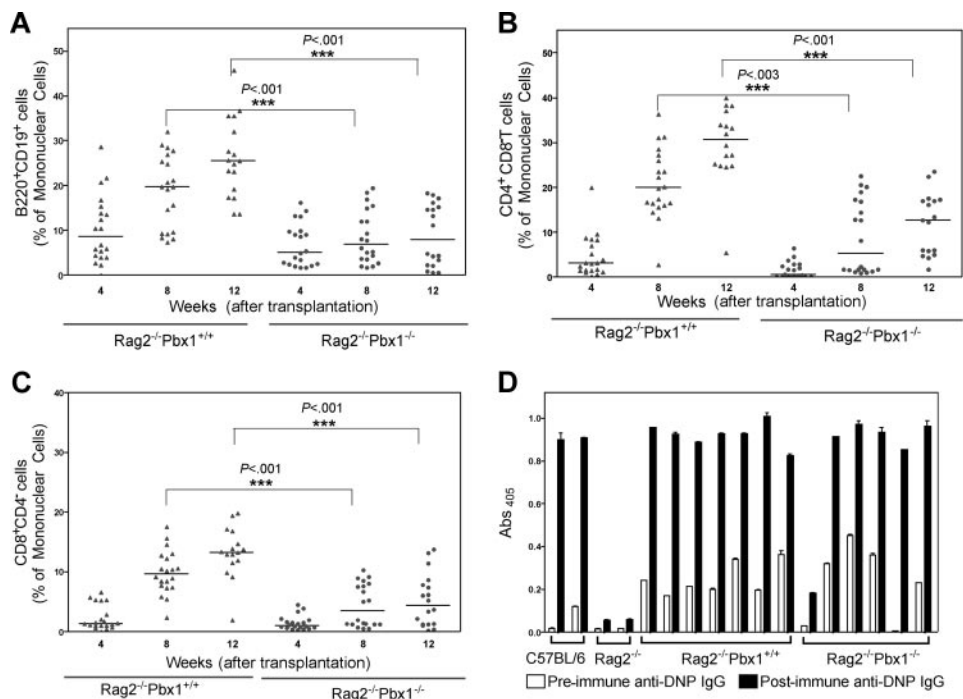
(Figure 7D). Therefore, the requirement for *Pbx1* in specification of the B-cell lineage occurs prior to the pro-B-cell stage of differentiation consistent with the lack of CLPs in *Pbx1*-deficient chimeras.

Discussion

Studies presented here demonstrate that *Pbx1*, a homeodomain transcription factor and pre-B-cell oncoprotein, is required in chimeric mice for the development of B cells initiating at CLPs. The lack of *Pbx1* completely depletes CLPs and results in the absence of detectable numbers of B and NK cells. T-cell numbers are also decreased, but residual T cells are clearly detectable in the absence of *Pbx1*.

These conclusions are based on the inability of *Pbx1*-deficient ES cells to generate CLPs and their progeny lymphocytes in chimeric *Rag1*-deficient mice. The experimental approach using chimeric mice was necessitated by the embryonic lethality of *Pbx1* null embryos prior to formation of a lymphoid compartment. A similar deficit of B-cell development was observed in *Pbx1*^{-/-} fetal

Figure 6. A transient and quantitatively reduced lymphoid development occurs in *Pbx1*^{-/-} fetal liver transplant recipients. (A) Wild-type or *Pbx1*^{-/-} fetal liver cells (1 × 10⁶) were transferred to lethally irradiated *Rag2*^{-/-} recipients. At the indicated time points, FACS analysis was performed to assess B-cell reconstitution in peripheral blood from chimeric mice using fluorochrome-conjugated B cell-specific (B220 and CD19) antibodies. Very few B220⁺CD19⁺ cells were observed in *Pbx1*^{-/-} as well as *Pbx1*^{+/+} fetal liver recipients at 4 weeks after transplantation. The numbers of B cells steadily increased at later time points in *Pbx1*^{+/+} *Rag2*^{-/-} fetal liver chimeras (▲), but not in the *Pbx1*^{-/-} chimeras (●). Median values (of 20 recipients) are represented by a solid horizontal line at each time point. (B) FACS analysis was performed to monitor T-cell reconstitution in the peripheral blood of chimeric mice using fluorochrome-conjugated anti-CD4 and CD8 antibodies. Reconstitution of CD4⁺CD8⁻ T cells in the *Pbx1*^{+/+} and *Pbx1*^{-/-} fetal liver chimeras followed similar patterns as B cells. (C) The kinetics of reconstitution of CD8⁺CD4⁻ was also similar to B cells indicating significantly reduced lymphopoiesis in the *Pbx1*^{-/-} *Rag2*^{-/-} fetal liver chimeras. (D) Chimeric mice were immunized with KLH-DNP (100 μg, intraperitoneally) and serum anti-DNP IgG antibodies were measured by ELISA. *Pbx1*^{-/-} chimeras mounted comparable DNP-specific IgG response with wild-type chimeras. Error bars represent SEMs for triplicate analyses.



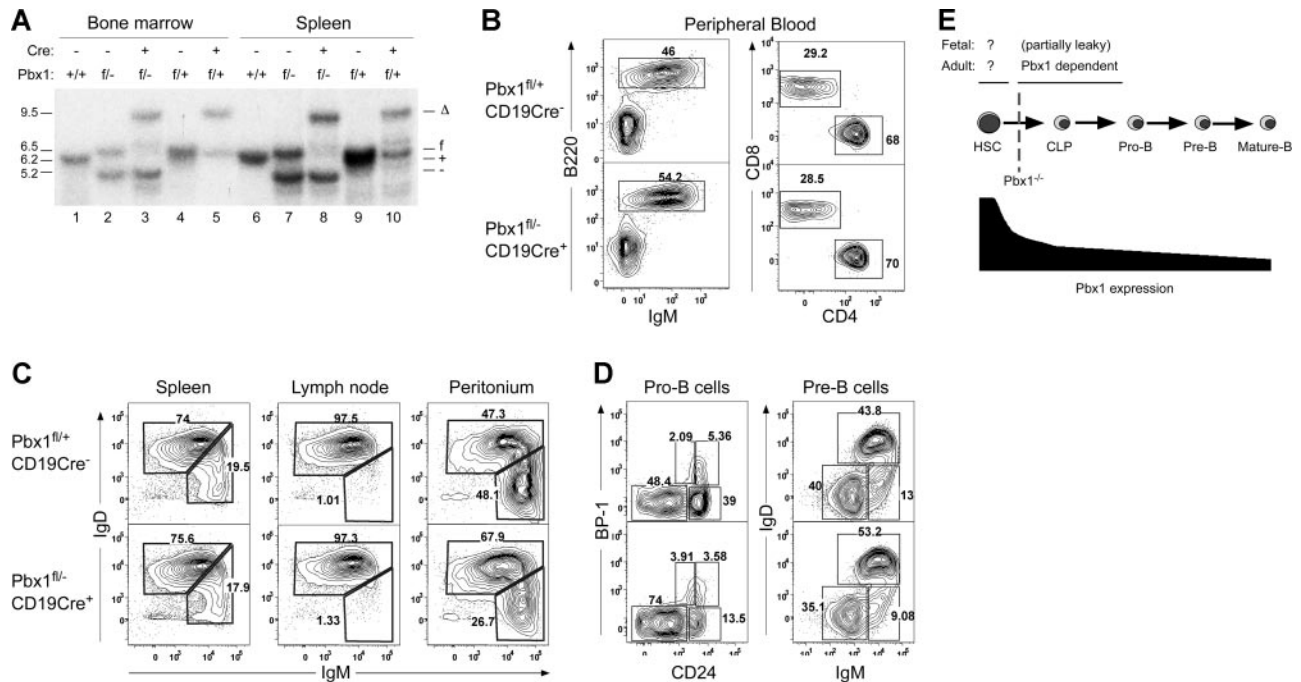


Figure 7. Conditional inactivation of *Pbx1* in pro-B cells using CD19Cre does not prevent B-cell development. (A) Southern blot analysis of *Pbx1* gene configurations in FACS-purified B cells (CD19⁺) in the bone marrow (left) and spleen (right) was performed on mice with the genotypes indicated above the gel lanes. The respective migrations of *Pbx1* DNA fragments corresponding to the deleted (Δ), floxed (f), wild-type (+), and null (–) alleles are indicated to the right of the panel. The absence of floxed and wild-type alleles in CD19⁺ cells of *Pbx1*^{fl/-} CD19Cre mice indicates complete *Pbx1* deletion in B cells (lanes 3 and 8). (B) FACS analysis shows normal numbers of B220⁺IgM⁺ B cells and CD4⁺ and CD8⁺ single-positive T cells in the peripheral blood of *Pbx1*^{fl/-} CD19Cre transgenic mice. (C) FACS analysis demonstrates that IgM^{hi}IgD^{lo} and IgM^{lo}IgD^{hi} B-cell subsets are present at normal levels in the secondary lymphoid organs of *Pbx1*^{fl/-} CD19Cre mice. (D) B-cell developmental subsets (Hardy fractions A–F) are normally represented in the bone marrow of *Pbx1*^{fl/-} CD19Cre mice. (E) Role of *Pbx1* during B-cell development. The B-cell developmental pathway originating from upstream HSCs and CLPs is shown schematically. Relative *Pbx1* expression levels are illustrated below and depict the marked down-regulation of *Pbx1* with progressive differentiation along the B-cell lineage. Genetic analyses in this report define a *Pbx1*-dependent stage of B-cell development, with a critical requirement for *Pbx1* function at a point between HSCs and B-cell commitment.

liver recipients where transient lymphocyte development was observed and also suggesting that the requirement is not absolute. Thus, *Pbx1* joins a small cohort of transcriptional regulators that are cell intrinsic factors specifically required for development of the B-lymphoid lineage. However, our results distinguish *Pbx1* from these critical regulators as a more upstream B-lineage requirement and one of the first factors known to contribute to development of CLPs.

Specification of the B-cell lineage and differentiation of committed pro-B cells depend critically on the coordinated and sequential actions of transcription factors E2A, EBF, and Pax5.²⁷ The E2a protein isoforms E12 and E47²⁸ appear to be the most upstream acting of these B-lineage regulators and together with HEB and E2-2 constitute a family of related basic helix-loop-helix (bHLH) proteins (known as E proteins). B lymphopoiesis is dependent on the combined dosage of E proteins, because pro-B–cell development at or prior to the Hardy fraction A stage is severely affected in the absence of either E12 or E47,^{10,11} whereas it is reduced 2-fold in mice lacking HEB or E2-2.²⁹ E proteins regulate expression of several B cell-specific genes including EBF, which together with E2a proteins, is required for transition of pro-B cells to the Hardy fraction B stage. *EBF* null mice accumulate B lineage-committed progenitors that express the IL-7 receptor and germline μ transcripts.³⁰ Pax5 appears to regulate a slightly later stage transition as evidenced by maturation arrest of progenitors (CD19⁺ λ 5⁺VpreB⁺) just prior to the pre-B–cell stage in *Pax5* null mice.³¹

Pbx1, in contrast, is required upstream of all 3 of the described transcription factors during B lymphopoiesis since *Pbx1*-deficient chimeric mice lack all (B220⁺) B-lineage intermediates as well as CLPs, the upstream progenitors of the earliest B-cell progenitors.

Our demonstration in this study that *Pbx1* is required for CLP development introduces this transcription factor as a key regulator in B-cell development. Recently, the zinc-finger transcription factor Bcl11a (Evi9) was shown to serve an essential role in early B lymphopoiesis upstream of EBF and Pax5.³² *Bcl11a* mutant embryos lack B cells and have alterations in several types of T cells. Effects on CLPs remain to be determined. Ikaros-deficient mice,³³ which also show severe defects in B-cell development, may also lack CLPs.³⁴ However, this study used AA4.1 to phenotypically identify CLPs, a marker that has been reported to be expressed mainly on B cell-restricted lymphoid cells. Thus, *Pbx1* is one of the first transcriptional regulators whose function is clearly required for CLP development.

CLPs were originally identified and defined as precursors of both B- and T-lineage lymphocytes as well as NK and dendritic cells.¹⁹ Consistent with lack of CLPs we also show the chimeras that are generated from *Pbx1* null ES cells lack NK cells. NK cells are bone marrow-derived lymphocytes distinct from B and T cells and can be distinguished from other lymphocytes by the absence of B- and T-cell antigen receptors, and their development does not require events that are necessary for antigen receptor gene rearrangement.³⁵ Indeed, they are present in *scid* and *Rag-1*^{-/-} or *Rag-2*^{-/-} deficient mice (Figure 4A).^{22,36,37} Thus, lack of NK cells in *Pbx1*-deficient chimeras cannot be attributed to a defective bone marrow microenvironment, and the chimeras generated from control wild-type ES cells produced significant percentages of NK cells (Figure 4A). The lack of NK cells in *Pbx1*^{-/-}*Rag1*^{-/-} chimeras could be due to a competitive disadvantage of *Pbx1*^{-/-} NK cells compared to *Rag1*^{-/-} NK cells. However, the complete absence of CLPs in *Pbx1*^{-/-}*Rag1*^{-/-} chimeras suggests that NK

cells may not have developed at all as is the case for B cells and conventional T cells. In mouse adult bone marrow, NK cells develop from CLPs through an intermediate step identified as NK precursor (NKP).²⁶ A subset of transcription factors, which includes Ikaros, ETS family members, Id2, and Id3 have been shown to be involved with development of NK cells.³⁸⁻⁴¹ In our study, we propose that the absence of NK cells in *Pbx1*^{-/-} chimeras is a consequence of lack of CLP development.

Dendritic cells can be derived from both lymphoid as well as myeloid precursors.^{42,43} Analysis of dendritic cell lineage in our chimeras was limited by the fact that the Ly9.1 marker is not very well expressed on these cells. Nevertheless, we have shown that despite the absence of detectable CLP, B, and NK cells, HSCs along with T- and NKT-lineage cells developed from *Pbx1*^{-/-} ES cells in *Rag1*-deficient mice. This supports the existence of alternative pathways for T-cell generation, including extrathymic pathways,^{44,45} or pathways independent of CLPs.³⁴ We cannot rule out the possibility that T cells arose during fetal development and persisted postnatally; however, the presence of T cells in mice receiving transplants of *Pbx1*^{-/-} bone marrow cells is most consistent with de novo T-cell production independent of CLPs. Thus, although CLPs are capable of generating B-, T-, and NK-lineage cells, they are likely not the exclusive progenitor for T cells, which may also arise from HSCs or other upstream multipotent progenitors. The substantial reduction of T-cell numbers in the absence of *Pbx1* supports the hypothesis that CLPs represent a major pathway and a crucial intermediate in the development of both B and T cells. The development of T cells in conjunction with the presence of a significant percent of donor-derived (Ly9.1⁺) cells in all lymphoid locations in *Pbx1*^{-/-}*Rag1*^{-/-} chimeric mice strongly suggests that HSCs generated in the absence of *Pbx1* are functional. Thus, the failure of B-lineage lymphopoiesis is unlikely to simply reflect a general HSC failure, although our studies cannot exclude HSC defects as a possible contributing factor to the failure of B-cell development. Conversely, tissue-specific inactivation of *Pbx1* at the pro-B-cell stage demonstrated that this transcription factor is not required at later stages (beyond Hardy fraction B) of B-cell development. Taken together, the data are most consistent with a *Pbx1* requirement between hematopoietic stem cells and commitment to B-cell development at or upstream of CLPs (Figure 7E). However, this requirement is partially leaky in fetal lymphopoiesis.

Our studies provide a developmental context for considering the oncogenic contributions of *PBX1*, a proto-oncogene that was originally discovered by virtue of its location at the site of chromosomal translocations in acute pre-B-cell leukemia. Interestingly, chromosomal translocations result in the fusion of *PBX1* with the *E2A* gene, which is also required for B-cell development, and resultant production of E2a-Pbx1 chimeric proteins that retain the DNA-binding homeodomain of Pbx1.^{3,4} The oncogenic fusion of 2 transcriptional regulators that are required for early-stage B-lineage development raises the intriguing possibility that they may normally function collaboratively in B-lineage commitment.

There is precedent for physical and functional interactions of E proteins and TALE homeodomain proteins. Pbx1 binds DNA

cooperatively in vitro with the myogenic hHLH proteins MyoD, myf5, and others.⁴⁶ During muscle differentiation, MyoD regulates expression of *myogenin* through a divergent E box element in its promoter as a complex with Pbx and Meis TALE proteins.⁴⁷ Because Pbx-Meis heterodimers occupy this element in undifferentiated cells in the absence of MyoD, it has been proposed that they act as a DNA-bound marker for subsequent recruitment of MyoD.

The generality of the model described is not clear. However, we propose that a similar functional relationship may serve a critical role in establishment of the B lineage by facilitating the recruitment and targeting of E proteins to initiate B-cell commitment. Forced expression of Pbx1 as a fusion protein under control of the *E2A* promoter may perturb their normal regulated interactive roles and prevent down-regulation of Pbx1 expression that normally accompanies progressive differentiation of B-cell progenitors. The characterization of B cell-specific enhancers occupied by Pbx1 or E proteins in CLPs may support this hypothetical model and provide further insights into the transcriptional regulatory networks that underlie B-cell commitment and are corrupted in a subset of B-cell precursor leukemia that harbor *Pbx1* and *E2A* mutations.

Acknowledgments

We thank Richard Hardy for critical reading of the manuscript; Francesca Ficara for helpful discussions; Michael White for assistance in establishing Rag-deficient blastocyst complementation techniques; Patricia Labosky for providing the TL1 cell line; Laurie Ailles for providing lentiviral vectors; Yanru Chen-Tsai, Jennifer Lin, and Denise Leong for blastocyst injections; Cita Nicolas and Maria Ambrus for expert technical assistance; and Omira Herman and Sue Sheppard for tissue processing for flow cytometric analysis.

This work was supported by National Institutes of Health grants CA42971 and CA90735 (M.L.C.), HD43997 (L.S.), and AI047458 (I.L.W.), as well as grant 6-FY03-071 from the March of Dimes and Birth Defects Foundation (L.S.). L.S. is an Irma T. Hirschl Scholar.

Authorship

Contribution: M.S. designed and performed experiments, analyzed data, and wrote the manuscript; J.W.T. performed experiments and analyzed data; H.K. designed and performed experiments, analyzed data, and edited the manuscript; H.Z. performed experiments; L.S. designed and performed experiments; I.L.W. designed and directed experiments; L.A.H. designed and directed experiments and edited the manuscript; M.C. designed and directed experiments and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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B-Cell development fails in the absence of the *Pbx1* proto-oncogene

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Supplemental materials for : Sanyal et al

Files in this Data Supplement

Table S1. Antibodies used for flow cytometric analysis (PDF, 30.2 KB)

Antigen	Clone	Conjugation	Application
Ly9.1	30C7	FITC, Biotin	Congenetic marker
B220	RA3-6B2	APC, Cy5PE, PerCP-Cy5.5	B cells, Hardy profile
CD19	1D3	Fluorescein, Biotin	B cells
CD43	S7	PE	B cells, Hardy profile
CD24 30-F1		Biotin	Hardy profile
BP-1 6C31BP1		Alexa594	Hardy profile
IgM (Igh-6a)	DS-1	PE	Mature B cells
IgM (Igh-6b)	AF6-78	APC-Cy7	Mature B cells
IgM	331	APC-Cy7	Mature B cells, Hardy profile
IgD	11-26	PE-Cy7	Mature B cells, Hardy profile
CD5	53-7.3	PE-Cy5	B1 B cells
CD21	7G6	Fluorescein	Mature B cells
CD23	B3-B4	Alexa594	Mature B cells
TCR β	H57-597	APC	T cells
NK1.1 PK136		PE	NK cells
CD3 145-2C11		Cascade Blue	Lineage
CD4	GK1.5	APC-Cy7,Cascade Blue	T cells
CD8	53-6.7	PE-Cy7, Cascade Blue	T cells
Gr1	RB6-8C5	Cascade Blue	Lineage
CD11b Mac-1	Cascade	Blue	Lineage

Figure S1. Configurations of *Pbx1* alleles (JPG, 35.1 KB) -

Gene targeting of the *Pbx1* wt allele (top line) resulted in the insertion of a *LoxP*-PGK-hygromycin-GFP fusion cassette at the unique *Bst*BI site downstream of *Pbx1* exon 3 and placement of a *LoxP* site upstream of exon 3 at the *Bam*HI site (second line). The floxed exon 3 was excised by *in vitro* Cre recombination to produce an inactivated *Pbx1* allele (third line). Correctly targeted clones were identified by the presence of a 16 kb *Ssp*I fragment on Southern blot analysis (3' probe). The *Pbx1* null allele generated by insertion of a PGK-neo cassette into exon 3 (bottom line) was described previously⁸.

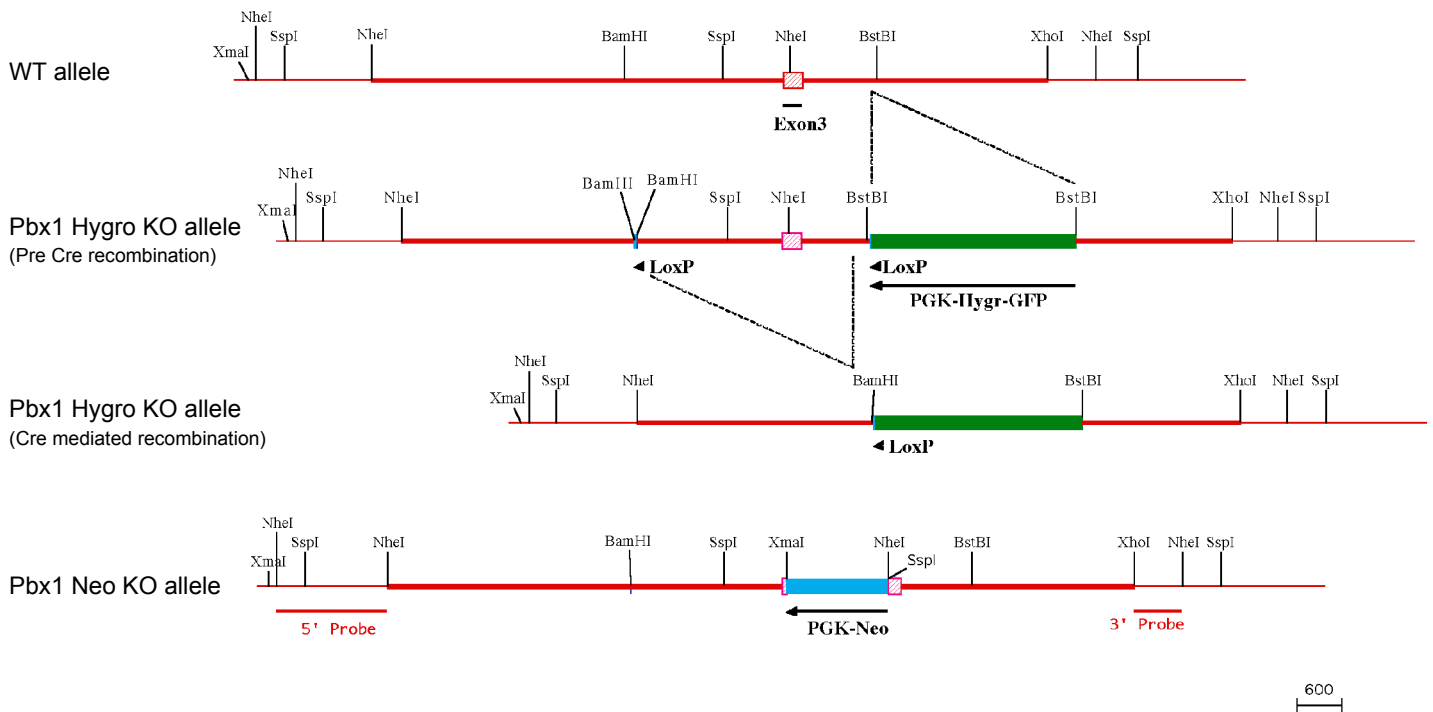


Figure S2. Lentiviral vector for rescue of *Pbx1b* expression in *Pbx1*^{-/-} ES cells (JPG, 9.02 KB) -

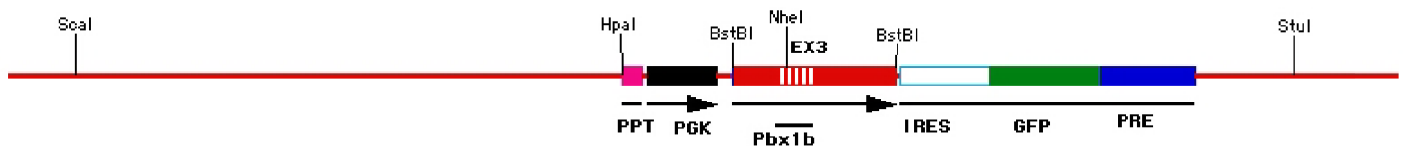


Figure S3. Cellular architecture of the spleens from FL transplanted mice (JPG, 106 KB) -

H&E preparations of the spleens were analyzed for cellular architecture (left panels). A portion of the spleen was processed for cryosection and stained with Texas Red conjugate d anti-B220 monoclonal antibody (middle panels). Single cell preparations of the spleens were stained with fluorochrome conjugated anti-B220, IgM and IgD anti bodies and analyzed by flow cytometry (right panels).

Spleens of Pbx1^{-/-} and control fetal liver transplanted chimeras

