The *Justy* mutation identifies *Gon4-like* as a gene that is essential for B lymphopoiesis

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A recessive mutation named *Justy* was found that abolishes B lymphopoiesis but does not impair other major aspects of hematopoiesis. Transplantation experiments showed that homozygosity for *Justy* prevented hematopoietic progenitors from generating B cells but did not affect the ability of bone marrow stroma to support B lymphopoiesis. In bone marrow from mutant mice, common lymphoid progenitors and pre-pro–B cells appeared normal, but cells at subsequent stages of B lymphopoiesis were dramatically reduced in number. Under culture conditions that promoted B lymphopoiesis, mutant pre-pro–B cells remained alive and began expressing the B cell marker CD19 but failed to proliferate. In contrast, these cells were able to generate myeloid or T/NK precursors. Genetic and molecular analysis demonstrated that *Justy* is a point mutation within the *Gon4-like* (*Gon4l*) gene, which encodes a protein with homology to transcriptional regulators. This mutation was found to disrupt Gon4l pre-mRNA splicing and dramatically reduce expression of wild-type Gon4l RNA and protein. Consistent with a role for Gon4l in transcriptional regulation, the levels of RNA encoding C/EBP\(\alpha\) and PU.1 were abnormally high in mutant B cell progenitors. Our findings indicate that the Gon4l protein is required for B lymphopoiesis and may function to regulate gene expression during this process.

Hematopoietic progenitor cells progress through pathways that restrict developmental plasticity and enforce commitment to specific lineages (Laiosa et al., 2006; Brown et al., 2007). These processes coincide with gene expression remodeling as orchestrated by a transcription factor network (Laiosa et al., 2006; Nutt and Kee, 2007). Many DNA-binding components of this network are known, but how these proteins coordinately regulate gene expression is poorly understood. Also, disruption of the hematopoietic transcription factor network is strongly associated with oncogenesis (O’Neil and Look, 2007; Rosenbauer and Tenen, 2007). Thus, further characterizing the mechanisms that control hematopoiesis could provide insights regarding neoplasia in this system.

Among hematopoietic developmental pathways, B lymphopoiesis is well defined (Hardy et al., 2007; Welner et al., 2008). During this process, the transcription factors Ikaros, E2A, EBF, and Pax5 likely function to recruit protein complexes that activate B-lineage genes or repress genes associated with other lineages (Ng et al., 2007; Nutt and Kee, 2007). Consistent with this idea, gene activation by EBF and Pax5 involves the chromatin-remodeling complex SWI–SNF, whereas Ikaros or Pax5 can interact with transcriptional coresspressors (Eberhard et al., 2000; Koipally and Georgopoulos, 2002; Gao et al., 2009). Interactions between B cell transcription factors and unknown cofactors are
F1 males were screened by flow cytometry to identify mice with normal peripheral blood that might be carriers of recessive mutations. These mice were mated to wild-type C3H females and then to the resulting F2 females. Flow cytometric analysis identified F3 mice that lacked blood cells expressing the B-lineage marker CD45R/B220 (B220). These mice were crossed to wild-type C3H mice, and the progeny were used for brother–sister matings. Offspring lacking B220+ cells were crossed to wild-type C3H mice, and the progeny were used for brother–sister matings to recover mice that were homozygous for the causal mutation. These mice are fertile and have a normal life span but lack serum immunoglobulin and fail to generate antibodies when challenged with antigens (unpublished data). The mutant strain was named Justy (just T cells).

In the text that follows, mice homozygous for the mutation are called Justy mice.

Spleens from Justy mice were abnormally small, had hypoplastic white pulp, and yielded significantly fewer cells than wild-type organs (Fig. 1, a and b). Flow cytometric analysis showed that the frequency and number of CD19+ cells in Justy spleens were dramatically decreased (Fig. 1, c and d) and that lymph nodes or peritoneal lavages from Justy mice lacked CD19+ cells (not depicted). T and NK cell populations were characterized by flow cytometry (unpublished data). The number of CD3+ cells in Justy spleens was consistently decreased by approximately twofold, but otherwise no abnormalities affecting T cells were found. Likewise, NK cells in thymus and spleen appeared normal in Justy mice. The cellularity of also likely important for immunoglobulin gene rearrangement (Fuxa et al., 2004; Reynaud et al., 2008). Thus, although much has been defined, significant gaps remain in our knowledge of how gene expression is regulated during B lymphopoiesis.

Mice bearing random mutations are powerful tools for identifying genes with roles in the immune system (Cook et al., 2006; Beutler et al., 2007). In this paper, we describe a chemically induced recessive mutation that blocks B lymphopoiesis at an early stage without perturbing the development of other hematopoietic lineages. This lesion is a point mutation that reduces RNA and protein expression from Gon4-like (Gon4l), which likely encodes a novel regulator of gene expression. The mutation had little impact on B-lineage gene expression but impaired the regulation of genes associated with myeloid cell development. Our findings demonstrate that Gon4l is critical for B lymphopoiesis.

RESULTS AND DISCUSSION

Justy mice lack peripheral B cells

Male C3HeB/FeJ (C3H) mice were injected with a dose of n-ethyl-n-nitrosourea that resulted in \~1 mutation per 2.7 megabase pairs of DNA (Hrabé de Angelis et al., 2000; Augustin et al., 2005) and were mated to wild-type C3H females.
The few host-derived cells present lacked B220 indicating that B220−esis when transplanted into marrow stroma. Wild-type bone marrow restored hematopoietic progenitors and bone marrow stroma can support B lymphopoiesis from Justy cells (Fig. 3 e). However, the frequency and number of B220− cells in Justy bone marrow were close to normal (Fig. 3, a and b). This population contains cells defined as pro–B and pre–B cells but also includes NK cell precursors and plasmacytoid DCs (pDCs). To distinguish these subsets, expression of CD19, the NK marker CD49b (clone DX5), and the pDC markers CD11c and Ly6C by B220− cells was assessed (Fig. 3 c). Relative to wild type, the frequency and number of pro–B cells (CD49b−CD11c−Ly6C−CD19+) in Justy bone marrow were greatly decreased, but these parameters were normal for pre–B cells (quadruple-negative cells; Fig. 3 c and d). Surface levels of IL-7 receptor α and AA4.1/CD93 on Justy pre–B cells were similar to that on wild type (Fig. 3 e). Common lymphoid progenitors (CLPs) are a primary source of B cell progenitors were also examined. B220−CD43− cells comprising B and immature B cells were decreased ~300-fold in Justy bone marrow relative to wild type (Fig. 3, a and b). However, the frequency and number of B220−CD43− cells in Justy bone marrow were close to normal (Fig. 3, a and b). This population contains cells defined as pro–B and pre–B cells but also includes NK cell precursors and plasmacytoid DCs (pDCs). To distinguish these subsets, expression of CD19, the NK marker CD49b (clone DX5), and the pDC markers CD11c and Ly6C by B220−CD43− cells was assessed (Fig. 3 c). Relative to wild type, the frequency and number of pro–B cells (CD49b−CD11c−Ly6C−CD19+) in Justy bone marrow were greatly decreased, but these parameters were normal for pre–B cells (quadruple-negative cells; Fig. 3, c and d). Surface levels of IL-7 receptor α and AA4.1/CD93 on Justy pro–B cells were similar to that on wild type (Fig. 3 e). Common lymphoid progenitors (CLPs) are a primary source of B cell progenitors. Therefore, these cells were also characterized (Fig. S2 a). Markers examined included Ly6d, which identifies CLPs with potent B cell potential (Inlay et al., 2009; Mannson et al., 2010). These studies demonstrated that the phenotype and number (Fig. S2 b) of CLPs in Justy bone marrow were normal (Fig. S1, b and d), suggesting that the multipotent stem cell pool is not grossly perturbed.

B cell progenitors were also examined. B220−CD43− cells comprising B and immature B cells were decreased ~300-fold in Justy bone marrow relative to wild type (Fig. 3, a and b). However, the frequency and number of B220−CD43− cells in Justy bone marrow were close to normal (Fig. 3, a and b). This population contains cells defined as pro–B and pre–B cells but also includes NK cell precursors and plasmacytoid DCs (pDCs). To distinguish these subsets, expression of CD19, the NK marker CD49b (clone DX5), and the pDC markers CD11c and Ly6C by B220−CD43− cells was assessed (Fig. 3 c). Relative to wild type, the frequency and number of pro–B cells (CD49b−CD11c−Ly6C−CD19+) in Justy bone marrow were greatly decreased, but these parameters were normal for pre–B cells (quadruple-negative cells; Fig. 3 c and d). Surface levels of IL-7 receptor α and AA4.1/CD93 on Justy pro–B cells were similar to that on wild type (Fig. 3 e). Common lymphoid progenitors (CLPs) are a primary source of B cell progenitors. Therefore, these cells were also characterized (Fig. S2 a). Markers examined included Ly6d, which identifies CLPs with potent B cell potential (Inlay et al., 2009; Mannson et al., 2010). These studies demonstrated that the phenotype and number (Fig. S2 b) of CLPs in Justy bone marrow were normal. 

Differential expression of BP-1 subdivides the pro–B cell compartment into late pro–B cells (HSA+BP-1+) and early pro–B cells (HSA+BP-1−), whereas differential expression of CD24/HSA subdivides late pro–B cells into fractions C and C′.
with the latter containing cells expressing pre-B cell receptors. Fraction C' cells were absent from justy bone marrow, but fraction C and early pro-B cells were detectable (Fig. 3 f). However, relative to wild type, the numbers of these cells were dramatically decreased (Fig. 3 g). These data demonstrate that B lymphopoiesis in justy mice is disrupted starting at the early pro-B cell stage.

Justy B cell progenitors specifically lack B cell potential

Pre-pro-B cells, as defined in the previous section, can give rise to early pro-B cells in culture (Rumfelt et al., 2006). To assess how the mutation affects the developmental potential of pre-pro-B cells, these cells were sort purified from wild-type or justy bone marrow and added to stromal cell monolayers under conditions that promote B lymphopoiesis. 10 d later, surface marker phenotypes and cell yields were assessed by flow cytometry (Fig. 4, a and c). Wild-type pre-pro-B cells proliferated extensively and gave rise to B220+CD19+ cells. In contrast, although justy cells could generate B220+CD19+ cells, the yield was much lower compared with that from wild type. The responses of sort-purified early pro-B cells to these conditions were also assessed (Fig. 4, b and c). Wild-type cells underwent robust proliferation, as expected, whereas justy cells remained alive, as judged by vital dye exclusion, but failed to expand. IL-7/Stat5 signaling drives B cell proliferation in the culture system. When analyzed immediately after removal from mice, similar levels of surface IL-7 receptor α and intracellular tyrosine-phosphorylated Stat5 were detected in wild-type and justy B cell progenitors (Fig. 3 e; Fig. S3), suggesting that the mutation does not impair receptor-proximal IL-7 signaling. These data indicate that the justy mutation impairs the development of early pro-B cells from progenitors. Alternatively (or in addition), the mutation may prevent early pro-B cells that develop from responding to the appropriate stimuli.

Pre-pro-B cells can generate cells expressing myeloid or T/NK markers (Balciniuate et al., 2005; Rumfelt et al., 2006). Under conditions that favor B lymphopoiesis but permit myeloid cell development, wild-type and justy pre-pro-B cells both generated cells expressing the macrophage/pDC marker Ly6C (Fig. 4, d and f). Likewise, wild-type and justy pre-pro-B cells both produced B220-Thy1.2+CD49b+ cells under conditions that promote T/NK cell precursor development (Fig. 4, e and f). Thus, justy pre-pro-B cells retain the capacity to differentiate toward these alternative lineages.

The justy mutation disrupts splicing of RNA transcribed from the gon4l gene

To identify the genetic basis for the phenotype, C3H justy mice were crossed to wild-type B6 mice and F1 progeny were interbred. Genomic DNA from F2 mice that lacked or contained B cells was screened with a panel of PCR primers that detect simple sequence length polymorphisms (SSLPs) between C3H and B6 and thus define the strain of origin for discrete genomic intervals. This analysis established a complete association between a lack of B cells and a 617-kb-pair region on chromosome 3 from C3H between SSLPs D3Mit49 and D3Mit175 (bps 88353984 and 88971024; University of California, Santa Cruz (UCSC) assembly, July 2007). The SSLPs were used to transfer the C3H segment into the B6 background via serial backcrossing. Intercrossing F10 B6 mice generated offspring that were homozygous for the C3H region with Mendelian frequency. Flow cytometric analysis showed that B lymphopoiesis in these mice is blocked, as seen in the original mutant strain (unpublished data), confirming that the transferred C3H DNA segment contained the causative lesion.

The identified region encodes 13 genes (Fig. 5 a and Table S1). Quantitative (Q) RT-PCR analysis of RNA from bone marrow lineage-negative cells showed that all of these genes were expressed normally in justy cells except for gon4l, which was expressed at approximately twofold lower levels relative to wild type (Fig. 5 b). RT-PCR analysis was performed to define the structure of gon4l mRNA expressed in wild-type and justy cells, which revealed a striking difference (Fig. 5 c). Primers targeting exons 24 and 26 amplified a 446-bp product from wild-type cDNA with the expected sequence. The same product was amplified from justy cDNA.

Figure 4. Justy B cell progenitors lack B cell potential but retain myeloid and T cell potential. (a and b) Flow cytometric analysis of cells harvested 10 d after adding 2,000 pre-pro-B cells (a) or early pro-B cells (b) in media containing SCF, Flt3L, and IL-7 to S17 stromal cell monolayers. (c) Yields of CD19+B220+ cells from cultures started with pre-pro-B or early pro-B cells as indicated below the graph. (d) Flow cytometric analysis of non-B cells in the CD19+B220+ gates in a. (e) Flow cytometric analysis of cells obtained 15 d after adding 2,000 pre-pro-B cells in media containing IL-6, -7, and -15 and Flt3L to OP9-DL1 stromal cell monolayers. All Thy1.2+CD49b+ cells expressed CD25 (not depicted). (f) Yields of cells obtained after plating pre-pro-B cells onto the indicated stromal cells. All data are representative of three independent experiments.
but the amount obtained was generally lower. In addition, PCR amplification of Justy cDNA gave rise to a prominent and unique 527-bp product that contained an 81-bp sequence inserted between exons 24 and 25. BLAT analysis (UCSC Genome Browser) showed that the insertion was derived from the intron between Gon4l exons 24 and 25. Gon4l mRNA containing the insertion was otherwise unaffected based on sequence analysis of PCR products anchored to the insertion.

The data described in the previous paragraph posited that intron 24 of Gon4l in Justy mice contains a mutation that causes the 81-bp element to be recognized as an exon. Sequence analysis of the Justy genome identified a T-to-A substitution immediately downstream of the 81-nt sequence that increases homology between the adjoining region and the consensus donor pre-mRNA splice site (Fig. 5 d). The structure of the 527-bp Justy-specific PCR product confirmed that this region is used as a donor splice site, resulting in fusion of the 81-nt sequence to exon 25. The structure of the 527-bp product also showed that a cryptic acceptor splice site in intron 24 mediates fusion of the 81-nt sequence to exon 24. Notably, inclusion of the 81-nt sequence into Gon4l mRNA places two premature termination codons into the open reading frame. However, the encoded protein was not detected when Justy cell lysates were immunoblotted with antibodies capable of recognizing it (unpublished data). This result suggests that inclusion of the 81-nt sequence, and thus the premature termination codons, activates the nonsense-mediated decay RNA surveillance pathway (Chang et al., 2007), preventing translation of the encoded protein.

Aberrant splicing of Gon4l RNA results in decreased expression of protein

It was not feasible to assess how the mutation affects Gon4l protein expression in the few B-lineage cells present in Justy mice. However, Northern blot analysis showed that Gon4l RNA was abundant in wild-type and Justy thymocytes (unpublished data). Immunoblotting of thymocyte lysates with antibodies specific for the C terminus of Gon4l showed that wild-type cells express this protein and that the level of Gon4l in Justy cells was greatly reduced (Fig. 5 e). To correlate this effect with altered Gon4l RNA splicing, Q RT-PCR analysis of thymocyte RNA was performed using primer pairs specific for either aberrantly spliced Gon4l RNA (i.e., containing the 81-nt insertion) or for wild-type Gon4l RNA (Fig. 5 f). Aberrantly
spliced RNA was detected in Justy cells but not in wild type. Wild-type Gon4l RNA was observed in both wild-type and Justy thymocytes but the level in Justy cells was ~10-fold lower. These data demonstrate that disruption of Gon4l RNA splicing as a result of the Justy mutation greatly reduces expression of wild-type Gon4l mRNA and thus protein.

Wild-type Gon4l RNA levels are greatly decreased in Justy B cell progenitors
Gon4l expression in B cell progenitors was assessed. Q RT-PCR analysis showed that Gon4l RNA is present at all stages of B lymphopoiesis and at elevated levels in later stages (Fig. 6 a). Immunoblot analysis of lysates prepared from wild-type pro–B cells grown in culture demonstrated that Gon4l was present (Fig. 6 b), which is consistent with the Justy phenotype. Gon4l was also detected in lysates from the mouse B cell line M12 (Fig. 6 b) or those from other mouse B cell lines (not depicted). Q RT-PCR analysis was performed to determine the levels of wild-type and aberrantly spliced Gon4l RNA in sort-purified pre-pro–B and early pro–B cells (Fig. 6 c). Similar to thymocytes, aberrantly spliced Gon4l RNA was detected only in Justy B cell progenitors. Wild-type Gon4l RNA was detected in both wild-type and Justy cells but the levels in Justy cells were greatly and significantly reduced. These data show that the Justy mutation dramatically reduces expression of wild-type Gon4l mRNA in B cell progenitors, an effect which was demonstrated to decrease Gon4l protein expression.

Gon4l encodes a protein with homology to transcriptional corepressors
As described for its orthologues (Liu et al., 2007; Ohtomo et al., 2007), mouse Gon4l contains regions with homology to transcriptional regulators (Fig. 6 d). A portion of Gon4l is 78% similar to human YY1AP (YY1-associated protein), so named because it interacts with the transcription factor YY1 (Wang et al., 2004). Two regions near the C terminus of Gon4l bear strong homology (E-values of 2.2 and 3.4 × 10^{-12}; from the SMART database) to the consensus paired amphipathic helix (PAH) repeat sequence, which forms a four-helix bundle (Brubaker et al., 2000). The mammalian transcriptional corepressors Sin3a and Sin3b each contain four PAH repeats, which mediate interactions with chromatin-modifying factors or DNA-binding proteins (Grzenda et al., 2009). NMR analysis (protein data bank accession code 1UG2) has shown that the C terminus of Gon4l forms a helix-loop-helix structure called a SANT domain (SWI3, ADA2, N-CoR, and TFIIB; Boyer et al., 2004). Proteins containing this domain include the corepressor proteins SMRT and N-CoR. Lastly, the zebrafish and C. elegans Gon4l proteins can localize to the nucleus (Friedman et al., 2000; Liu et al., 2007). These features implicate Gon4l in pathways that mediate transcriptional regulation.

Regulation of gene expression is altered in Justy B cell progenitors
Q RT-PCR analysis was performed to assess how the Justy mutation affects gene expression in B cell progenitors. The development of pro–B cells requires the transcription factors E2A, EBF, and Pax5. Justy pre–pro–B and early pro–B cells expressed normal levels of RNA encoding these factors, except that the level of Pax5 RNA in Justy early pro–B cells was consistently but modestly elevated (Fig. 6 e). Consistent with these data, Justy cells expressed normal or slightly decreased levels of RNA encoding other B-lineage factors (Fig. S4).
These findings suggest that decreased expression of E2A, EBF, or Pax5 is not responsible for the phenotype.

Progression of B lymphopoiesis has been correlated with decreased expression of RNA encoding PU.1 or C/EBPα (Rumfelt et al., 2006). Either of these transcription factors inhibits B lymphopoiesis when expressed at inappropriately high levels (DeKoter and Singh, 2000; Heavey et al., 2003; Xie et al., 2004; Hsu et al., 2006). As reported previously (Rumfelt et al., 2006), Q RT-PCR analysis of wild-type cells suggested that expression of PU.1 and C/EBPα RNA is down-regulated during the pre–pro–B to early pro–B cell transition (by factors of ∼4 and 46, respectively; unpublished data). In Justy pre–pro–B cells, the level of PU.1 RNA was slightly increased (∼1.4-fold), whereas that of C/EBPα RNA was significantly elevated (∼3.5-fold; P < 0.025) relative to that detected in wild-type cells. In Justy early pro–B cells, the levels of PU.1 and C/EBPα RNA were both significantly higher than those in wild-type cells, with the latter being increased by a factor of ∼20 (Fig. 6 f). Furthermore, comparison of Q RT-PCR data from Justy pre–pro–B and early pro–B cells suggested that mechanisms that down-regulate PU.1 and C/EBPα RNA expression are impaired. Consistent with these abnormalities, RNA encoded by the PU.1 and C/EBPα target gene Csfr was expressed at elevated levels in Justy early pro–B cells relative to wild type (Fig. 6 f). These results suggest that loss of Gon4l expression impairs pathways that inhibit expression of PU.1 and C/EBPα RNA in developing B cells.

Gon4l orthologues are present in the genomes of plants, invertebrates, and vertebrates (Kuryshev et al., 2006) and is highly conserved among the latter. Similar to our study, forward–genetic studies involving C. elegans and zebrafish have demonstrated that Gon4l is important for specific developmental pathways (Friedman et al., 2000; Liu et al., 2007). Homozygosity for loss-of-function Gon4l alleles in zebrafish cause embryologic abnormalities and death soon after conception (Liu et al., 2007). Furthermore, such mutations disrupt erythropoiesis and the expression of key erythroid genes (Liu et al., 2007), suggesting that Gon4l is required for hematopoietic development. In addition, loss of Gon4l expression in zebrafish appears to affect pathways that control cell cycle progression (Liu et al., 2007; Lim et al., 2009). Ours is the first study to demonstrate that Gon4l has an important role in mammalian hematopoiesis.

Gon4l RNA is expressed throughout zebrafish embryos (Liu et al., 2007) and is broadly expressed in mammals (Kuryshev et al., 2006; Ohtomo et al., 2007, 2008). Our data show that Gon4l RNA and protein are expressed in B–lineage cells and thymocytes, indicating that Gon4l is not a B–lineage–specific factor. Why the Justy mutation seems to affect only B lymphopoiesis is not clear. Perhaps the role of Gon4l varies between cell lineages and tissues, which suggests that additional but subtler phenotypes might exist. Another factor that may contribute to the specificity of the phenotype is variation between tissues in the extent of aberrant Gon4l pre-mRNA splicing and, thus, the decrease in wild-type Gon4l RNA and protein expression. Indeed, Q RT-PCR analysis of wild-type Gon4l RNA levels in tissues from wild-type and Justy mice showed that such variation occurs (Fig. S5). These data suggest the Justy mutation has created a conditional loss-of-function Gon4l allele rather than a completely nonfunctional one. Further analysis of Justy mice and the Gon4l protein will address these issues and will likely yield novel insights regarding mechanisms that regulate B lymphopoiesis.

MATERIALS AND METHODS
Mice. Mutagenesis, breeding, and positional cloning have been previously described (Hrabé de Angelis et al., 2000; Augustin et al., 2005). Mice were used in compliance with the United States Department of Health and Human Services Guide for Use of Laboratory Animals, as documented in writing and approved by the University of Iowa Animal Care and Use Committee.

Bone marrow transplantation. Recipients were given 1,100 rads of irradiation. 1 h later, 107 red blood cell–depleted bone marrow cells were injected into the retro-orbital plexus. Recipients were given drinking water containing antibiotic (Baytril) for 3 wk afterward and analyzed 5 wk later.

Flow cytometric analysis and cell sorting. Cells were suspended in PBS containing 3% FBS and preincubated with anti-CD16/32. Cells were incubated with fluorochrome– or biotin-conjugated antibodies for 30 min on ice and washed. When necessary, cells were resuspended in buffer containing streptavidin– or avidin-conjugated fluorochrome, incubated for 30 min on ice, and washed. Flow cytometric analysis was performed using an LSR II or a FACSDiva (BD) and data were analyzed using FlowJo (Tree Star, Inc.). A FACSDiva was used to sort purified cells. To isolate cells at the pre–B cell stage onward, splenocytes or bone marrow cells were stained and sorted. To isolate pre–pro–B and early pro–B cells from bone marrow, a negative selection step was included. Cells were incubated with rat anti–mouse Ter-119, CD5, Ly6C, and IgM, washed, and then incubated with magnetic beads coated with sheep anti–rat antibodies (Invitrogen). Beads and bound cells were removed and remaining cells were stained and sorted. Intracellular staining for phospho-Stat5 was performed using a PE-labeled mouse anti–phospho–Stat5 antibody according to the manufacturer’s (BD) instructions.

Antibodies. The following fluorochrome-conjugated antibodies were purchased from the lists of sources or generated in the Waldschmidt Laboratory: Alexa Fluor 700-B220 (RA3-6B2), APC-c-Kit (2B8), and APC-CD24/HA5 (M1/69, BioLegend); Sca-1 (5D7; Pacific Blue); APC conjugates B220 (RA3-6B2) and CD3e (145–2C11), FITC conjugates CD11c (N418) and CD49b (DX5), PE conjugates BP-1 (6C3) and DC25 (PC65.1), and PE-Cy7 conjugates CD11c (N418) and CD19 (1D3; ebioscience); APC-12.2 (53–2–1), APC-Cy7-B220 (RA3-6B2), PE-Gr-1 (RB6-8C5), and PerCP-B220 (RA3-6B2) and Biotin conjugates CD11c/Mac-1 (M1/70), Gr-1 (RB6-8C5), CD43 (S7), CD49b (DX5), CD11c (N418), Ter-119, CD3e (145–2C11), and CD19 (APC-H7; BD); Avidin-Texas Red and Streptavidin Per-CP (BD); Streptavidin Qdot 605 (Invitrogen); FITC-Ly5d (RGRSL 141.8.1; Sigma-Aldrich); Cy5–FITC–, PE–, and PE-Cy7–B220 (RA3-6B2) and FITC-CD3e (145–2C11), Texas Red-CD45.1 (A20), FITC-CD45.2 (104), PE-CD4 (GK1.5), Cy5–CD64 (53–6–7), FITC-CD21 (7E5), PE-CD23 (B3B4), Cy5–IgM (B76), and FITC-Ly5c6 (Waldschmidt Laboratory).

Stromal cell co-cultures. To test for B and myeloid potential, 2,000 sorted cells were added to 24-well plates containing S17 stromal cells (Collins and Dorshkind, 1987) in media containing SCF, Flt3L, and IL-7 at 10 ng/ml each. To test for T/NK cell potential, 2,000 sorted cells were added to wells containing OP9-DL1 cultures of stromal cells (Schmitt and Zúñiga-Pflücker, 2002) in media containing 1 ng/ml IL-6, 10 ng/ml IL-7, 10 ng/ml Flt3L, and 25 ng/ml IL-15. 10 or 15 d later, cells were harvested and analyzed by flow cytometry. S17 cultures were stained for Ly6C, Gr-1, CD11b, B220, and CD19; OP9-DL1 cultures were stained for Thy-1.2, CD25, CD49b (DX5), B220, and CD19.

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Brief Definitive Report
Pro-B cells were generated by adding Lin− cells to layers of OP9 stromal cells in media containing SCF, Flt3L, and IL-7 at 10 ng/ml each. Lin− cells were isolated by negative selection (as described in the Flow cytometric analysis and cell sorting section) except that antibodies for CD19 and B220 were included. Cultures were started in 24-well plates and expanded over a 3-wk period. > 90% of the cells obtained were B220+CD19+.

RT-PCR analysis. RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using SuperScript III (Invitrogen). Conventional PCR was performed using Amplitaq Gold (Applied Biosystems) and a MyCycler (Bio-Rad Laboratories). Quantitative real-time PCR was performed using POWER SYBR Green Master Mix and the PRISM 7700 Detection System (Applied Biosystems) to obtain cycle threshold (Ct) values for target and internal reference (Hprt) cDNAs. Target cDNA levels were normalized to Hprt cDNA levels using the Eq. 2 \[ \frac{C_{\text{target}}}{C_{\text{Hprt}}} \]

Antibody production and immunoblot analysis. cDNA encoding Gon4l aa 938–1364 or 1746–2260 was cloned into the plasmid pGEX-5-1 (GE Healthcare). GST-Gon4l fusion proteins were expressed in bacteria and purified using glutathione-agarose beads. Fusion proteins were cleaved with Factor Xa (GE Healthcare) and resolved by SDS-PAGE. Rabbits were immunized three to five times with gel slices containing Gon4l protein. Immunoglobulins from pooled bleeds were purified by protein A-Sepharose and affinity selection and used for immunoblot analysis. Protein lysates were prepared by resuspending cells in RIPA buffer containing protease inhibitors (Roche). Immunoblot analysis used standard protocols.

Online supplemental material. Fig. S1 shows flow cytometric analysis and quantification of hematopoietic and myeloid progenitors in bone marrow. Fig. S2 shows flow cytometric analysis and quantification of CLPs in bone marrow. Fig. S3 shows flow cytometric analysis of IL-7Rα expression and phospho-Stat5 levels in B cell progenitors. Fig. S4 shows Q RT-PCR analysis of B lineage gene expression in B cell progenitors. Fig. S5 shows the ratio of normal Gon4l RNA detected in tissues from Justy or wild-type mice as determined by conventional PCR and real-time PCR analysis.

Online supplemental material. The sequential determination model of hematopoiesis. Trends Immunol. 28:442–448. doi:10.1016/j.it.2007.07.007.

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