



## Regulation of B Cell Linker Protein Transcription by PU.1 and Spi-B in Murine B Cell Acute Lymphoblastic Leukemia

This information is current as of March 6, 2022.

Li S. Xu, Kristen M. Sokalski, Kathryn Hotke, Darah A. Christie, Oren Zarnett, Jan Piskorz, Gobi Thillainadesan, Joseph Torchia and Rodney P. DeKoter

*J Immunol* 2012; 189:3347-3354; Prepublished online 5 September 2012;

doi: 10.4049/jimmunol.1201267

<http://www.jimmunol.org/content/189/7/3347>

**Supplementary Material** <http://www.jimmunol.org/content/suppl/2012/09/05/jimmunol.1201267.DC1>

**References** This article **cites 59 articles**, 26 of which you can access for free at:  
<http://www.jimmunol.org/content/189/7/3347.full#ref-list-1>

**Why *The JI*? Submit online.**

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*\*average*

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:  
<http://jimmunol.org/subscription>

**Permissions** Submit copyright permission requests at:  
<http://www.aai.org/About/Publications/JI/copyright.html>

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:  
<http://jimmunol.org/alerts>



# Regulation of B Cell Linker Protein Transcription by PU.1 and Spi-B in Murine B Cell Acute Lymphoblastic Leukemia

Li S. Xu,<sup>\*,†</sup> Kristen M. Sokalski,<sup>\*,†</sup> Kathryn Hotke,<sup>\*,†</sup> Darah A. Christie,<sup>\*,†</sup> Oren Zarnett,<sup>\*,†</sup> Jan Piskorz,<sup>\*,†</sup> Gobi Thillainadesan,<sup>‡,§</sup> Joseph Torchia,<sup>‡,§</sup> and Rodney P. DeKoter<sup>\*,†,¶</sup>

B cell acute lymphoblastic leukemia (B-ALL) is frequently associated with mutations or chromosomal translocations of genes encoding transcription factors. Conditional deletion of genes encoding the E26-transformation-specific transcription factors, PU.1 and Spi-B, in B cells ( $\Delta$ BP mice) leads to B-ALL in mice at 100% incidence rate and with a median survival of 21 wk. We hypothesized that PU.1 and Spi-B may redundantly activate transcription of genes encoding tumor suppressors in the B cell lineage. Characterization of aging  $\Delta$ BP mice showed that leukemia cells expressing IL-7R were found in enlarged thymuses. IL-7R-expressing B-ALL cells grew in culture in response to IL-7 and could be maintained as cell lines. Cultured  $\Delta$ BP cells expressed reduced levels of B cell linker protein (BLNK), a known tumor suppressor gene, compared with controls. The *Blnk* promoter contained a predicted PU.1 and/or Spi-B binding site that was required for promoter activity and occupied by PU.1 and/or Spi-B as determined by chromatin immunoprecipitation. Restoration of BLNK expression in cultured  $\Delta$ BP cells opposed IL-7-dependent proliferation and induced early apoptosis. We conclude that the tumor suppressor BLNK is a target of transcriptional activation by PU.1 and Spi-B in the B cell lineage. *The Journal of Immunology*, 2012, 189: 3347–3354.

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in young children (1). Despite a better than 80% cure rate, ALL represents a leading cause of leukemia-related deaths in children and upon relapse in adults (1). The majority of human ALLs are cancers of the B lymphocyte lineage (B cell ALL [B-ALL]) and are frequently associated with mutations or chromosomal translocations targeting genes encoding transcription factors (2). For example, 25% of cases of pre-B ALL have the t(12;21) chromosomal translocation, resulting in a fusion between the *ETV6* and *RUNX1* genes encoding the transcription factors TEL and AML1, respectively (1). The E26-transformation-specific transcription factor PU.1, encoded by the gene *Spi1* in mice and *SP1* in humans, is required for generating lymphoid progenitor cells and is a key regulator of B cell fate specification (3, 4). *SP1* mutations are associated with human AML (5, 6) and B-ALL (7). Reduced PU.1 expression is sufficient to induce AML in mice (8, 9). Reduced PU.1 levels are also associated with human lymphoid leukemia and lymphoma (10, 11).

Spi-B (encoded by *Spib*) is expressed in developing B cells (12), and it interacts with DNA binding sites thought to be identical to those recognized by PU.1 (13). Reduced Spi-B expression was recently associated with human B-ALL (14). Our laboratory previously showed that mice that lack both PU.1 and Spi-B in the B cell lineage (*CD19<sup>+</sup>Cre Spi1<sup>lox/lox</sup> Spib<sup>-/-</sup>* mice, henceforth known as  $\Delta$ BP mice) have impaired B cell development and develop B-ALL with 100% incidence rate by 21 wk of age (15). These results demonstrate that PU.1 and Spi-B have tumor-suppressor function in the B cell lineage and suggest that mutation of the *SP1* and *SP1B* genes, or upstream activators of these genes, could be oncogenic drivers in leukemia-initiating cells. Because these proteins are transcription factors, it is important to identify target genes that could explain the role of PU.1 and Spi-B in lymphoid leukemogenesis.

B cell linker protein (BLNK, also known as SLP65 or BASH) is an adaptor protein that is required for B cell development as a consequence of its important role in BCR signaling (16–18). BLNK links BCR signaling with enforcement of B cell differentiation by mediating interactions between Syk, Vav, PLC $\gamma$ 2, Grb2, and Bruton's tyrosine kinase (Btk) upon phosphorylation by Syk (19, 20). Inactivating mutations of *BLNK* are associated with B-ALL in human patients (21, 22), and reduced expression of BLNK has also been associated with B-ALL (23). In mice, mutation of *Blnk* is sufficient to induce B-ALL with a 10% incidence rate (23, 24). Therefore, BLNK has important roles in enforcing B cell differentiation and functions as a tumor-suppressor gene.

We previously noted that levels of *Blnk* mRNA transcripts were reduced in sorted splenic  $\Delta$ BP B-ALL cells compared with control B cells (15). Therefore, we hypothesized that *Blnk* is a target of transcriptional activation by PU.1 and/or Spi-B. To test this hypothesis, we performed a detailed characterization of B-ALL cells from  $\Delta$ BP mice.  $\Delta$ BP B-ALL cells expressed high levels of IL-7R $\alpha$  and grew in culture in response to IL-7. Cultured  $\Delta$ BP B-ALL cells expressed reduced levels of BLNK mRNA transcripts and protein compared with control cells. The *Blnk* promoter was

\*Department of Microbiology and Immunology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada N6A 5C1;

†Centre for Human Immunology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada N6A 5C1; ‡Department of Oncology, London Regional Cancer Program, London, Ontario, Canada N6A 4L6; §Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1; and ¶Division of Genetics and Development, Children's Health Research Institute, London, Ontario, Canada N6C 2V5

Received for publication May 2, 2012. Accepted for publication July 26, 2012.

This work was supported by the Canadian Institutes of Health Research (Grant MOP-106581) and the National Sciences and Engineering Research Council (Grant 386046).

Address correspondence and reprint requests to Dr. Rodney P. DeKoter, Department of Microbiology and Immunology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, ON N6A 5C1, Canada. E-mail address: rdekoter@schulich.uwo.ca

The online version of this article contains supplemental material.

Abbreviations used in this article: ALL, acute lymphoblastic leukemia; B-ALL, B cell ALL; BLNK, B cell linker protein; Btk, Bruton's tyrosine kinase; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/\$16.00

confirmed as a direct target of activation by PU.1 and/or Spi-B using transient transfection and chromatin immunoprecipitation (ChIP) analysis. Finally, we showed that forced expression of BLNK in cultured  $\Delta$ BP B-ALL cells opposed proliferation by inducing early apoptosis. We conclude that the tumor suppressor BLNK is a target of transcriptional activation by PU.1 and Spi-B in the B cell lineage.

## Materials and Methods

### Breeding and care of mice

Mice used in this study were on the C57BL/6 background and were generated by mating  $CD19^{+/Cre}Sfpil^{lox/lox}Spib^{-/-}$  ( $\Delta$ BP) males to  $CD19^{+/+}Sfpil^{lox/lox}Spib^{-/-}$  ( $\Delta$ B) females, and genotyped as previously described (15). Mouse care was monitored under an approved animal use protocol in accord with the University of Western Ontario Council on Animal Care.

### Cell culture

$\Delta$ BP B-ALL cells were cultured in IMDM (Lonza, Shawinigan, QC, Canada) containing 5% IL-7-containing conditioned media from the J558L-IL-7 cell line (25), 10% FBS (Biologos, Montgomery, IL),  $1 \times$  penicillin/streptomycin/L-glutamine (Lonza, Shawinigan, Quebec, Canada), and  $5 \times 10^{-5}$  M 2-ME (Sigma-Aldrich, St. Louis, MO). Purified recombinant growth factors were purchased from PeproTech (Embrun, ON, Canada) and used at the following concentrations: murine stem cell factor (100 ng/ml), murine M-CSF (10 ng/ml), murine IL-3 (10 ng/ml), murine IL-6 (10 ng/ml), murine GM-CSF (1 ng/ml), murine Flt-3 ligand (20 ng/ml), murine IL-7 (100 ng/ml), and murine G-CSF (10 ng/ml). WEHI-279 B cell lymphoma cells were maintained in complete DMEM (Lonza) containing 4.5 g/l glucose. ST2 stromal cells were maintained in complete IMDM. Plat-E retroviral packaging cells and NIH-3T3 cells were maintained in complete DMEM containing 1.5 g/l glucose. All cell lines were maintained in 5% CO<sub>2</sub> atmosphere and at 37°C.

### Colony-forming assays

$\Delta$ BP cells or retrovirally infected  $\Delta$ BP cells were plated in methylcellulose media containing recombinant murine IL-7 (MethoCult 03630; Stem Cell Technologies, Vancouver, BC, Canada) and incubated for 7 d at 37°C and an atmosphere of 5% CO<sub>2</sub>. Colonies were scored and visualized using a Zeiss A-plan 5×/0.12 Ph20 441021-9910 objective lens on an Axio Observer A1 microscope (Carl Zeiss MicroImaging, Thornwood, NY).

### Flow cytometric analysis and immunoblotting

Abs used in this study were purchased from eBioscience (San Diego, CA) and included biotin anti-pre-BCR (clone SL156), biotin anti-IgM (II/41), PE anti-c-Kit/CD117 (2B8), biotin anti-CD45R/B220 (2A3-6B2), biotin anti-IL-7R $\alpha$ /CD127 (B12-1), PE-conjugated anti-CD93 (AA4.1), FITC-conjugated anti-BP-1 (6C3), and PE-, allophycocyanin-, or Cy5-conjugated streptavidin as secondary reagents. Alexa Fluor 647-Annexin V was purchased from BioLegend (San Diego, CA). Flow cytometric analysis was performed using a BD FACSCalibur system (BD Biosciences, Franklin Lakes, NJ). Data analysis was performed using FlowJo 9.4.10 Software (Tree Star, Ashland, OR). All analyses shown are gated on a viable lymphocyte population based on forward and side scatter values. Immunoblotting was performed using standard methods or as previously published (26). Abs used included rabbit anti-Akt, rabbit anti-phospho-Akt, rabbit anti-BLNK (Cell Signaling Technology, Beverly, MA), rabbit anti-PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti- $\beta$ -actin peptide Ab (Santa Cruz), HRP-conjugated anti-rabbit or anti-goat secondary Abs (Pierce Biotechnology, Nepean, ON, Canada), and Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

### Bioinformatics analysis

Phylogenetic comparison of the upstream region of the *Blnk* gene from human and mouse was performed using MacVector 11.0 (Accelrys, San Diego, CA) using sequences obtained from the Ensembl.org database. Identification of potential E26-transformation-specific binding sites within the upstream region of the *Blnk* gene was performed using the application MATINSPECTOR (Genomatix, Munich, Germany) (27).

### Plasmid construction

A 405-bp region upstream of the translation start site in the mouse *Blnk* promoter was amplified by PCR using LA-TAQ (TaKaRa; Fisher Scientific, Toronto, ON, Canada), and 5' and 3' HindIII recognition sites were

added to the PCR primers. The PCR fragment was cloned using the StrataClone PCR Cloning Kit (Agilent Technologies Canada, Mississauga, ON, Canada). A HindIII fragment containing the *Blnk* promoter was ligated in forward and reverse orientation into the HindIII site of pGL3-basic (Promega, Madison, WI). To obtain pGL3-*Blnk*-MUT plasmid, we subjected the pGL3-*Blnk*-FWD to site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). *Blnk* cDNA was generated from RNA isolated from spleen of a C57B1/6 mouse using the RNA-Bee isolation reagent (Tel-Test, Friendswood, TX) and reverse transcribed using the iScript kit (Bio-Rad Canada, Mississauga, ON, Canada). The 1501-bp *Blnk* cDNA was amplified by PCR using LA-TAQ (TaKaRa) and cloned using the StrataClone PCR Cloning Kit (Agilent). An EcoRI fragment containing the *Blnk* cDNA was ligated into the EcoRI site of MIGR1. To generate MIG-3XFLAG-PU.1 and MIG-3XFLAG-Spi-B, we used PCR to amplify the 3XFLAG coding sequence from the plasmid vector pBICEP-CMV-2 (Sigma-Aldrich) to introduce a 5' XhoI site and retain an in-frame NotI site. This DNA fragment was cloned and ligated into the retroviral vectors MIG-HA-PU.1 and MIG-HA-Spi-B (28) using XhoI and NotI sites to replace the hemagglutinin epitope tag sequence of this vector. All constructs were confirmed by DNA sequencing. Oligonucleotide sequences are listed in Supplemental Table I.

### Retroviral production and infection

MIGR1, MIG-3XFLAG-PU.1, MIG-3XFLAG-Spi-B, and MIG-BLNK retroviruses were generated by transient transfection of Plat-E packaging cells (29) using polyethylenimine transfection at a 3:1 polyethylenimine/DNA ratio (30). Virus-containing supernatants were collected at 48 h posttransfection, and viral titers were measured by infection of NIH-3T3 cells. Infections were performed by "spinoculation" with high-titer virus by centrifugation at  $2000 \times g$  for 3 h at 32°C in the presence of 8  $\mu$ g/ml polybrene. After centrifugation, cells were washed and cultured for 48 h to promote retroviral integration and gene expression. Infection frequencies were detected by flow cytometric analysis of GFP.

### Transient transfection and ChIP analysis

Transient transfection of WEHI-279 cells was performed using electroporation as previously described (31). MIGR1, MIG-3XFLAG-PU.1, and MIG-3XFLAG-Spi-B-infected WEHI-279 clonal cell lines were cross-linked with 1% formaldehyde for 10 min at room temperature. ChIP was performed as previously described (31). Enrichment was measured using quantitative PCR (qPCR) of DNA immunoprecipitated with anti-FLAG magnetic beads (Sigma-Aldrich), using primers indicated in Supplemental Table I. Percentage of input was calculated using the comparative threshold cycle method (32).

### Statistical analysis

All data are reported as mean  $\pm$  SD of the mean. Statistical significance was determined using a Student *t* test unless otherwise indicated. The *p* values <0.05 were considered significant. Statistical analysis was performed using Prism 5.0 (GraphPad Software, La Jolla, CA).

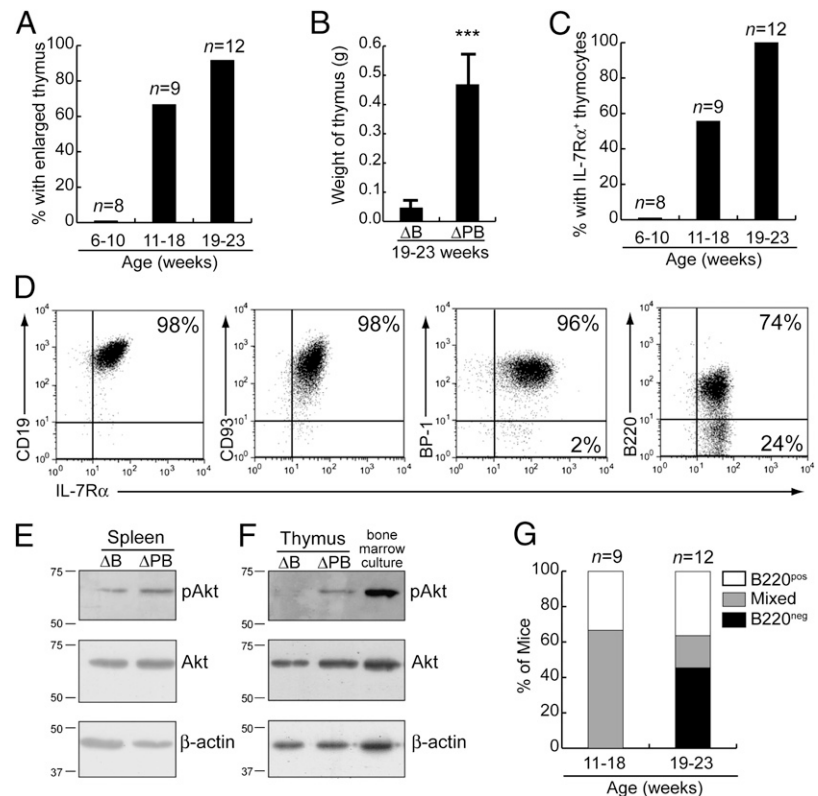
## Results

### Leukemia cells in the thymus of $\Delta$ BP mice express IL-7R $\alpha$

Mice that lack PU.1 and Spi-B in the B cell lineage ( $\Delta$ BP mice) require euthanasia at a median age of 21 wk as a consequence of lethal B-ALL (15). Leukemia cells infiltrate all lymphoid tissues in these mice, but the proximal cause of death is dyspnea associated with an enlarged thymus (15). To determine whether thymus enlargement is age dependent, groups of  $\Delta$ BP mice were analyzed at various ages between 6 and 23 wk. Thymuses were never enlarged in mice aged 6–10 wk (0/8). However, thymuses were enlarged in 6 of 9 mice aged 11–18 wk and 11 of 12 mice aged 19–23 wk (Fig. 1A). The weight of enlarged thymuses in 19- to 23-wk-old  $\Delta$ BP mice was >10-fold greater than that of littermates that lack only Spi-B ( $\Delta$ B; Fig. 1B). To confirm the identity of cells in enlarged thymuses of  $\Delta$ BP mice, we performed flow cytometric analysis. Every enlarged thymus, regardless of age, contained >90% CD19<sup>+</sup> cells, indicating that they were of the B cell lineage (Fig. 1D). All CD19<sup>+</sup> B-ALL cells also expressed cell-surface IL-7R $\alpha$  (CD127; Fig. 1C, 1D). The one thymus that was not enlarged in the 19- to 23-wk age group (Fig. 1A, right



**FIGURE 1.** Age-dependent appearance of IL-7R $\alpha$ -expressing B-ALL cells in the thymus of  $\Delta$ BP mice. **(A)** Enlarged thymuses in  $\Delta$ BP mice at various ages. The y-axis indicates percentage of mice with enlarged thymuses in the age groups indicated on the x-axis. **(B)** Increased thymic weight in  $\Delta$ BP mice. The total weight of the thymus in grams was compared in  $\Delta$ B ( $n = 7$ ) and  $\Delta$ BP mice ( $n = 9$ ) between 19 and 23 wk of age. \*\*\* $p < 0.001$  by Student  $t$  test. **(C)** Expression of IL-7R $\alpha$  by tumor cells in enlarged thymuses. The y-axis indicates percentage of mice in which the frequency of IL-7R $\alpha$ -expressing thymocytes was  $>60\%$ , in age groups indicated on the x-axis. **(D)** Coexpression of IL-7R $\alpha$  with immature B cell markers in  $\Delta$ BP mice. Flow cytometric analysis was performed to determine expression of the indicated markers. Increased brightness of IL-7R $\alpha$  in the third panel is a consequence of using Cy7 rather than PE as a fluorophore. **(E and F)** Increased levels of total and phosphorylated Akt (pAkt) in  $\Delta$ BP B-ALL cells. Immunoblotting for pAkt, total Akt (Akt), or  $\beta$ -actin was performed on lysates prepared from the spleen or thymus of a 16-wk-old  $\Delta$ BP mouse, as well as from a  $\Delta$ B control. Bone marrow cells cultured in IL-7 were used as a positive control for Akt activation. Result shown is representative of two experiments performed. **(G)** Progressive loss of B220 expression on leukemic B cells in enlarged thymus of  $\Delta$ BP mice. The y-axis indicates the percentage of mice in each age group in which the enlarged thymus contained either B220 $^{pos}$  cells (B220 $^{pos}$ ), B220 $^{-}$  cells (B220 $^{neg}$ ), or a mixture of both B220 $^{pos}$  and B220 $^{-}$  cells (mixed).



bar) was still extensively infiltrated with CD19 $^{+}$  IL-7R $\alpha$  $^{+}$  cells. CD19 $^{+}$  B-ALL cells were negative for c-Kit and expressed low levels of cell-surface IgM (data not shown). IL-7R $\alpha$  $^{+}$  B-ALL cells expressed the pre-B cell markers BP-1 (33), AA4.1/CD93 (34), and variable levels of B220 (Fig. 1D). To determine whether there was functional IL-7R signaling in IL-7R $\alpha$  $^{+}$  B-ALL cells, we determined the status of Akt phosphorylation (35). Immunoblotting of lysates prepared from the tissues of 11- to 18-wk-old  $\Delta$ BP mice showed increased levels of phosphorylated and total Akt protein compared with  $\Delta$ B mice in either spleen (Fig. 1E) or thymus (Fig. 1F), suggesting active IL-7R signaling in these cells. B-ALL cells from 11- to 18-wk-old  $\Delta$ BP mice were B220 $^{+}$  or were a mixture of B220 $^{+}$  and B220 $^{-}$  cells (Fig. 1D, right panel; Fig. 1G). However,  $>40\%$  of B-ALLs from mice between 19 and 23 wk of age were exclusively B220 $^{-}$  (Fig. 1G). These results suggest that expression of B220 on B-ALL cells was progressively lost as  $\Delta$ BP mice aged. *Cd45*, encoding B220, has been shown to be a PU.1 target gene, so it is expected that the loss of B220 is a consequence of *Sfp1* deletion (36). In summary, the incidence of thymus enlargement in  $\Delta$ BP mice increased with age, and the B-ALL cells that infiltrated the thymus in  $\Delta$ BP mice expressed functional IL-7R.

#### IL-7-dependent pre-B cell lines can be established from the thymus of $\Delta$ BP mice

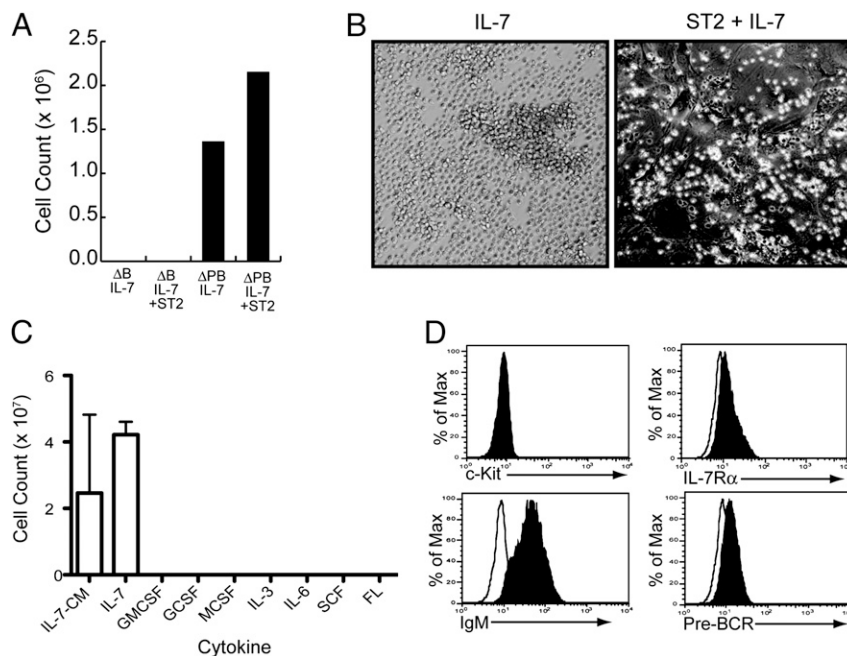
Expression of functional IL-7R by B-ALL cells in  $\Delta$ BP thymus suggested that these cells might proliferate in response to IL-7 in culture. We tested whether B-ALL cells from the enlarged thymus of 19- to 23-wk-old  $\Delta$ BP mice could proliferate in response to complete media, ST2 stromal cells, IL-7, or ST2 stromal cells and IL-7. As controls, thymocytes from age-matched littermate  $\Delta$ B mice were cultured under the same conditions. Control  $\Delta$ B thymocytes did not grow in culture in any condition. In contrast,  $\Delta$ BP cells proliferated in response to IL-7 alone or ST2 + IL-7

(Fig. 2A). Cells from the enlarged thymuses of 19- to 23-wk-old  $\Delta$ BP mice generated colonies in IL-7-containing methylcellulose at a frequency of  $\sim 50$  per 50,000 cells, suggesting that IL-7 was sufficient to induce proliferation in a manner that did not require cell-cell contact. After expansion, these cells had the typical small and highly refractile appearance of pro-B/pre-B cells, and could be efficiently expanded and propagated as cell lines (Fig. 2B).  $\Delta$ BP B-ALL cells proliferated in response to either IL-7-conditioned media or purified rIL-7 (Fig. 2C). However, established  $\Delta$ BP B-ALL cell lines did not proliferate in response to cell culture media alone, stem cell factor, Flt3 ligand, IL-3, M-CSF, or GM-CSF (Fig. 2C). Cultured  $\Delta$ BP B-ALL cell lines expressed CD19, BP-1, and AA4.1/CD93 (data not shown), as well as IL-7R $\alpha$  and surface IgM (Fig. 2D). Cultured cells did not express detectable c-Kit but did express cell-surface pre-BCR as detected by Ab SL156 (Fig. 2D). Because c-Kit is considered a marker for pro-B cells that is lost on differentiation into pre-B cells (37), these results are most consistent with a description of IL-7-dependent cell lines established from  $\Delta$ BP B-ALL as pre-B cells. In summary, IL-7 is both necessary and sufficient for the ex vivo growth of pre-B-like cell lines from the thymus of 19- to 23-wk-old  $\Delta$ BP mice.

#### The *Blnk* gene is activated by PU.1 and/or Spi-B

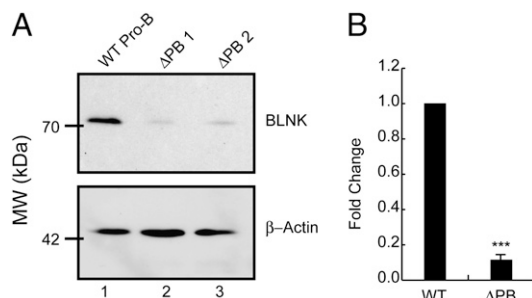
We previously reported that B-ALL cells from  $\Delta$ BP mice express reduced levels of mRNA transcripts encoding BLNK relative to control B cells (15). Because mutation of *Blnk* is sufficient to induce B-ALL in mice (23, 24), we sought to determine whether *Blnk* is a target of gene activation by PU.1, and Spi-B. IL-7-dependent  $\Delta$ BP B-ALL cell lines expressed reduced levels of BLNK protein compared with wild type IL-7-dependent pro-B cells (Fig. 3A).  $\Delta$ BP B-ALL cell lines expressed 10-fold reduced steady-state levels of *Blnk* mRNA transcripts compared with wild-type IL-7-dependent pro-B cells (Fig. 3B). Phylogenetic comparisons of

**FIGURE 2.** Establishment of IL-7-dependent cell lines from thymocytes of  $\Delta$ PB mice. **(A)** IL-7 is sufficient to promote proliferation of  $\Delta$ PB B-ALL cells. B-ALL cells from the enlarged thymus of 17-wk-old  $\Delta$ PB or  $\Delta$ B mice were placed in culture with IL-7 or IL-7 and ST2 stromal cells, and counted after 7 d. One representative experiment of five performed is shown. **(B)** Appearance of cultured  $\Delta$ PB cells. Cells were photographed at  $\times 40$  original magnification using a Zeiss AxioVert inverted microscope under phase-contrast illumination. **(C)** IL-7 is both necessary and sufficient for proliferation of  $\Delta$ PB B-ALL cells. Cells that had been grown for several passages in IL-7 were washed and cultured at  $10^6$  cells/flask in media containing cytokines indicated on the x-axis and counted after 4 d. **(D)** Cell-surface phenotype of IL-7-dependent  $\Delta$ PB B-ALL cells. Flow cytometry was used to determine expression of the markers indicated on the x-axis. The open histogram indicates staining with isotype control Ab, whereas the filled histogram indicates staining with the indicated Ab.



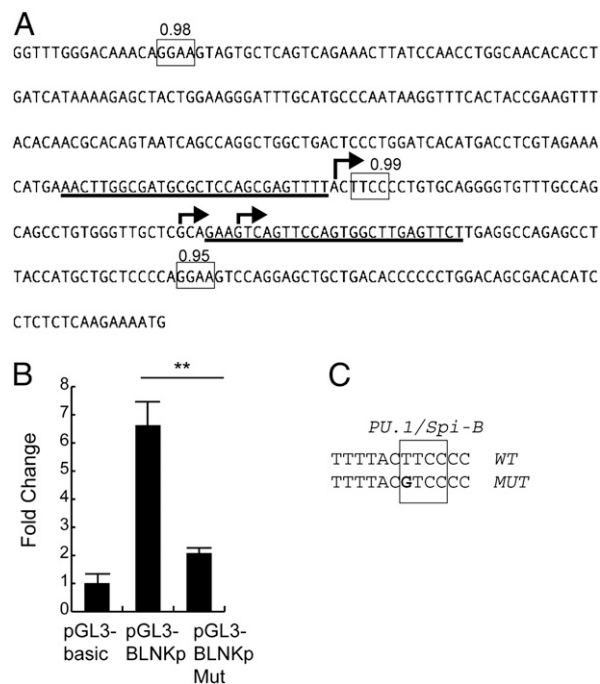
DNA sequences in the previously described mouse *Blnk* promoter region (38), as well as analysis using a position weight matrix algorithm (27), were used to identify predicted PU.1/Spi-B binding sites. Three PU.1 and/or Spi-B binding sites were predicted based on high matrix similarity scores (Fig. 4A). Interestingly, the site with the highest matrix similarity score was located near the published transcription start sites of the *Blnk* gene and near binding sites for Pax5 (38) (Fig. 4A). A 418-bp segment of the murine *Blnk* promoter that displayed high mouse-human similarity was amplified and cloned from C57BL/6 B cell genomic DNA. The *Blnk* promoter was tested for activity by transient transfection in WEHI-279 B lymphoma cells and showed activity only in the forward orientation (Fig. 4B and data not shown). Mutation of the highest scoring predicted binding site from GGAA to GGAC (Fig. 4C) reduced *Blnk* promoter activity by 3-fold (Fig. 4B). Therefore, the *Blnk* promoter contains a potential PU.1 and/or Spi-B binding site.

To determine whether PU.1 or Spi-B can directly interact with the *Blnk* promoter in B cells, we performed ChIP experiments.

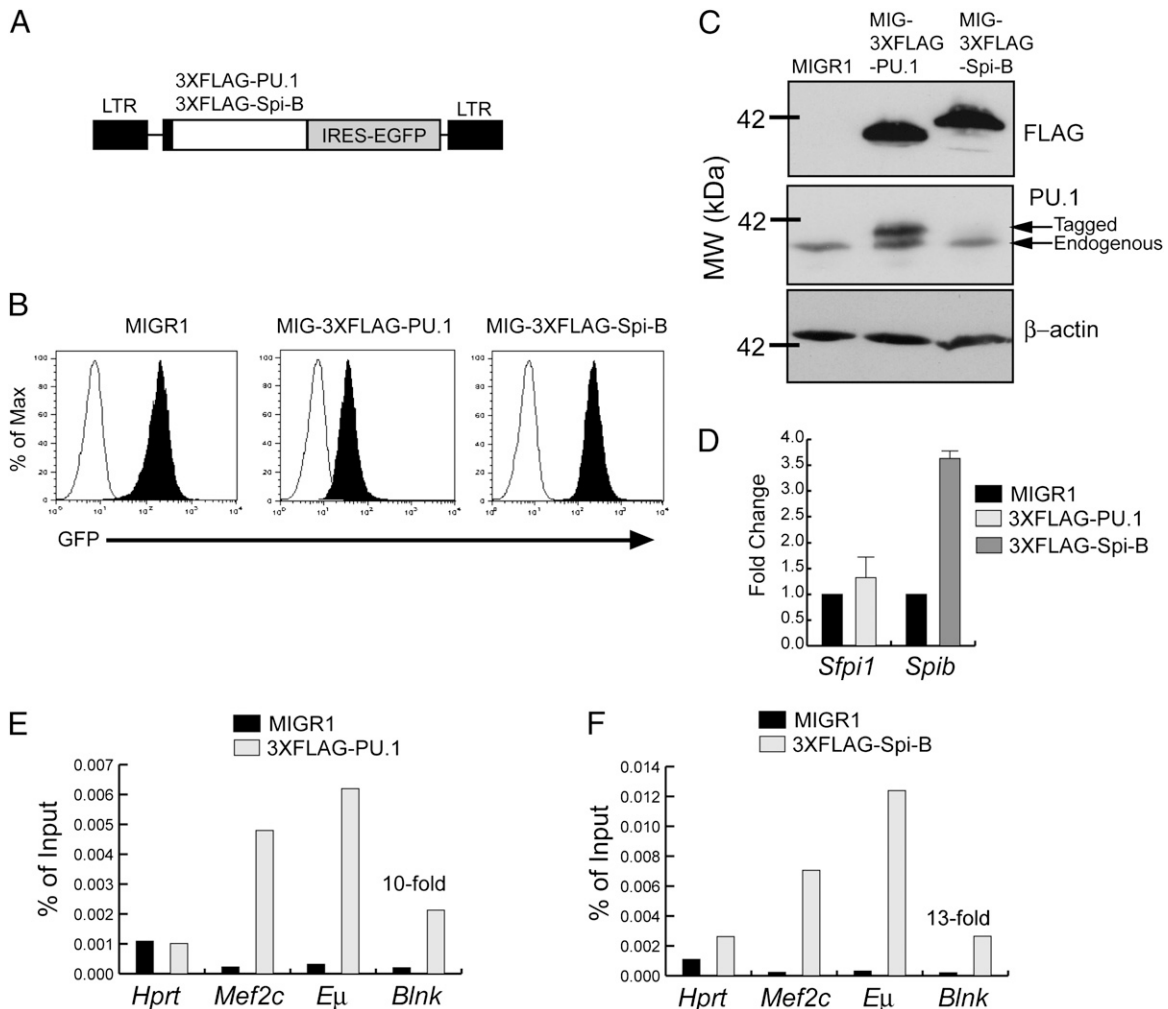


**FIGURE 3.**  $\Delta$ PB B-ALL cells express reduced levels of BLNK. **(A)** Reduced levels of BLNK protein in  $\Delta$ PB B-ALL cells. Immunoblot was used to compare relative levels of BLNK protein in two independent  $\Delta$ PB B-ALL cell lines relative to IL-7-dependent wild-type pro-B cells. As a loading control, levels of  $\beta$ -actin protein were determined by immunoblot (lower panel). **(B)** Reduced levels of *Blnk* mRNA transcripts in  $\Delta$ PB B-ALL cells. RT-qPCR was used to compare relative levels of *Blnk* mRNA transcripts in  $\Delta$ PB B-ALL cells compared with IL-7-dependent wild-type pro-B cells. Transcript levels were normalized to  $\beta_2$ -microglobulin mRNA transcript levels. \*\*\* $p < 0.001$  by Student *t* test.

Because anti-Spi-B Abs were not available, WEHI-279 B lymphoma clones were generated expressing GFP and 3XFLAG-tagged full-length PU.1 or Spi-B protein under the control of a retroviral long terminal repeat promoter (Fig. 5A, 5B). As a negative control, a WEHI-279 clone expressing only GFP (MIGR1)



**FIGURE 4.** *Blnk* is transcriptionally activated by PU.1 and/or Spi-B. **(A)** Schematic of the *Blnk* promoter region. Major transcription start sites are indicated by arrowheads. Predicted PU.1/Spi-B binding sites are indicated by boxes. Numbers indicate matrix similarity scores. Underlined sequences represent previously identified Pax5 binding sites. **(B)** Reduced *Blnk* promoter activity after mutagenesis of a predicted PU.1/Spi-B binding site. The *Blnk* promoter fragment shown in (A) was tested for activity in a luciferase assay with either an intact PU.1/Spi-B binding site (BLNKp) or the site mutated as shown in (C) (BLNKp-Mut). \*\* $p < 0.01$  by Student *t* test. **(C)** Site-directed mutagenesis of a predicted PU.1/Spi-B binding site. MUT, Mutated DNA sequence.



**FIGURE 5.** Interaction of PU.1 and Spi-B with the *Blnk* promoter. **(A)** Schematic of retroviral vectors. **(B)** Flow cytometric analysis of WEHI-279 clones expressing GFP only (MIGR1), FLAG-tagged PU.1 and GFP, or Spi-B and GFP. **(C)** Expression of FLAG-tagged proteins by WEHI-279 clones. Immunoblot was performed on lysates from the clones described in **(A)** using anti-FLAG Ab (upper panel), anti-PU.1 Ab (center panel), or anti- $\beta$ -actin Ab (lower panel). **(D)** Increase in steady-state levels of transcripts encoding PU.1 and Spi-B in infected WEHI-279 cells. RT-qPCR was performed to determine relative levels of the indicated transcripts in WEHI-279 cells infected with control retrovirus (MIGR1) or retrovirus encoding PU.1 (3XFLAG-PU.1) or Spi-B (3XFLAG-Spi-B). **(E and F)** ChIP analysis. Chromatin prepared from the clones described in **(A)–(D)** was immunoprecipitated with anti-FLAG Ab. Immunoprecipitated DNA was quantified by relative qPCR using primers recognizing the hypoxanthine-guanine phosphoribosyltransferase promoter (*Hprt*), *Mef2c* enhancer (*Mef2c*), *IgH* intronic enhancer (*E $\mu$* ), or *Blnk* promoter (*Blnk*). Amounts of immunoprecipitated DNA are expressed as percentage of input. Enrichment was calculated as the fold change in percentage of input. Results shown are representative of three independent experiments.

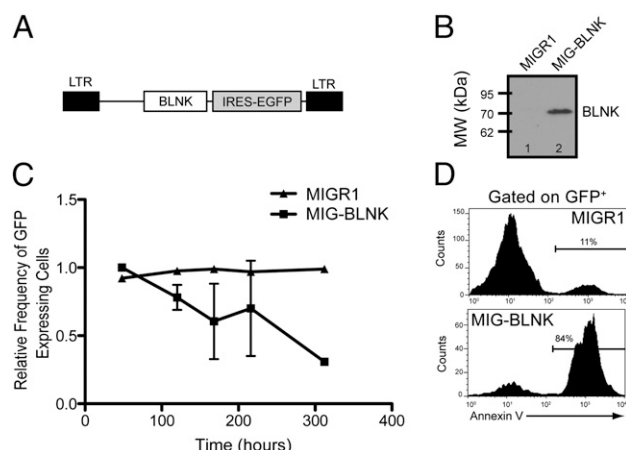
was also generated (Fig. 5B). WEHI-279 cells infected with MIG-3XFLAG-PU.1 retrovirus expressed 2- to 3-fold higher PU.1 protein levels and 1.3-fold higher *Sfpi1* mRNA transcript levels than controls (Fig. 5C, 5D). A WEHI-279 clone infected with MIG-3XFLAG-Spi-B was selected for expression of FLAG-tagged Spi-B at levels comparable with FLAG-tagged PU.1, and was found to express *SpiB* mRNA transcripts at 3.6-fold higher levels than endogenous *SpiB* transcript levels in control cells (Fig. 5C, 5D). ChIP was performed on fixed WEHI-279 clonal cell lines using anti-FLAG mAb. qPCR was performed on immunoprecipitated DNA to determine association of regulatory regions with 3XFLAG-tagged PU.1 or Spi-B. Enrichment was compared with DNA immunoprecipitated from MIGR1 cells using anti-FLAG Ab. The promoter of the *Hprt* gene, which was not expected to be associated with PU.1 or Spi-B, was not significantly enriched by immunoprecipitation with anti-FLAG Ab (Fig. 5D, 5E). In contrast, intronic enhancers of the *Mef2c* and *IgH* loci, which are highly expressed in the B cell lineage under control of PU.1 (39, 40), were enriched in cells expressing FLAG-tagged

PU.1 and Spi-B (Fig. 5D, 5E). PU.1 and Spi-B were associated with the *Blnk* promoter, showing enrichment by 10- and 13-fold compared with the negative control, respectively (Fig. 5D, 5E). Reanalysis of published ChIP-sequencing data using anti-PU.1 in murine B cells (41) confirmed that endogenous PU.1 interacts with the site we identified in the *Blnk* promoter (data not shown). Taken together, these results suggest that PU.1 and Spi-B activate *Blnk* transcription by direct interaction with the *Blnk* promoter.

#### Forced expression of BLNK opposes the growth of cultured $\Delta$ PB B-ALL cells

If transformation of  $\Delta$ PB cells is, in part, a consequence of reduced BLNK expression, then restoration of BLNK expression might be expected to oppose growth of these cells in vitro. To test this idea, we used RT-PCR to amplify the full *Blnk* coding sequence from C57BL/6 splenic B cell RNA. *Blnk* cDNA was cloned and ligated into the retroviral vector MIGR1 to promote expression of BLNK protein, as well as enhanced GFP under control of the retroviral long terminal repeat promoter (Fig. 6A).





**FIGURE 6.** Forced expression of BLNK opposes IL-7-dependent proliferation of  $\Delta$ PB B-ALL cells. **(A)** Schematic of the MIG-BLNK retroviral vector. **(B)** Expression of BLNK protein in NIH-3T3 cells infected with retroviral vectors. Immunoblot was used to determine BLNK expression in NIH-3T3 cells infected with either MIGR1 or MIG-BLNK. **(C)** Reduced frequency of MIG-BLNK-infected  $\Delta$ PB B-ALL cells upon serial passage. Flow cytometry was used to determine the percentage of cells infected with MIGR1 (triangles) or MIG-BLNK (squares) at the time points shown on the x-axis. The y-axis shows relative infection frequencies normalized to the frequency of infected cells at 48 h. **(D)** Increased apoptosis of MIG-BLNK-infected  $\Delta$ PB B-ALL cells 48 h postinfection. Cells were stained with Annexin V and analyzed using flow cytometry. Results are shown gated on GFP<sup>+</sup> cells.

BLNK protein was expressed in NIH-3T3 cells infected with the MIG-BLNK retrovirus (Fig. 6B). To determine whether forced BLNK expression opposes proliferation of cultured  $\Delta$ PB B-ALL cells, we infected cultured  $\Delta$ PB cells with MIG-BLNK retrovirus or with MIGR1 retrovirus as a control. Infected cells were cultured 48 h to promote retroviral integration, and the frequency of cells expressing GFP was determined by flow cytometry on passage every 72–96 h. The relative frequency of MIGR1-infected cells changed no more than 1.2-fold for the duration of the experiment. In contrast, the relative frequency of MIG-BLNK-infected cells was reduced with each passage until termination of the experiment (Fig. 6C). This result suggested that BLNK-infected  $\Delta$ PB cells proliferated poorly compared with MIGR1-infected cells. To confirm this finding, we placed MIGR1- or MIG-BLNK-infected  $\Delta$ PB cells in methylcellulose colony-forming assays with IL-7. After 7 d, GFP<sup>+</sup> and GFP<sup>−</sup> colonies were counted using an inverted fluorescence microscope. The frequency of GFP<sup>+</sup> colonies generated by MIGR1-infected cells was similar to the frequency of GFP<sup>+</sup> cells placed in the assay as determined by flow cytometry. In contrast, no GFP<sup>+</sup> colonies were generated from MIG-BLNK-infected  $\Delta$ PB cells. To determine why BLNK-infected  $\Delta$ PB cells failed to proliferate, we measured early apoptosis using Annexin V staining 48 h after retroviral infection. A high frequency of MIG-BLNK-infected cells stained positively with Annexin V compared with MIGR1-infected cells (Fig. 6D). We conclude that restoration of BLNK expression using a retroviral vector opposes IL-7-dependent proliferation of  $\Delta$ PB B-ALL cell lines, at least, in part, by inducing early apoptosis.

## Discussion

This study explored the mechanism by which deletion of genes encoding PU.1 and Spi-B induces B-ALL in mice. We showed that B-ALL cells from the enlarged thymus of  $\Delta$ PB mice express IL-7R on their surface and can be grown ex vivo in response to IL-7. Second, we showed that the gene encoding BLNK is a direct target

of activation by PU.1 and/or Spi-B. These results suggest that PU.1 and Spi-B are complementary activators of *Blnk* transcription, and that B-ALL is induced in  $\Delta$ PB mice, at least in part, as a consequence of reduced BLNK levels.

PU.1 and Spi-B have both been implicated as oncogenes or tumor suppressors, depending on the circumstance. PU.1 was discovered as a gene upregulated in murine erythroleukemia as a consequence of proviral insertion by the murine SFFV retrovirus (42). Overexpression of PU.1 has also been implicated as a cause of T cell leukemia in genetically modified mice (43). In contrast, reduced levels of PU.1 caused by mutation or repression of the *Spi1* gene are sufficient to induce acute myeloid leukemia (8, 44). Spi-B is frequently overexpressed in diffuse large B cell lymphoma (45). Therefore, PU.1 and Spi-B have known functions as either oncogenes or tumor suppressors, but little is known about the mechanism of these functions. We expect that PU.1 and Spi-B transcriptionally activate downstream target genes with tumor-suppressor function.

BLNK is considered a tumor suppressor because: 1) point mutations or reduced levels of BLNK are associated with human B-ALL (21–23), and 2) mutation of *Blnk* is sufficient to induce B-ALL in mice (24, 46). However, BLNK is required for B cell development in mice and humans, causing primary human B cell deficiency when mutated (16, 20). BLNK is required for B cell development because it links signals from the pre-BCR or BCR with B cell differentiation by functioning as an adaptor or scaffold to promote interaction of key signaling proteins (20). Several recent studies suggest that BLNK has a dual function of enforcing BCR signaling whereas blocking IL-7 signaling (35, 46, 47). BLNK interacts directly with JAK3, promoting the uncoupling of JAK3 from IL-7 signaling (46). BLNK also inhibits activation of the PI3K-Akt pathway downstream of IL-7R signaling (35, 47). Therefore, reduced BLNK levels, as might be induced by mutation, repression, or reduced transcriptional activation, would be expected to result in increased IL-7R signaling, as well as reduced BCR signaling, promoting proliferation and impairing differentiation (35, 46).

Addition to IL-7 signaling may be a common feature of pediatric leukemia. Human B-ALL tumor cells frequently express functional IL-7R on their surface and can respond to IL-7 in culture (48). Gain-of-function mutations in the *IL7R* gene (encoding IL-7R $\alpha$ ) are associated with pediatric ALL (49). In mice, overexpression of IL-7 is sufficient to induce lymphoproliferation leading to leukemia (50). As described in *Results*, B-ALL cells in  $\Delta$ PB mice express high levels of IL-7R on their surface and grow readily in response to IL-7 ex vivo. These results suggest that IL-7R expression and IL-7 signaling plays a role in development of leukemia in  $\Delta$ PB mice. Developing B cells express high levels of IL-7R $\alpha$ , but this receptor is not expressed by mature B cells (51). Little is known about what factors control transcriptional downregulation of *IL7R* in developing B cells; however, we speculate that BLNK might be involved in regulation of IL-7R $\alpha$  expression, as well as downstream signaling. B-ALL cells from *Blnk*<sup>−/−</sup> mice express IL-7R on their surface and can be efficiently propagated in culture in response to IL-7 (23, 24). In addition, *Blnk*<sup>−/−</sup> B-ALL cells grow in autocrine fashion, both producing and proliferating in response to IL-7 (46). Therefore, in the absence of BLNK, pre-B cells might have sustained IL-7R signaling as a consequence of a failure to inhibit IL-7R signaling, as well as a failure to appropriately downregulate IL-7R $\alpha$  expression.

Our findings suggest that activation of BLNK expression is an important pathway by which PU.1 and/or Spi-B regulate B cell differentiation. B cells deficient in PU.1 and/or Spi-B have severely impaired BCR signaling (52) and, as a consequence, cannot

efficiently differentiate into follicular B cells (31). PU.1 and Spi-B regulate a number of genes required for BCR signaling, including P2Y10, Grap2, and Btk (53, 54). Interestingly, mutation of *Btk* strongly cooperates with mutations in *Blnk* to induce B-ALL in mice (55). These results suggest that PU.1 and Spi-B increase BLNK and Btk levels to enforce B cell differentiation and oppose oncogenic transformation.

B-ALL is frequently associated with chromosomal translocations that result in generation of abnormal transcription factors. Twenty-five percent of cases of pre-B ALL involve the t(12;21) chromosomal translocation that results in an *ETV6-RUNX1* (also known as *TEL-AML1*) fusion gene (1). The significance of this observation to this study is that RUNX1 is an important upstream activator of the *SP11* gene in humans and the *Sfp11* gene in mice (56). PU.1 levels are reduced upon expression of RUNX1 fusion proteins such as ETO-RUNX1 (57, 58). Recently, it was also shown that B-ALL cells harboring *ETV6-RUNX1* fusions express reduced levels of Spi-B (14). Based on these observations, we wish to propose a hypothetical model for B-ALL leukemogenesis involving PU.1 and Spi-B. First, chromosomal translocations resulting in the generation of *ETV6-RUNX1* fusion protein in 25% of all B-ALL patients may result in reduced PU.1 and Spi-B levels as a consequence of reduced *SP11* and *SP1B* transcription. Reduced levels might additionally cooperate with loss-of-function *SP11* mutations that have been observed (7). Reduced levels of PU.1 and Spi-B in pre-B cells would result in changes in gene expression, including reduced *BLNK*, leading to increased IL-7-dependent proliferation. Increased proliferation and/or expansion of pre-B cells may lead to B-ALL by additional mechanisms including genomic instability. Interestingly, BLNK has also been implicated in the maintenance of genomic integrity (59). Further consideration of this pathway of leukemic transformation may result in identification of checkpoints that could be evaluated for molecular targeted therapy.

## Acknowledgments

We thank Dr. Kristin Chadwick of the London Regional Flow Cytometry facility for cell sorting. We thank Dr. Ian Welch and Heather-Anne T. Cadieux-Pitre for excellent animal care and advice.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Pui, C. H., L. L. Robison, and A. T. Look. 2008. Acute lymphoblastic leukaemia. *Lancet* 371: 1030–1043.
- Cobaleda, C., and I. Sánchez-García. 2009. B-cell acute lymphoblastic leukaemia: towards understanding its cellular origin. *Bioessays* 31: 600–609.
- Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265: 1573–1577.
- Iwasaki, H., C. Somoza, H. Shigematsu, E. A. Duprez, J. Iwasaki-Arai, S. Mizuno, Y. Arinobu, K. Geary, P. Zhang, T. Dayaram, et al. 2005. Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* 106: 1590–1600.
- Mueller, B. U., T. Pabst, M. Osato, N. Asou, L. M. Johansen, M. D. Minden, G. Behre, W. Hiddemann, Y. Ito, and D. G. Tenen. 2002. Heterozygous PU.1 mutations are associated with acute myeloid leukemia. *Blood* 100: 998–1007.
- Link, D. C., G. Kunter, Y. Kasai, Y. Zhao, T. Miner, M. D. McLellan, R. E. Ries, D. Kapur, R. Nagarajan, D. C. Dale, et al. 2007. Distinct patterns of mutations occurring in de novo AML versus AML arising in the setting of severe congenital neutropenia. *Blood* 110: 1648–1655.
- Mullighan, C. G., J. Zhang, L. H. Kasper, S. Lerach, D. Payne-Turner, L. A. Phillips, S. L. Heatley, L. Holmfeldt, J. R. Collins-Underwood, J. Ma, et al. 2011. CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature* 471: 235–239.
- Rosenbauer, F., K. Wagner, J. L. Kutok, H. Iwasaki, M. M. Le Beau, Y. Okuno, K. Akashi, S. Fiering, and D. G. Tenen. 2004. Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat. Genet.* 36: 624–630.
- Walter, M. J., J. S. Park, R. E. Ries, S. K. Lau, M. McLellan, S. Jaeger, R. K. Wilson, E. R. Mardis, and T. J. Ley. 2005. Reduced PU.1 expression causes myeloid progenitor expansion and increased leukemia penetrance in mice expressing PML-RARalpha. *Proc. Natl. Acad. Sci. USA* 102: 12513–12518.
- Nishii, K., K. Kita, H. Miwa, M. Shikami, M. Taniguchi, E. Usui, N. Katayama, and H. Shiku. 2000. Expression of B cell-associated transcription factors in B-cell precursor acute lymphoblastic leukemia cells: association with PU.1 expression, phenotype, and immunogenotype. *Int. J. Hematol.* 71: 372–378.
- Jundt, F., K. Kley, I. Anagnostopoulos, K. Schulze Pröbsting, A. Greiner, S. Mathas, C. Scheidereit, T. Wirth, H. Stein, and B. Dörken. 2002. Loss of PU.1 expression is associated with defective immunoglobulin transcription in Hodgkin and Reed-Sternberg cells of classical Hodgkin disease. *Blood* 99: 3060–3062.
- Ray, D., R. Bosselut, J. Ghysdael, M. G. Mattei, A. Tavittian, and F. Moreau-Gachelin. 1992. Characterization of Spi-B, a transcription factor related to the putative oncoprotein Spi-1/PU.1. *Mol. Cell. Biol.* 12: 4297–4304.
- Wei, G. H., G. Badis, M. F. Berger, T. Kivioja, K. Palin, M. Enge, M. Bonke, A. Jolma, M. Varjosalo, A. R. Gehrke, et al. 2010. Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. *EMBO J.* 29: 2147–2160.
- Fuka, G., M. Kauer, R. Kofler, O. A. Haas, and R. Panzer-Grümayer. 2011. The leukemia-specific fusion gene ETV6/RUNX1 perturbs distinct key biological functions primarily by gene repression. *PLoS ONE* 6: e26348.
- Sokalski, K. M., S. K. Li, I. Welch, H. A. Cadieux-Pitre, M. R. Gruca, and R. P. DeKoter. 2011. Deletion of genes encoding PU.1 and Spi-B in B cells impairs differentiation and induces pre-B cell acute lymphoblastic leukemia. *Blood* 118: 2801–2808.
- Minegishi, Y., J. Rohrer, E. Coustan-Smith, H. M. Lederman, R. Pappu, D. Campana, A. C. Chan, and M. E. Conley. 1999. An essential role for BLNK in human B cell development. *Science* 286: 1954–1957.
- Pappu, R., A. M. Cheng, B. Li, Q. Gong, C. Chiu, N. Griffin, M. White, B. P. Sleckman, and A. C. Chan. 1999. Requirement for B cell linker protein (BLNK) in B cell development. *Science* 286: 1949–1954.
- Jumaa, H., B. Wollscheid, M. Mitterer, J. Wienands, M. Reth, and P. J. Nielsen. 1999. Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. *Immunity* 11: 547–554.
- Wienands, J., J. Schweikert, B. Wollscheid, H. Jumaa, P. J. Nielsen, and M. Reth. 1998. SLP-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. *J. Exp. Med.* 188: 791–795.
- Fu, C., C. W. Turck, T. Kurosaki, and A. C. Chan. 1998. BLNK: a central linker protein in B cell activation. *Immunity* 9: 93–103.
- Mullighan, C. G., S. Goorha, I. Radtke, C. B. Miller, E. Coustan-Smith, J. D. Dalton, K. Girtman, S. Mathew, J. Ma, S. B. Pounds, et al. 2007. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 446: 758–764.
- Zhang, J., C. G. Mullighan, R. C. Harvey, G. Wu, X. Chen, M. Edmonson, K. H. Buetow, W. L. Carroll, I. M. Chen, M. Devidas, et al. 2011. Key pathways are frequently mutated in high-risk childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Blood* 118: 3080–3087.
- Jumaa, H., L. Bossaller, K. Portugal, B. Storch, M. Lotz, A. Flemming, M. Schrappe, V. Postila, P. Riikonen, J. Pelkonen, et al. 2003. Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia. *Nature* 423: 452–456.
- Flemming, A., T. Brummer, M. Reth, and H. Jumaa. 2003. The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. *Nat. Immunol.* 4: 38–43.
- Winkler, T. H., F. Melchers, and A. G. Rolink. 1995. Interleukin-3 and interleukin-7 are alternative growth factors for the same B-cell precursors in the mouse. *Blood* 85: 2045–2051.
- Christie, D. A., C. D. Lemke, I. M. Elias, L. A. Chau, M. G. Kirchhof, B. Li, E. H. Ball, S. D. Dunn, G. M. Hatch, and J. Madrenas. 2011. Stomatin-like protein 2 binds cardiolipin and regulates mitochondrial biogenesis and function. *Mol. Cell. Biol.* 31: 3845–3856.
- Cartharius, K., K. Frech, K. Grote, B. Klocke, M. Haltmeier, A. Klingenhoff, M. Frisch, M. Bayerlein, and T. Werner. 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 21: 2933–2942.
- DeKoter, R. P., H. J. Lee, and H. Singh. 2002. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity* 16: 297–309.
- Morita, S., T. Kojima, and T. Kitamura. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* 7: 1063–1066.
- Godbey, W. T., K. K. Wu, and A. G. Mikos. 1999. Poly(ethyleneimine) and its role in gene delivery. *J. Control. Release* 60: 149–160.
- DeKoter, R. P., M. Geadah, S. Khoosal, L. S. Xu, G. Thillainadesan, J. Torchia, S. S. Chin, and L. A. Garrett-Sinha. 2010. Regulation of follicular B cell differentiation by the related E26 transformation-specific transcription factors PU.1, Spi-B, and Spi-C. *J. Immunol.* 185: 7374–7384.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: e45.
- Cooper, M. D., D. Mulvaney, A. Coutinho, and P. A. Cazenave. 1986. A novel cell surface molecule on early B-lineage cells. *Nature* 321: 616–618.
- McKearn, J. P., C. Baum, and J. M. Davie. 1984. Cell surface antigens expressed by subsets of pre-B cells and B cells. *J. Immunol.* 132: 332–339.
- Ochiai, K., M. Maienschein-Cline, M. Mandal, J. R. Triggs, E. Bertolino, R. Sciammas, A. R. Dinner, M. R. Clark, and H. Singh. 2012. A self-reinforcing regulatory network triggered by limiting IL-7 activates pre-BCR signaling and differentiation. *Nat. Immunol.* 13: 300–307.



36. 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.
37. Rolink, A. G., E. ten Boekel, T. Yamagami, R. Ceredig, J. Andersson, and F. Melchers. 1999. B cell development in the mouse from early progenitors to mature B cells. *Immunol. Lett.* 68: 89–93.
38. Schebesta, M., P. L. Pfeffer, and M. Busslinger. 2002. Control of pre-BCR signaling by Pax5-dependent activation of the BLNK gene. *Immunity* 17: 473–485.
39. Stehling-Sun, S., J. Dade, S. L. Nutt, R. P. DeKoter, and F. D. Camargo. 2009. Regulation of lymphoid versus myeloid fate 'choice' by Mef2c. *Nat. Immunol.* 10: 289–296.
40. Nelsen, B., G. Tian, B. Erman, J. Gregoire, R. Maki, B. Graves, and R. Sen. 1993. Regulation of lymphoid-specific immunoglobulin mu heavy chain gene enhancer by ETS-domain proteins. *Science* 261: 82–86.
41. Heinz, S., C. Benner, N. Spann, E. Bertolino, Y. C. Lin, P. Laslo, J. X. Cheng, C. Murre, H. Singh, and C. K. Glass. 2010. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* 38: 576–589.
42. Moreau-Gachelin, F., A. Tavittian, and P. Tambourin. 1988. Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. *Nature* 331: 277–280.
43. Rosenbauer, F., B. M. Owens, L. Yu, J. R. Tumang, U. Steidl, J. L. Kutok, L. K. Clayton, K. Wagner, M. Scheller, H. Iwasaki, et al. 2006. Lymphoid cell growth and transformation are suppressed by a key regulatory element of the gene encoding PU.1. *Nat. Genet.* 38: 27–37.
44. Metcalf, D., A. Dakic, S. Mifsud, L. Di Rago, L. Wu, and S. Nutt. 2006. Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia. *Proc. Natl. Acad. Sci. USA* 103: 1486–1491.
45. Lenz, G., G. W. Wright, N. C. Emre, H. Kohlhammer, S. S. Dave, R. E. Davis, S. Carty, L. T. Lam, A. L. Shaffer, W. Xiao, et al. 2008. Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. *Proc. Natl. Acad. Sci. USA* 105: 13520–13525.
46. Nakayama, J., M. Yamamoto, K. Hayashi, H. Satoh, K. Bundo, M. Kubo, R. Goitsuka, M. A. Farrar, and D. Kitamura. 2009. BLNK suppresses pre-B-cell leukemogenesis through inhibition of JAK3. *Blood* 113: 1483–1492.
47. Herzog, S., E. Hug, S. Meixlsperger, J. H. Paik, R. A. DePinho, M. Reth, and H. Jumaa. 2008. SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway. *Nat. Immunol.* 9: 623–631.
48. Touw, I., K. Pouwels, T. van Agthoven, R. van Gurp, L. Budel, H. Hoogerbrugge, R. Delwel, R. Goodwin, A. Namen, and B. Löwenberg. 1990. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. *Blood* 75: 2097–2101.
49. Shochat, C., N. Tal, O. R. Bandapalli, C. Palmi, I. Ganmore, G. te Kronnie, G. Cario, G. Cazzaniga, A. E. Kulozik, M. Stanulla, et al. 2011. Gain-of-function mutations in interleukin-7 receptor- $\alpha$  (IL7R) in childhood acute lymphoblastic leukemias. *J. Exp. Med.* 208: 901–908.
50. Rich, B. E., J. Campos-Torres, R. I. Tepper, R. W. Moreadith, and P. Leder. 1993. Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. *J. Exp. Med.* 177: 305–316.
51. Sudo, T., S. Nishikawa, N. Ohno, N. Akiyama, M. Tamakoshi, H. Yoshida, and S. Nishikawa. 1993. Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc. Natl. Acad. Sci. USA* 90: 9125–9129.
52. Garrett-Sinha, L. A., G. H. Su, S. Rao, S. Kabak, Z. Hao, M. R. Clark, and M. C. Simon. 1999. PU.1 and Spi-B are required for normal B cell receptor-mediated signal transduction. *Immunity* 10: 399–408.
53. Garrett-Sinha, L. A., P. Hou, D. Wang, B. Grabiner, E. Araujo, S. Rao, T. J. Yun, E. A. Clark, M. C. Simon, and M. R. Clark. 2005. Spi-1 and Spi-B control the expression of the Grap2 gene in B cells. *Gene* 353: 134–146.
54. Müller, S., A. Maas, T. C. Islam, P. Sideras, G. Suske, S. Philipsen, K. G. Xanthopoulos, R. W. Hendriks, and C. I. Smith. 1999. Synergistic activation of the human Btk promoter by transcription factors Sp1/3 and PU.1. *Biochem. Biophys. Res. Commun.* 259: 364–369.
55. Ta, V. B., A. B. de Haan, M. J. de Bruijn, G. M. Dingjan, and R. W. Hendriks. 2011. Pre-B-cell leukemias in Btk/Slp65-deficient mice arise independently of ongoing V(D)J recombination activity. *Leukemia* 25: 48–56.
56. Huang, G., P. Zhang, H. Hirai, S. Elf, X. Yan, Z. Chen, S. Koschmieder, Y. Okuno, T. Dayaram, J. D. Growney, et al. 2008. PU.1 is a major downstream target of AML1 (RUNX1) in adult mouse hematopoiesis. *Nat. Genet.* 40: 51–60.
57. Vangala, R. K., M. S. Heiss-Neumann, J. S. Rangatia, S. M. Singh, C. Schoch, D. G. Tenen, W. Hiddemann, and G. Behre. 2003. The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. *Blood* 101: 270–277.
58. Wang, L., A. Gural, X. J. Sun, X. Zhao, F. Perna, G. Huang, M. A. Hatlen, L. Vu, F. Liu, H. Xu, et al. 2011. The leukemogenicity of AML1-ETO is dependent on site-specific lysine acetylation. *Science* 333: 765–769.
59. Kamino, H., M. Futamura, Y. Nakamura, N. Kitamura, K. Kabu, and H. Arakawa. 2008. B-cell linker protein prevents aneuploidy by inhibiting cytokinesis. *Cancer Sci.* 99: 2444–2454.