Reduction in PU.1 Activity Results in a Block to B Cell Development, Abnormal Myeloid Proliferation, and Neonatal Lethality

Isaac B. Houston, **Meghana Kamath**, **Brock L. Schweitzer**, **Timothy M. Chlon**, and **Rodney P. DeKoter, Ph.D.**
From the Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH.

Abstract

Objective—It has been demonstrated that high concentration of the transcription factor PU.1 (encoded by *Sfpi1*) promotes macrophage development, whereas low concentration induces B cell development *in vitro*. This has led to the hypothesis that lower levels of PU.1 activity are required for B cell than for macrophage development *in vivo*. We utilized an allele of *Sfpi1* (termed BN) with a mutation in the first coding exon which resulted in a reduction of PU.1 expression in order to test this hypothesis.

Methods—Using gene targeting in ES cells, two ATG-start site codons of PU.1 were mutated, resulting in reduced PU.1 expression originating from a third start codon. Mice were assayed for phenotypic abnormalities using fluorescence activated cell sorting, microscopy, and colony forming ability. In addition, isolated cells were tested for their differentiation potential *in vitro* and *in vivo*.

Results—Lymphoid and myeloid cells derived from cultured $Sfpi1^{BN/BN}$ fetal liver cells had reduced levels of PU.1 expression and activity. B cell development was intrinsically blocked in cells isolated from $Sfpi1^{BN/BN}$ mice. In addition, myeloid development was impaired in $Sfpi1^{BN/BN}$ fetal liver. However, neonatal $Sfpi1^{BN/BN}$ mice had a dramatic expansion and infiltration of immature myeloid cells.

Conclusion—Contrary to our original hypothesis, high levels of PU.1 activity are required to induce both myeloid and B cell development. In addition, neonatal mice homozygous for the hypomorphic allele acquire a myeloproliferative disorder and die within 1 month of age.

Introduction

Hematopoiesis is a complex patterning process in which the various distinct cellular lineages of the blood system are generated from a pluripotent stem cell. Many key regulatory steps of hematopoietic development are controlled cell-intrinsically at the level of transcription. The genes involved in this process are regulated by an elaborate network of transcription factors. One transcription factor of particular interest is PU.1 (encoded by the gene *Sfpi1*), a tissue-specific Ets family member that is expressed in both lymphoid and myeloid cells of the immune system [1,2]. Gene targeting studies have demonstrated that PU.1 is required for the

Corresponding Author: Dr. Rodney DeKoter, Ph.D., Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, MSB 3304, 231 Albert Sabin Way, Cincinnati, OH 45267-0524, Phone (513) 558-3938, Fax (513) 558-8474, E-mail: Rodney.DeKoter@uc.edu

development of both myeloid and lymphoid lineages [3–5]. Previous research has suggested that differences in PU.1 concentration are involved in hematopoietic cell fate decisions. Specifically, PU.1 is expressed at high levels in myeloid lineage cells and at lower levels in B lymphocytes [1,2,6,7]. Based on retroviral gene transfer experiments performed *in vitro*, it was hypothesized that high concentration of PU.1 promotes differentiation into macrophages, whereas low concentration of PU.1 induces differentiation into B cells [8].

Several recently published studies have utilized a reporter gene encoding green fluorescent protein (GFP) inserted into the *Sfpi1* locus to monitor the level of PU.1 transcription in lymphoid and myeloid cells *in vivo*. These studies suggested that PU.1 is expressed at uniformly high levels in hematopoietic stem cells (HSCs) and common lymphoid progenitor cells (CLPs) [9,10]. During commitment to the B cell lineage, PU.1 expression is reduced to lower levels [10]. Conditional knockout studies demonstrated that deletion of *Sfpi1* in committed B cell progenitors minimally affects B cell development [11–13]. An important question that remains unanswered in these studies is whether high PU.1 activity is required for specification to the B lineage.

To test our hypothesis, we generated a novel hypomorphic allele of the *Sfpi1* gene generated by mutation of the first coding exon. Mice homozygous for this allele (termed BN) survive 1–3 weeks after birth and are osteopetrotic and runted. Splenic expression of PU.1 is dramatically reduced in $Sfpi1^{BN/BN}$ mice. Furthermore, PU.1 expression and activity are reduced in both lymphoid and myeloid cells cultured from $Sfpi1^{BN/BN}$ fetal liver. $Sfpi1^{BN/BN}$ neonatal mice are B cell deficient. In addition, fetal liver progenitors from $Sfpi1^{BN/BN}$ fetuses cannot reconstitute B cell development upon transplantation into immunodeficient recipient mice. Finally, we demonstrate that myeloid cell differentiation is impaired in $Sfpi1^{BN/BN}$ fetal liver. However, neonatal $Sfpi1^{BN/BN}$ mice have a dramatic proliferation of immature myeloid cells in their spleens and bone marrow. These results suggest that high levels of PU.1 activity are required for both lymphoid and myeloid development. In addition, these results demonstrate that reduced expression of PU.1 results in a neonatal myeloproliferative disorder.

Materials and Methods

Gene targeting and mice

3 kilobases (kb) upstream and 3.5 kb downstream of the two identified ATG-start codons (6.5 kb total) of the *Sfpi1* locus were inserted into the osdupdel2 vector along with *E. coli* TEM-1 β-lactamase coding sequence, obtained by digestion of BlaM(−)/pUC plasmid (Invitrogen, Carlsbad, CA, USA) with *SalI* and *KpnI*, to generate the 53Bladd targeting vector. Primers used to amplify the *Sfpi1* sequence from genomic DNA from 129SV mice were as follows: 5′-arm (5′-TCAGCACCACAGCAATAGTCTCGGG-3′ and 5′-TCCAGGTTGGTCAGATCCCCTGCTTCGGTG-3′), 3′-arm (5′-GAAGGGTTTTCCCTCACCGCCCCTGTGAG-3′ and 5′-GGCCAAGCAGAACGCAACACTGATGAACTC-3′). 129SV ES cells were electroporated with linearized targeting vector DNA and positive/negative selection of clones was performed using standard methods. Recombination events were screened by PCR and confirmed by Southern blot analysis on *HindIII* digested DNA and a probe directed against the neomycin gene. Additionally, DNA sequencing was performed on PCR-amplified genomic DNA. The mice used in this study were a primarily outbred mixture of C57Bl/6 and Black/Swiss (Taconic, Germantown, NY). For genotyping, PCR was performed with the following genotyping primers: 1 (5′-CGGCCAGAGACTTCCTGTAG-3′), 2 (5′-AAGTTGGCCGCAGTGTTATC-3′), 3 (5′-GCTCTTCGTCCAGATCATCC-3′), 4 (5′-ATGGTCACACATCCCAAAGC-3′).

Construction of Retroviral Vectors

MSCV-IRES-EGFP (MigR1) and MSCV-PU.1-IRES-EGFP (MigPU.1, HA-PU.1wt) retroviruses have been described previously [14]. Retroviral vectors encoding wild-type or *Sfpi1*[BN] transcripts were constructed by inserting either transcript between the *XhoI* and *EcoRI* sites of MigR1. Transcripts were amplified from cDNA prepared from pro-B cells using LA-TAQ (TaKaRa Bio Inc., Otsu Shiga, Japan) and cloned using the topoisomerase cloning kit (Stratagene, La Jolla CA). Primers used were as follows: wild-type forward (5′-ACCTGGAGCTCAGCTGGATGTTA-3′), BN forward (5′-CGTATCGATTGTACACTTAAGGGCCG-3′), common reverse (5′GGGCGACGGGTTAATGCTAT-3′). HA-PU.1ΔN31 retrovirus was constructed by insertion of ΔN31 transcript into the *Not I* and *Apa I* sites to replace the Spi-C sequence of MIG-Spi-C vector [15]. ΔN31 transcripts were amplified from pBluescript plasmid containing PU.1 cDNA lacking the N-terminal 31 amino acids and cloned as above. Primers used were as follows: DN31 forward (5′-CG<u>GCGGCCGC</u>ATGCATGACTACTACTCCTTC-3′) and DN31 reverse (5′-CC<u>GGGCCC</u>TCAGTGGGGCGGGAGGCGCCG-3′)

Cell Culture

Cell culture conditions and generation of pro-B cell lines have been described previously [16]. Cultured IL-3 myeloid cells were generated by culturing fetal liver progenitors (FLPs) in complete IMDM with IL-3 (5 ng/ml), IL-6 (10 ng/ml), and SCF (100 ng/ml) (Peprotech, Rocky Hill, NJ) for two days, followed by passage in complete IMDM containing IL-3 (5 ng/ml).

Day 14.5 *Sfpi1*^{+/+}, *Sfpi1*^{−/−}, *Sfpi1*[+/BN], or *Sfpi1*[BN/BN] FLPs were generated by timed matings of *Sfpi1*[+/BN] or *Sfpi1*^{+/−} mice as previously described [17]. For rescue experiments, *Sfpi1*^{−/−} FLPs were infected by spin-infection and analyzed by flow cytometry after two days of culture, as described [15]. Colony forming assays were performed as previously described [16] using recombinant murine GM-CSF (1 ng/ml), G-CSF (10 ng/ml), M-CSF (10 ng/ml) or IL-3 (5 ng/ml) + IL-6 (10 ng/ml) + SCF (100 ng/ml) (Peprotech).

Morphological, Flow Cytometric, and Immunoblot Analysis

For morphological analysis, 10^5 washed cells were cytospun onto glass slides and stained with Wright stain. Cells were viewed using an Olympus BH2 light microscope and photographed using an Olympus AX-70 microscope with a Photometrics digital camera. Flow cytometric analysis was performed on cells stained with the biotin-conjugated monoclonal antibodies (mAb) M1/70 (CD11b), RB6-8C5 (Gr-1), 2.4G2 (FcγRII/III); antibodies conjugated -Kit); orto antibodies conjugated to allophycocyanin (APC): A3-1 (F4/80), RA3-6B2 (B220) (BD Pharmingen). Biotin-conjugated antibodies were visualized by secondary staining with streptavidin-APC conjugate or streptavidin-PE conjugate (BD Pharmingen, San Diego, CA). Cells were analyzed using a BD FACSCalibur system. Immunoblotting was performed as previously described [13] using rabbit anti-PU.1 peptide Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and HRP-conjugated anti-rabbit secondary Ab (Pierce, Rockford, IL).

Transfection and Reporter Assays

An 1817 bp *FcγRIIb* promoter fragment was PCR amplified from C57Bl/6 genomic DNA and cloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI) as previously described [15]. A single nucleotide in the PU.1 binding site was mutated using the Stratagene QuickChange XL kit (Stratagene), using the oligonucleotides 5′-GACCGTTTCTTTTCAC*G<u>T</u>CC*CCATTTGGACTTCA-3′ and 5′-TGAAGTCCAAATGG*G<u>G</u>GAC*GTGAAAAGAAACGGTC-3′ (PU.1 binding site italicized, mutated nucleotide underlined). 38B9 and WEHI-231 cells were transfected with reporter DNA as well as a pRL-TK control vector using electroporation as previously described [15].

RAW264.7 cells were transfected using lipofectamine (Invitrogen). Lysates were prepared 24 hours after transfection and normalized luciferase activity was obtained using a Dual luciferase kit (Promega).

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed as previously described [15]. Synthetic complementary oligonucleotides were annealed and end-labeled with [α-^{32}P]dCTP using the Klenow fragment. Double-stranded oligonucleotide probes contained the PU.1-binding site in the *FcγRIIb* promoter, 5′-TCGATTCTTTTCAC*TTCC*CCAT-3′ or a mutated PU.1-binding site (mutant FcγRIIb), 5′-TCGATTCTTTTCAC*GTCC*CCAT-3′. An E2A consensus site oligonucleotide was used as a control, 5′-GATCTACATCTGTGCAGTTCGCTG-3′.

Transplantation into Immunodeficient Recipient Mice

Fetal liver cells (FLCs) were erythrocyte-depleted and two million FLCs were injected into the tail vein of sublethally irradiated (400 Rad, single dose) *Rag-2*$^{−/−}$ *Il2rg*$^{−/−}$immunodeficient recipient mice (Taconic emerging models program) [18]. Injections were performed within 4 hours of irradiation.

RT-PCR Analysis

RT-PCR was performed as previously described [13]. Primer sequences used are listed as follows: exon 1 forward (5′-TTTCCCTCACCGCCC-3′), exon 3 reverse (5′-TATCGAGGACGTGCATCTGTTCCA-3′), exon 3 forward (5′-ATCACTACTGGGATTTCTCCGCAC-3′), exon 4 reverse (5′-ATCAGCTTCTCCACACAGACACCTC-3′), exon 4 forward (5′-CGGATGTGCTTCCCTTATCAAAC-3′), exon 5 reverse (5′-TTTGTCCTTGTCCACCCACCAGAT-3′), *fcγRIIb* (5′-AAGCCTGTCACCATCACTGTCCAA-3′ and 5′-AGGGTTTCTCCCATTTCCCTGTGA-3′), *β-actin* (5′-TCCTTCGTTGCCGGTCCACA-3′ and 5′-CGTCTCCGGAGTCCATCACA-3′), *g6pdh* (5′-GAACATCATCCCTGCATCCA-3′ and 5′-CCAGTGAGCTTCCCGTTCA-3′). Real-time PCR was performed as previously described using a Cepheid SmartCycler system [13,19].

RESULTS

Generation of mice with a mutant *Sfpi1* allele

The first exon of *Sfpi1* has been reported to contain two translation start codons [1]. To generate a reporter allele for *Sfpi1* transcription, we designed a gene targeting strategy to replace both translation start codons in exon 1 with the *E. coli* TEM-1β-lactamase gene (Fig. 1A). Correctly targeted ES cells were injected into C57Bl/6 blastocysts to generate chimeric mice. One founder successfully transmitted the targeted allele (termed BN) to F1 offspring. Mice with this genotype were termed *Sfpi1*$^{+/BN}$. Genotypes were confirmed by PCR, Southern blotting, and DNA sequencing (Fig. 1B, 1C, and data not shown). *Sfpi1*$^{+/BN}$ mice were born at expected Mendelian frequencies (Table 1), had no phenotypic abnormalities, and were fertile.

We attempted to detect β-lactamase activity in *Sfpi1*$^{+/BN}$ mice using flow cytometry or immunoblotting [20]. However, to date we have not detected β-lactamase activity in cells or tissues prepared from these mice. RT-PCR analysis of transcription in the targeted allele suggests that RNA splicing occurs at high frequency from several consensus splice donor sites within the β-lactamase cDNA. The data suggest that there are likely various splice acceptor sites within the PGK-Neo cassette and within the *Sfpi1* gene (data not shown).

We mated *Sfpi1*[+/BN] heterozygous mice to generate *Sfpi1*^{+/+}, *Sfpi1*[+/BN] and *Sfpi1*[BN/BN] offspring. *Sfpi1*[BN/BN] mice were born at near Mendelian frequency and survived between 1 and 3 weeks, with an occasional mouse reaching weaning age (~3% of total mice at 21 days of age, Table 1). At 2–3 weeks of age, *Sfpi1*[BN/BN] mice were runted and had multiple symptoms of osteopetrosis, including failure of tooth eruption and partially occluded femurs (Fig. 1D and data not shown). These characteristics have been reported for *Sfpi1*^{−/−} mice [21] and suggest that these mice are macrophage-deficient. To determine if any PU.1 protein was generated by the BN allele, we prepared lysates from the spleens of two week-old wild-type or *Sfpi1*[BN/BN] mice. As shown in Fig. 1E, *Sfpi1*[BN/BN] mice express reduced levels of PU.1 protein compared to wild-type. In summary, these data suggest that the *Sfpi1*[BN] allele represents a hypomorphic, and not a null, allele.

Sfpi1[BN] allele results in reduced expression of PU.1

In order to analyze transcription of the *Sfpi1*[BN] allele in distinct lineages, we established IL-7 dependent pro-B cell or IL-3 dependent myeloid cell lines from mouse fetal liver as described in Materials and Methods. Using RNA prepared from these cells, we performed RT-PCR across all *Sfpi1* exons to examine the integrity of the *Sfpi1* transcript. *Sfpi1* transcripts were intact but at reduced levels in *Sfpi1*[BN/BN] pro-B cells compared to wild-type cells (Fig. 2A). To determine the levels of *Sfpi1* transcription, we performed real-time RT-PCR using primers specific to exons 3 and 4 of *Sfpi1*. Mutation of the *Sfpi1* allele resulted in a 6.7-fold reduction in transcripts from myeloid cells and a 2-fold reduction in transcripts from pro-B cells (Fig. 2B and 2C). PU. 1 protein was expressed at 20% of wild-type levels in *Sfpi1*[BN/BN] pro-B cells, as revealed by immunoblotting of serial dilutions of nuclear lysates (Fig. 2D and data not shown). These results suggest that transcription of the *Sfpi1^BN* allele is reduced in both lymphoid and myeloid cell lineages.

To determine whether the *Sfpi1*[BN] allele affects transcription of neighboring genes, we examined the expression of *Psmc3*, *Slc39a13*, *Mybpc3*, and *Madd* using real-time RT-PCR (Fig. 2E). Transcripts of *Madd* and *Psmc3* were slightly increased (~2 fold) in either cultured *Sfpi1*[BN/BN] pro-B cells or progenitors expanded in IL-3, IL-6, and SCF, relative to wild-type cells. Transcripts of *Slc39a13* or *Mybpc3* were not significantly altered. These changes are small and are likely to be a consequence of the phenotype imposed by hypomorphic PU.1.

The *Sfpi1*[BN] allele generates functional PU.1 protein

Upon further examination of the DNA sequence of the targeted allele, we identified a third potential ATG-start codon that might account for PU.1 expression (Fig. 3A). To determine if the mutated transcript has biological activity, we generated retroviruses containing either the wild-type or *Sfpi1^BN* full-length transcripts and tested their ability to rescue *Sfpi1*^{−/−} fetal liver progenitor cells (FLPs) in M-CSF-dependent colony-forming assays (Fig. 3B) [8]. Expression of PU.1 from the cloned BN transcript was substantially reduced compared to wild-type (Fig. 3C and 3D). *Sfpi1*^{−/−} FLPs infected with the cloned BN retrovirus generated colonies in response to M-CSF; however, the number of colonies was reduced compared to the number generated by infection with the cloned wild-type retrovirus (18 colonies per 10^5 cells plated compared to 345 colonies per 10^5 cells) (Fig. 3E). To determine whether this reduction in the ability of the *Sfpi1^BN* transcript-encoding retrovirus to rescue *Sfpi1*^{−/−} FLPs was due to reduced expression or altered function, we constructed an additional retrovirus encoding a truncated PU.1 protein (HA-PU.1ΔN31, Fig 3B). To optimize expression, this retroviral vector contains an HA-tag and Kozak sequence identical to a vector encoding full-length PU.1 protein (HA-PU.1wt, [14]). As expected, expression of PU.1 was readily detectable in western blots of HA-PU.1ΔN31-transfected cells (Fig. 3C and 3D). Furthermore, infection of *Sfpi1*^{−/−} FLPs with either HA-PU.1wt or HA-PU.1ΔN31 rescued M-CSF-dependent colony formation at near equivalent efficiency (643 and 711 colonies per 10^5 cells plated, respectively, Fig. 3E).

Macrophages from HA-PU.1ΔN31-infected *Sfpi1*$^{-/-}$FLPs resembled wild-type-rescued macrophages phenotypically when cytospun to glass slides and Wright stained (Fig. 2H). These results suggest that the reduction in PU.1 activity caused by the *Sfpi1*^{BN} allele is due to a reduction in expression and not altered function.

Reduction in PU.1 activity results in reduction of target gene expression

In order to determine whether hypomorphic expression of PU.1 affects transcription of target genes, we analyzed expression of a gene recently identified as PU.1-dependent, *FcγRIIb,* which encodes the low-affinity IgG Fc receptor [13,22]. We performed real-time RT-PCR analysis to measure steady-state levels of *FcγRIIb* transcripts in cells cultured from wild-type, *Sfpi1*$^{-/-}$, and *Sfpi1*$^{BN/BN}$ mice. *FcγRIIb* transcripts were reduced 7.8-fold in IL-3-dependent myeloid cells and 4-fold in IL-7-dependent pro-B cells. As expected, *FcγRIIb* transcripts were further reduced in *Sfpi1*$^{-/-}$IL-3 and pro-B cells (Fig. 4A and 4B).

To determine if PU.1 regulates the *FcγRIIb* gene directly or indirectly, we characterized the promoter of the gene. Using 5′ RACE analysis, we identified a number of transcription start sites in the region 50–70 bp upstream of the first coding exon (Fig. 4C). A predicted PU.1 binding site was located immediately upstream of the transcription start sites within a block of conserved DNA sequence (Fig. 4C). Electrophoretic mobility shift analysis (EMSA) showed that PU.1 in nuclear extracts from wild-type pro-B cells could interact with this site (Fig. 4D). Importantly, *Sfpi1*$^{BN/BN}$ pro-B cells contained reduced levels of PU.1 capable of interacting with this probe. No similar complex was formed using nuclear extracts from *Sfpi1*$^{-/-}$ pro-B cells. These complexes were demonstrated to contain PU.1 by supershift analysis with an anti-PU.1 antibody (data not shown). Furthermore, mutation of the PU.1 binding site reduced activity of a cloned segment of the *FcγRIIb* promoter as tested by transient transfection analysis of RAW264.7 macrophages, 38B9 pro-B cells, and WEHI-231 B cells (Fig. 4E). In summary, these data suggest that the *FcγRIIb* gene is directly regulated by PU.1. In addition, these data demonstrate that the *Sfpi1*^{BN} allele results in a reduction in PU.1 activity.

B cell development is blocked in *Sfpi1*$^{BN/BN}$ mice

To determine whether B cell development is affected by the BN mutation, we examined fetal liver or spleen cells of wild-type or *Sfpi1*$^{BN/BN}$ mice at various ages by flow cytometry. The spleens and fetal livers of *Sfpi1*$^{BN/BN}$ mice had a dramatic reduction in the number of B220$^+$CD19$^+$ cells relative to wild-type mice that was apparent from embryonic day 16.5 (E.D. 16.5) (Fig. 5A and 5B). To determine whether this defect is cell-intrinsic, we transplanted wild-type or *Sfpi1*$^{BN/BN}$ E.D.14.5 FLPs into sublethally irradiated immunodeficient mice lacking both Rag-2 and common gamma chain (*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice) [18]. Whereas wild-type FLPs generated B cells efficiently in recipient mice six weeks after transplantation, *Sfpi1*$^{BN/BN}$ FLPs failed to generate any detectable B lymphocytes in either spleen or bone marrow after six or nine weeks (Fig. 5C). These results suggest that *Sfpi1*$^{BN/BN}$ FLPs possess a cell-intrinsic block to B cell development.

Sfpi1$^{BN/BN}$ fetuses have reduced fetal myelopoiesis

Sfpi1$^{-/-}$ mice have a multilineage defect in the generation of monocytes, granulocytes, and B and T lymphocytes that is apparent by day 14.5 of fetal development [4]. Flow cytometric analysis of fetal livers from E.D. 14.5 *Sfpi1*$^{BN/BN}$ fetuses revealed a reduction in cells expressing the myeloid cell surface markers FcγRIIb/III, Gr-1, or CD11b (Fig. 6A), whereas the total cellularity of the fetal livers was increased (Fig. 6B). To determine the frequency of cytokine-responsive myeloid progenitor cells, we performed colony-forming assays on fetal liver cells (FLCs) from wild-type and *Sfpi1*$^{BN/BN}$ E.D. 14.5 fetuses. As shown in Fig. 6C, *Sfpi1*$^{BN/BN}$ fetal livers had a severe reduction in the frequency of progenitor cells able to respond to G-CSF (0.86 colonies/10^5 cells plated), M-CSF (0.11 colonies/10^5 cells plated),

GM-CSF (0.58 colonies/10^5 cells plated), and IL-3/IL-6/SCF (11.7 colonies/10^5 cells plated) compared to wild-type (36, 15, 41.5, 58.3 colonies/10^5 cells plated, respectively).

Due to the difference in colony generating ability of *Sfpi1*^BN/BN^ FLCs placed in IL-3/IL-6/SCF compared to other tested cytokines, we examined the nature of the colonies that grew under these conditions. Colonies that formed from *Sfpi1*^BN/BN^ FLCs in IL-3/IL-6/SCF had a nearly 2-fold increase in the number of cells per colony compared to wild-type (Fig. 6D). Additionally, wild-type colonies in IL-3/IL-6/SCF contained mature macrophages and granulocytes, whereas *Sfpi1*^BN/BN^ colonies contained promyelocytes and blast-like cells but no mature myeloid cells (Fig. 6E). These data therefore demonstrate that *Sfpi1*^BN/BN^ fetuses have a substantial reduction in myelopoiesis relative to wild-type fetuses. In addition, *Sfpi1*^BN/BN^ FLCs are capable of proliferating in response to IL-3/IL-6/SCF but are incapable of differentiation.

Neonatal *Sfpi1*^BN/BN^ mice acquire a myeloproliferative disorder

As mentioned above, *Sfpi1*^BN/BN^ mice are born at the expected Mendelian frequency and occasionally survive until weaning age. The spleens of 2–3 week old *Sfpi1*^BN/BN^ mice were moderately enlarged compared to wild-type littermates. Flow cytometric analysis of *Sfpi1*^BN/BN^ fetal liver, spleen, and bone marrow cells at various time points revealed a dramatic expansion of cells co-expressing the stem and progenitor cell marker c-Kit with the myeloid cell-surface markers FcγRII/III, Gr-1, and CD11b (Fig. 7A, 7B, and data not shown). This expansion was not apparent until after birth and was detectable as early as day 5 post birth (P.B.) (Fig. 7A). The percentage of cells containing the mature myeloid marker F4/80 was not increased (Fig. 7B). Cytospin analysis revealed that *Sfpi1*^BN/BN^ spleens contained almost exclusively immature myeloid cells (Fig. 7C). These results demonstrate that reduced PU.1 activity results in proliferation of immature myeloid cells in neonatal mice, but not in fetal liver.

Discussion

In this study, we addressed the hypothesis that lower levels of PU.1 activity might be required for B cell than for macrophage development *in vivo*. If this hypothesis is true, then system-wide reduction of PU.1 activity *in vivo* would be expected to induce B cell development while failing to promote macrophage development. We have tested this hypothesis by examining mice homozygous for a hypomorphic allele (termed BN) of the *Sfpi1* gene. Analysis of *Sfpi1*^BN/BN^ mice demonstrates that both B cell and macrophage development are impaired at an early stage. The block to B cell development appears more severe than the block to myeloid development, as an abnormal expansion of immature granulocytic precursors is apparent in the spleen and bone marrow of neonatal *Sfpi1*^BN/BN^ mice.

Studies utilizing a green florescent protein as a reporter gene "knocked-in" to the *Sfpi1* locus have revealed that PU.1 is expressed at uniformly high levels in HSCs as well as CLPs [9, 10]. However, upon commitment to the B cell lineage, PU.1 expression is reduced to eight-fold lower levels in B cells than in Mac-1^{+} myeloid cells [10]. Conditional knockout studies of the *Sfpi1* gene have also provided important insights about the role of PU.1 in B cell development. When *Sfpi1* is inactivated in HSCs, B cell development is profoundly blocked [5]. In contrast, when *Sfpi1* is inactivated in CLPs using a retrovirally expressed Cre recombinase or in committed B cells using Cre recombinase under control of the CD19 promoter, B cell development is minimally affected [5,11,12]. Taken together with our studies, these data suggest that high levels of PU.1 are essential for B cell development. However, these high levels are essential only at the earliest stages of B cell specification and may be dispensable after commitment to the B cell lineage. We expect that high levels of PU.1 are required to activate genes involved in B lineage specification such as IL-7Rα[14] and early B cell factor (EBF) [23].

Reporter gene studies show that PU.1 is expressed at high levels in a subset of common myeloid progenitors (CMPs) that is fated to develop into macrophages and granulocytes [10] and maintains high levels of expression throughout differentiation [9,10]. Studies of conditional *Sfpi1* knockout mice show that high levels of PU.1 expression are essential for proper differentiation of macrophages and granulocytes at all stages of development [5]. However, the results we have presented in this paper, as well as those published by others, suggest that reduced levels of PU.1 still permit specification to the myeloid lineages. Interestingly, reduced levels of PU.1 promote hyperproliferation of immature myeloid precursor cells during neonatal but not fetal development. Another study also concluded that inactivation of PU.1 in adult hematopoiesis led to increased proliferation of immature myeloid cells, and eventually acute myeloid leukemia [24,25]. An important future area of investigation will be to determine why low levels of PU.1 activity result in a hyperproliferation of immature myeloid cells *in vivo*.

Recently, studies have been published in which an upstream regulatory element (URE) in the *Sfpi1* locus was deleted using homologous recombination in ES cells [26–28]. The phenotype reported in these publications is quite distinct from the one that we report. Whereas *Sfpi1*$^{BN/BN}$ mice die within 1 month of age, ΔURE mice are healthy and phenotypically normal until 2–3 months of age [28]. However, between 3–8 months of age, ΔURE mice develop an aggressive acute myeloid leukemia. During the preleukemic phase, ΔURE mice have severely impaired B-2 B cell and myeloid development. The differences between these results and the ones we report might be due to differences in regulation of the mutated *Sfpi1* alleles.

In conclusion, the original hypothesis as presented above must be reconsidered. Our results suggest that both B cell and myeloid development require high levels of PU.1 activity *in vivo*. However, we suggest that the requirement for high levels of PU.1 during B cell development is during specification to the B cell lineage, and is not essential to maintain B cell identity after commitment. In contrast, in the myeloid lineage, high levels of PU.1 do not appear to be essential for specification, but are required for terminal differentiation. Finally, we demonstrate that reduction in PU.1 activity results in a myeloproliferative disorder in neonates. We expect that the hypomorphic allele of *Sfpi1* that we have characterized in this study will be of value to identify PU.1 target genes that mediate hematopoietic specification and commitment.

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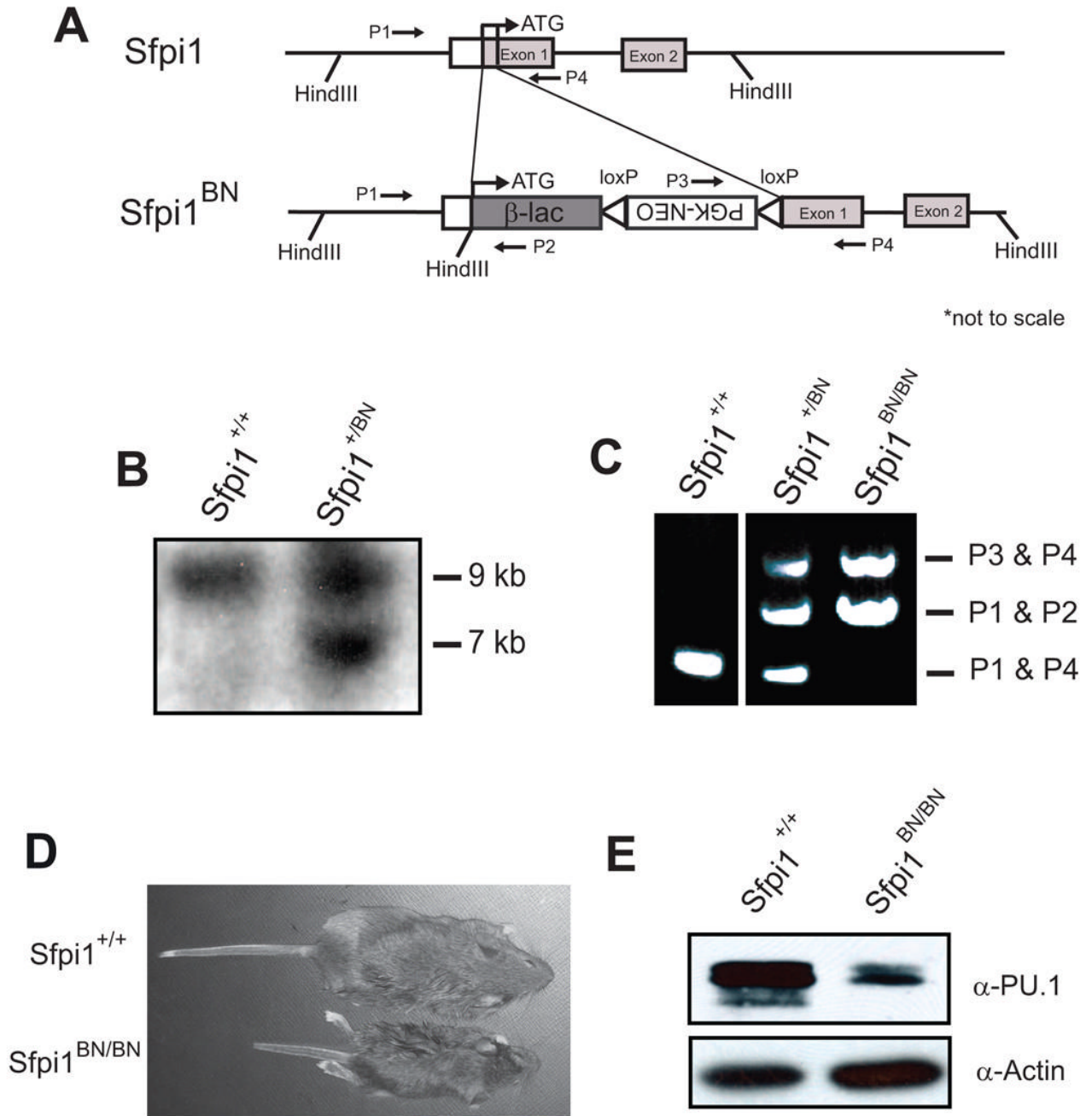
Figure 1. Generation of *Sfpi1*[+/BN] mice
(A) Targeting strategy used to replace the translation start sites of *Sfpi1* with β-lactamase (β-lac). LoxP sites are represented by open arrowheads. The locations of primers used in genotyping are indicated. (B) Southern blot analysis on HindIII-digested ES cell genomic DNA. The DNA was hybridized with a probe complementary to the PGK-NEO gene, which recognizes a 10.5 kb wild-type product and a 6.4 kb targeted fragment. The 9 kb product is due to the presence of the Neomycin gene in the feeder cell layer. (C) PCR results of a representative genotyping analysis using primers indicated above. (D) Photograph of 21 day-old littermates from *Sfpi1*[+/BN] heterozygous mating. (E) Western blot using α-PU.1 antibody demonstrating reduced levels of PU.1 in the spleens of 21-day *Sfpi1*[BN/BN] mice compared to wild-type mice.
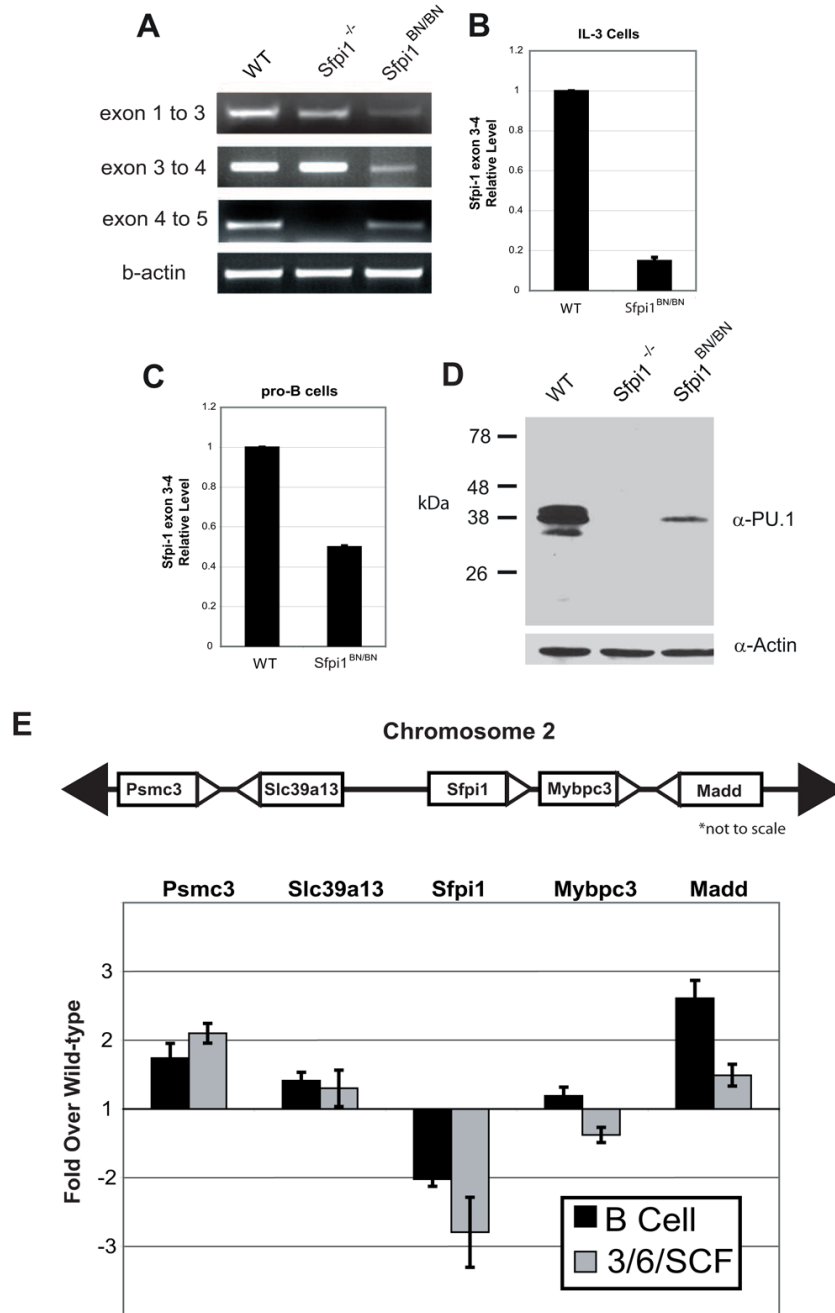
Figure 2. PU.1 expression is reduced by the BN mutation in both myeloid and lymphoid lineages
(A) RT-PCR analysis of transcription across the *Sfpi1* locus. PCR was performed using primers that reside within the exons indicated. The lack of product between exons 4 and 5 in the *Sfpi1*$^{-/-}$ cells is due to the partial deletion of exon 5 [4]. (B, C) Real-time RT-PCR was used to measure *Sfpi1* exon 3 to 4 transcripts in cultured IL-3 cells (B) or cultured IL-7 and stromal cell pro-B cells (C) from wild-type or *Sfpi1*$^{BN/BN}$ fetal liver. (D) Western blot for PU.1 protein expression in nuclear lysates of pro-B cells of the indicated genotype. Quantitative analysis demonstrated a 5-fold reduction in PU.1 expression. (E) Real-time RT-PCR analysis of RNA transcripts from pro-B cells or fetal liver progenitors cultured for 2 days in IL-3, IL-6, and SCF. Bars indicate the fold change in transcriptional expression in *Sfpi1*$^{BN/BN}$ cells compared

to wild-type cells using primers that recognize the genes indicated. Gene location in relation to *Sfpi1* is shown above. Error bars indicate the standard error.
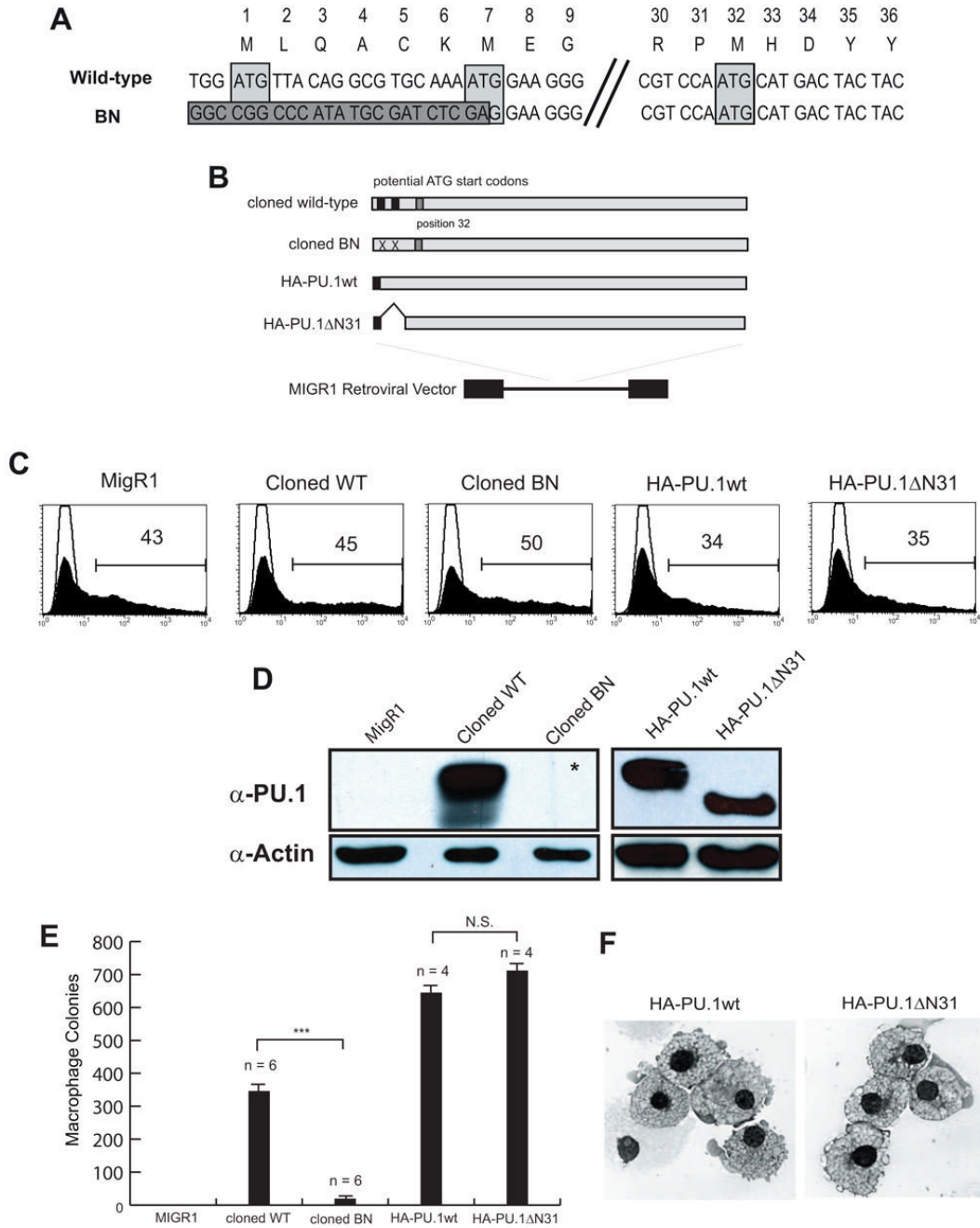
Figure 3. PU.1 activity is reduced by the BN mutation in both myeloid and lymphoid lineages
(A) Alignment of genomic DNA sequences of wild-type and targeted $Sfpi1^{BN/BN}$ including previously identified codon locations and their equivalent amino acids. The dark gray box indicates sequence following genomic DNA targeting resulting in the removal of both previously identified ATG-start codons (light gray boxes). Position 32 contains an in-frame potential ATG-start codon (3rd light gray box). (B) Diagram of cloning strategy to generate retroviruses. (C) Flow cytometric analysis of transfected Plat-E cells measuring GFP expression and demonstrating equivalent transfection efficiency of the indicated retroviruses. (D) Western blot analysis of transfected cells analyzed in (B) showing reduced expression of PU.1 protein from the cloned BN retrovirus. The asterisk indicates that a band was seen upon

longer exposure. (E) Colony forming ability of retrovirally-infected *Sfpi1*^{−/−} fetal liver progenitors (FLPs). *Sfpi1*^{−/−}FLPs were isolated according to Materials and Methods and spin-infected with the indicated retrovirus. Cells were plated in methylcellulose containing M-CSF and colonies were counted after 5 days. Numbers correspond to number of colonies per 10^5 infected cells plated (*** - p <= .001). (F) Cytospins of cells from methylcellulose plates from (E). Cytospun and Wright stained cells were visualized using an Olympus BH2 light microscope and photographed as described in Materials and Methods.
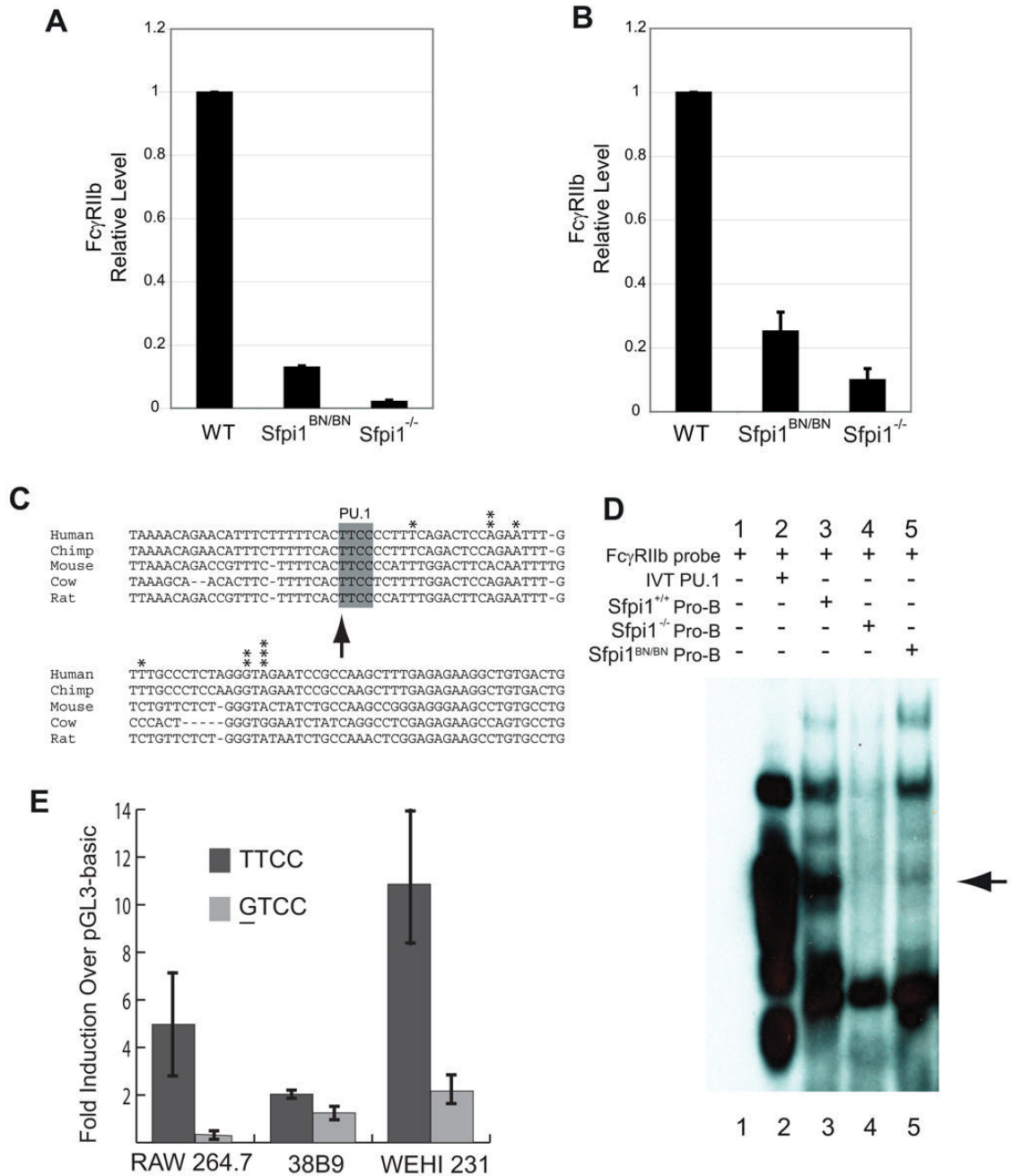
Figure 4. Reduction in PU.1 Activity as Measured by Transcription of the *FcγRIIb* gene
(A) Real-time RT-PCR analysis of *FcγRIIb* expression from RNA prepared from cultured IL-3-dependent myeloid cells of the indicated genotype. (B) Real-time RT-PCR analysis of *FcγRIIb* expression from RNA samples obtained from cultured IL-7-dependent pro-B cells of the indicated genotype. (C) Sequence comparison of the *FcγRIIb* promoter among various species. Arrow indicates PU.1-binding site. Asterisks indicate identified transcription start sites. (D) Electrophoretic mobility shift assay of nuclear lysates using an *FcγRIIb* probe. Arrow indicates the band corresponding to PU.1 interacting with the DNA probe. (E) Luciferase reporter assays using wild-type (TTCC) or mutated (GTCC) PU.1 binding site indicated in (C). Cell lines used include RAW264.7 (macrophage), 38B9 (pro-B), WEHI-231 (B cells).
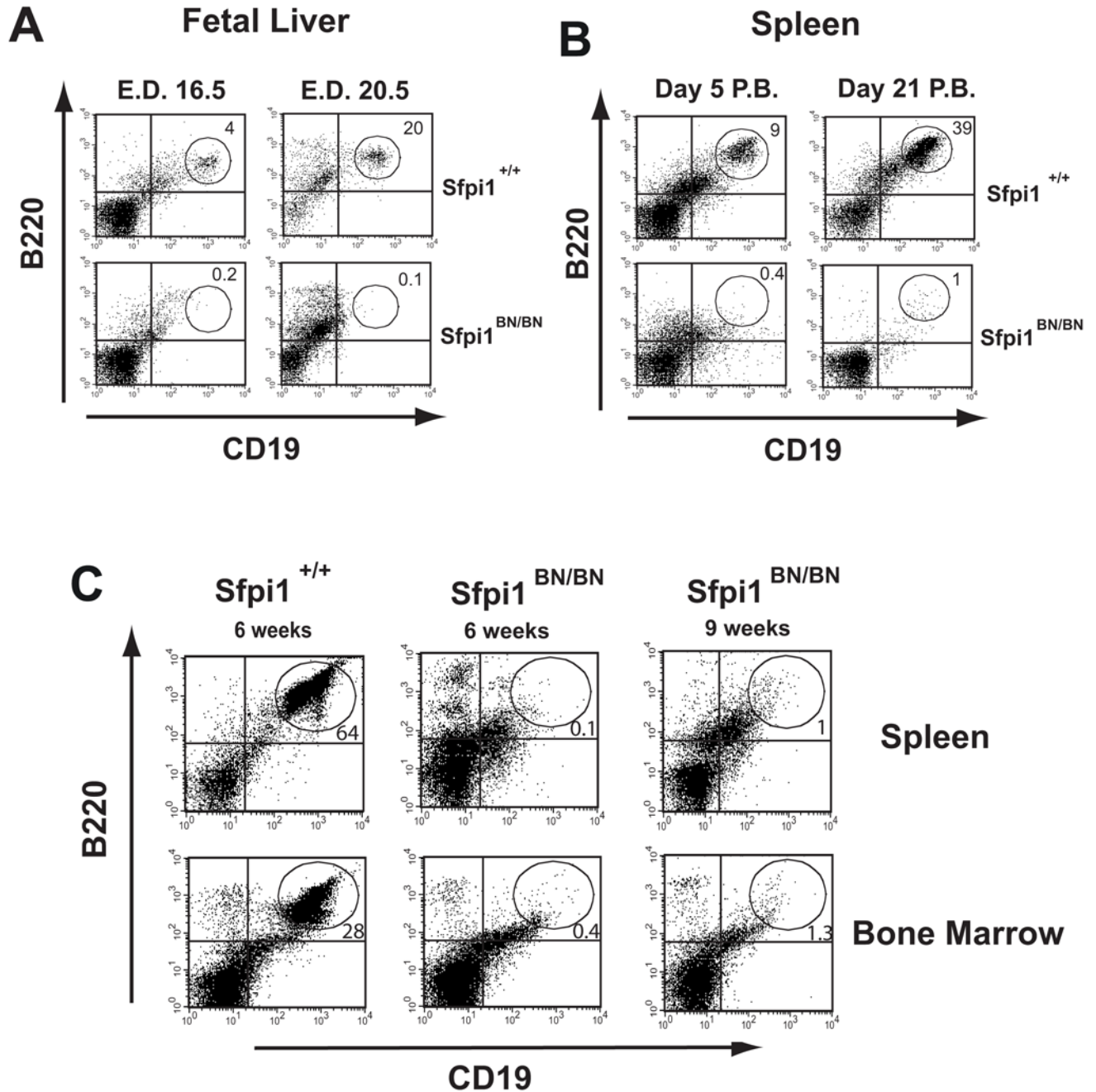
Figure 5. Cell Intrinsic Block to B Cell Development in *Sfpi1*^BN/BN mice
(A, B) Flow cytometric analysis of single-cell suspensions from either fetal livers or spleens of wild-type and *Sfpi1*^BN/BN mice at the indicated age. Isolated spleen cells were analyzed by flow cytometry using gating for size and granularity and antibodies to the indicated cell surface markers. Numbers indicate percentage of gated cells in the indicated region. E.D. = emybryonic day, P.B. = post birth. (C) Flow cytometric analysis of single-cell suspensions from spleens or bone marrow of recipient mice transplanted with wild-type or *Sfpi1*^BN/BN fetal liver cells. Fetal liver cells were isolated from wild-type or *Sfpi1*^BN/BN mice and transplanted by tail vein injection into sublethally irradiated *Rag2*^−/− *Il2rg*^−/−recipient mice. Analysis was performed after the indicated time following transplantation.
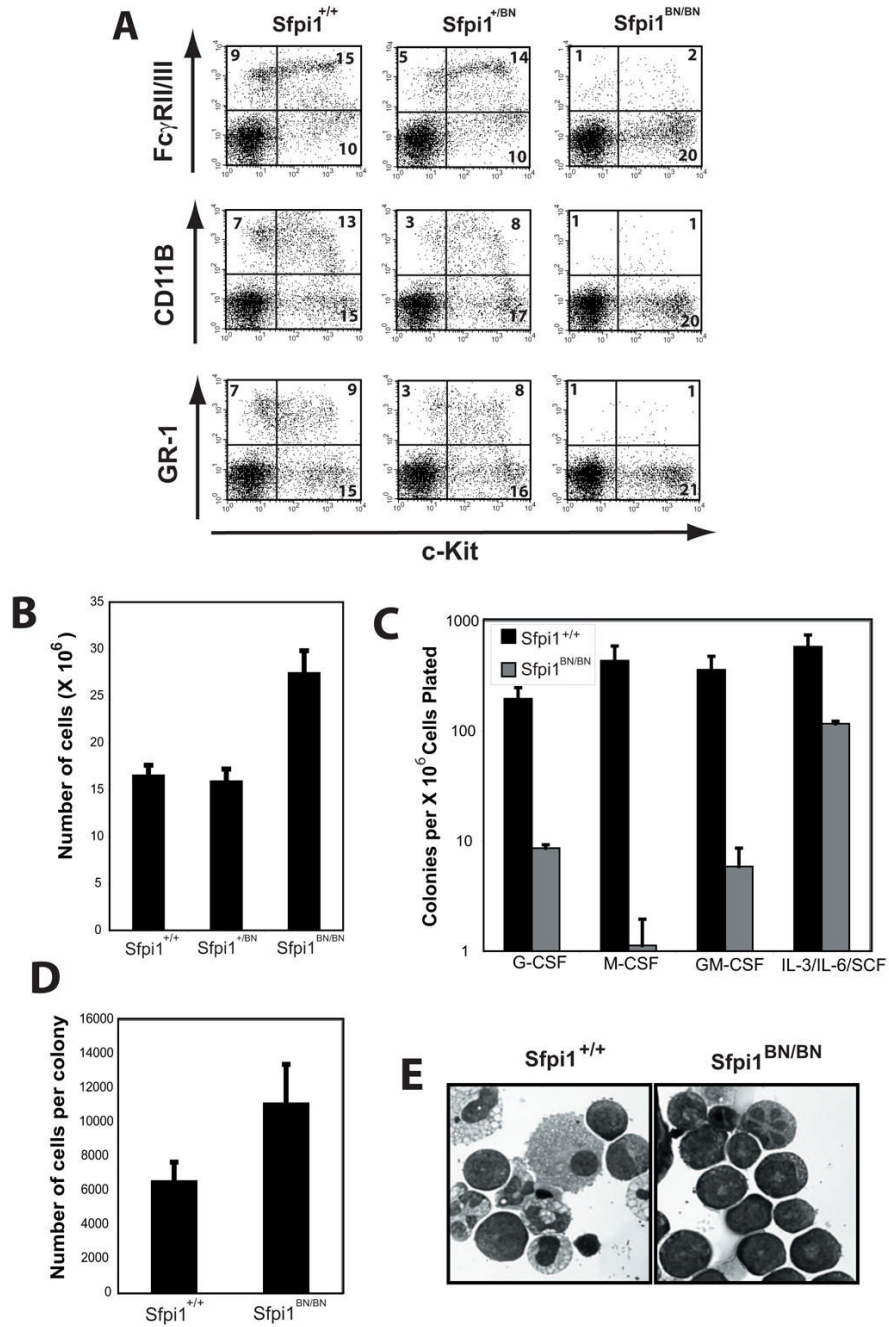
Figure 6. – Fetal Liver Myelopoiesis is Severely Reduced in *Sfpi1*^{BN/BN} Mice

(A) Flow cytometric analysis of single-cell suspensions from day 14.5 fetal liver cells. The cell surface markers analyzed are shown. Numbers indicate the percentage of total cells. (B) Total number of cells per fetal liver of wild-type, heterozygous, and *Sfpi1*^{BN/BN} mice. (C) Colony assays from fetal liver progenitors isolated from wild-type and *Sfpi1*^{BN/BN} mice. Fetal liver cells were isolated from the indicated mouse and plated at various concentrations in methylcellulose with the indicated cytokines. Colonies were scored following 7 days of culture. Error bars represent the standard error of a minimum of three plates. (D) Calculated cells per colony of either wild-type or *Sfpi1*^{BN/BN} fetal liver progenitors. Cells in methylcellulose

cultures from (C) were washed and counted. (E) Cytospins of cells from methylcellulose plates described in (C and D).
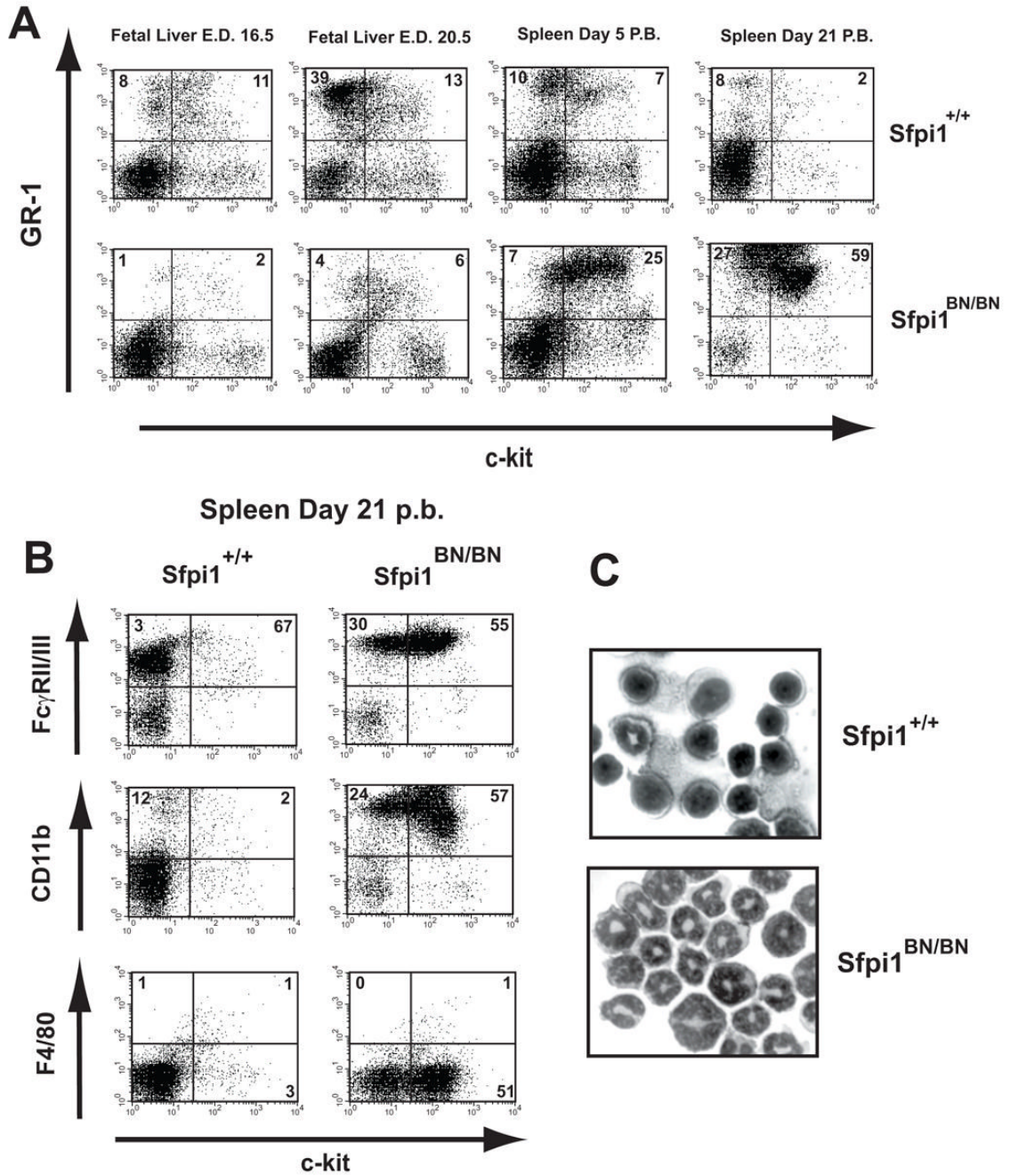
Figure 7. Myeloproliferative Disorder in *Sfpi1*[BN/BN] neonates
(A) Flow cytometric analysis of single-cell suspensions from either fetal liver or spleens of mice at the age indicated. Isolated cells were gated for proper size and granularity and analyzed by flow cytometry using antibodies to the indicated cell surface markers. Numbers indicate the percentage of gated cells. (B) Flow cytometric analysis of single-cell suspensions from spleens of 21 day-old wild-type and *Sfpi1*[BN/BN] mice, performed as described in (A). (C) Cytospins of spleen cells from 18 day-old littermates demonstrating infiltration of immature myeloid cells in the spleens of *Sfpi1*[BN/BN] mice. Isolated spleen cells were cytospun onto glass slides, Wright stained, and photographed as described in Materials and Methods.

Table 1

Frequency of Live Births from Matings of *Sfpi1*[+/BN] Mice BN Matings

AGE	Total	PU.1 +/+	PU.1 +/BN	PU.1 BN/BN
E 14.5	32	9	14	9
Day 1	119	29	61	29
Day 8	165	54	88	23
Day 21	259	104	146	9

Genomic DNA was prepared from d14.5 fetuses, from newborns one day after birth, from mice one week after birth, or at the time of weaning (21 days). Genomic DNA was used to identify genotypes by PCR as described in Materials and Methods.