Repression of *Flt3* by Pax5 is crucial for B-cell lineage commitment

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Early B-lymphopoiesis requires the growth-factor receptors, IL-7R and Flt3, and the activity of a number of transcription factors. One factor, Pax5, is required for commitment to the B-cell lineage, although the molecular mechanism by which this occurs is unknown. We demonstrate here that an important function of Pax5 is to repress *Flt3* transcription in B-cell progenitors, as Pax5-deficient pro-B cells express abundant *Flt3* that is rapidly silenced upon the reintroduction of Pax5, whereas enforced expression of *Flt3* in wild-type progenitors significantly impairs B-cell development. These findings demonstrate that the repression of *Flt3* by Pax5 is essential for normal B-lymphopoiesis.

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Hematopoietic stem cells (HSCs) have the dual capacity to self-renew and to give rise to cells of all the blood lineages. Although the identity and diversity of the intermediaries is still not completely understood, it is clear that an early differentiation step from the HSC gives rise to multipotent progenitors that have reduced capacity to self-renew. These progenitors up-regulate the tyrosine kinase receptor Flt3 (also termed flk2 and CD135) (Adolfsson et al. 2001) and efficiently reconstitute the lympho-myeloid but not the erythro-megakaryocytic lineages (Adolfsson et al. 2005). Flt3 is later expressed on a subset of common myeloid progenitors, with dendritic cell (DC) potential (D'Amico and Wu 2003), and common lymphoid progenitors (CLPs) (Sitnicka et al. 2002).

Flt3$^{-/-}$ mice have a severe deficiency in B-cell progenitors, and the mutant HSCs are impaired in their ability to reconstitute lymphoid and myeloid cells in recipient mice (Mackarehtschian et al. 1995). Similarly, Flt3L-deficient mice have significantly reduced numbers of CLPs, B-cell progenitors, DCs, and natural killer (NK) cells (McKenna et al. 2000). Flt3L expressed by bone marrow stroma (Lisovsky et al. 1996), is a weak growth stimulator of Flt3^{+} CLPs in vitro and synergizes with stem cell factor (SCF) and IL-7 to promote the proliferation of lymphocytes and, most potently, DCs (Ray et al. 1996; Saunders et al. 1996; Veiby et al. 1996). The ability of Flt3 to promote the expression of the IL-7 receptor on CLPs provides a mechanism by which this synergy occurs in early lymphopoiesis (Borge et al. 1999).

B-cell specification is controlled by the coordinate activity of a number of transcription factors, including E2A and EBF, which regulate rearrangement of the *IgH* locus and pre-B-cell receptor expression (for review, see Busslinger 2004; Singh et al. 2005). These factors also induce *Pax5*, which is essential for the restriction of lymphoid progenitors to the B-cell fate (Nutt et al. 1999). In the absence of Pax5, B lymphopoiesis in the bone marrow is blocked at the early pro-B (or pre-BI)-cell stage (Urbanek et al. 1994; Nutt et al. 1997). Analysis of *Pax5*$^{-/-}$ pro-B cells has demonstrated that Pax5 plays a dual role in B-cell commitment, activating lineage-specific genes such as *CD19* and *mb-1* while repressing lineage inappropriate genes such as *M-CSR*, and *Notch1* (Nutt et al. 1998; Souabni et al. 2002). *Pax5*$^{-/-}$ pro-B cells also have the capacity to self-renew indefinitely and to differentiate into multiple hematopoietic lineages (Nutt et al. 1999; Rolink et al. 1999). This similarity in developmental potential between *Pax5*$^{-/-}$ pro-B cells and multipotent progenitors suggested that Pax5 normally represses genes necessary for the maintenance of the undifferentiated state. Here we show that one key function of Pax5 in B-lineage commitment is to directly repress *Flt3* expression, a process that is required for normal B-cell development.

Results and Discussion

Flt3 expression on pro-B cells

As Pax5 is essential for the commitment of progenitors to the B-cell fate, we hypothesized that Pax5 would directly repress genes associated with multipotency. *Flt3* represented an attractive candidate for Pax5 repression, as it is required for the repopulation capacity of multipotent cells (Adolfsson et al. 2001), and its expression is extinguished early in B-cell differentiation (Ogawa et al. 2000). Analysis of the c-kit^{+}B220^{+} fraction (termed pro-B cells) isolated from the bone marrow of young mice (day 14) demonstrated only residual cell surface Flt3, whereas all *Pax5*$^{-/-}$ pro-B cells expressed Flt3 (Fig. 1A). *Flt3* expression could also be seen at the RNA level and was maintained in long-term *Pax5*$^{-/-}$ pro-B-cell cultures (Fig. 1B,C). Thus *Flt3* expression is maintained in the absence of Pax5. To determine whether the ectopic Flt3 is functional, we cultured *Pax5*$^{-/-}$ pro-B cells for 3 d in a range of Flt3L concentrations. These experiments demonstrated a clear dose-dependent proliferation of *Pax5*$^{-/-}$ pro-B cells in response to Flt3L (Fig. 1D).

Induction of Pax5 in pro-B cells represses Flt3 expression

The continued expression of Flt3 on *Pax5*$^{-/-}$ pro-B cells suggested that one function of Pax5 in wild-type cells is to repress the *Flt3* gene. To test this hypothesis, we used a retroviral vector that expressed Pax5 fused to the ligand-binding domain of human estrogen receptor (Pax5ER) (Nutt et al. 1998). *CD19* is a direct transcrip-

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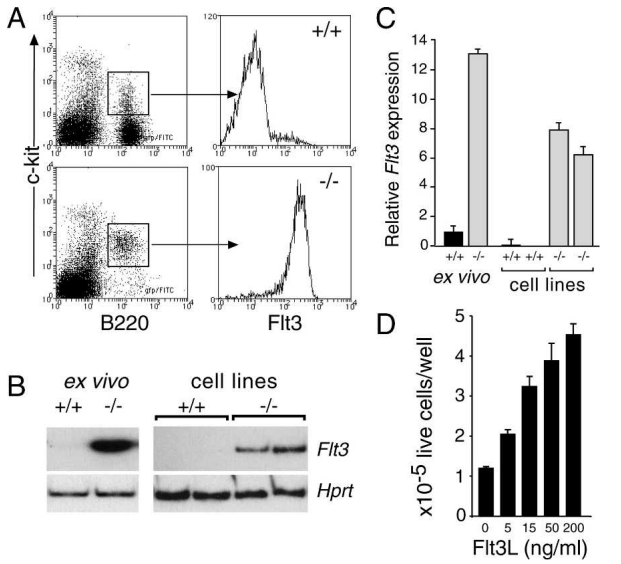

Figure 1. *Pax5*$^{-/-}$ pro-B cells express Flt3. (*A*) FACS analysis of Flt3 on bone marrow c-kit$^+$B220$^+$ pro-B cells from 14-d-old wild-type (+/+) and *Pax5*$^{-/-}$ (–/–) mice. c-kit$^+$B220$^+$ pro-B cells and established pro-B cell lines were analyzed for *Flt3* expression by RT–PCR (*B*) and real-time PCR (*C*). *Hprt* was used to normalize cDNA input. (*D*) *Pax5*$^{-/-}$ pro-B cells proliferate in response to Flt3L. Cells were cultured in IL-7 plus the indicated amounts of Flt3L for 72 h. The mean live cell number of triplicate wells ± SD is shown.

tional target that requires Pax5 for its expression (Nutt et al. 1998). As expected, *Pax5*$^{-/-}$ pro-B cells transduced with Pax5ER were Flt3$^+$CD19$^-$, indicating that the fusion protein was inactive in the absence of ligand. Upon the addition of β-estradiol (E2), CD19 expression was strongly induced on the Pax5ER, but not control ER expressing cells. Importantly cell-surface Flt3 levels significantly declined with Pax5ER induction (Fig. 2A). RT–PCR analysis of the kinetics of the repression indicated that *Flt3* mRNA levels had dropped significantly within 4 h of Pax5 induction, indicating that this effect was likely to be direct (Fig. 2B,C).

To examine the timing of *Flt3* repression during early B-cell differentiation, we have examined the c-kit$^+$B220$^+$ pro-B-cell compartment of wild-type mice. Interestingly, while the majority of pro-B cells were CD19$^+$Flt3$^-$, indicating Pax5 expression, a small fraction of Flt3$^+$CD19$^-$ and Flt3^{low}CD19$^+$ cells was also present. This suggests that the progenitors progress from Flt3$^+$CD19$^-$ to Flt3^{low}CD19$^+$ and Flt3$^-$CD19$^+$ stages with the onset of *Pax5* expression, while in its absence, only Flt3$^+$CD19$^-$ cells were generated (Fig. 2D). This conclusion is supported by a recent study that described a B220$^+$c-kit$^+$CD19$^-$NK1.1$^-$ bone marrow cell population that can efficiently produce both lymphoid and myeloid cells in vitro (Balciunaite et al. 2005).

Pax5 binds to the Flt3 promoter

Pax5 regulates the expression of a number of genes by direct binding to their proximal promoter elements. As the *Flt3* transcriptional start site and promoter region have not been reported previously, we used RNase protection assays to identify the *Flt3* transcription initiation site. Analysis of the protected fragment placed the transcriptional start site at 47 nucleotides (nt) upstream of the initiating methionine, a finding that was independently confirmed using 5′ RACE (Supplementary Fig. 1).

The conservation of genomic sequences between species is generally indicative of functional importance. The putative *Flt3* promoter sequence was aligned with the corresponding human, rat, and canine sequences. A region including the exon-1 and extending ~250 nt upstream of the transcriptional start site was highly conserved between these four species (Supplementary Fig. 1B). Beyond this, the sequences differed considerably. A search for transcription factor-binding sites in this region revealed two potential Pax5-binding sites (Supplementary Fig. 1B,C) (Czerny et al. 1993).

To determine whether Pax5 could bind to these sites within the *Flt3* promoter, we performed electrophoretic mobility shift assays (EMSAs) using nuclear extracts and labeled oligonucleotides corresponding to either of the putative sites (Px1 and Px2). A high-affinity site from the human *CD19* promoter served as a positive control (Fig. 3A; Kozmik et al. 1992). A DNA-binding complex was identified with each of the three probes that could be competed away with an unlabeled Pax5-binding site probe, but not with a fragment harboring a mutated Pax5

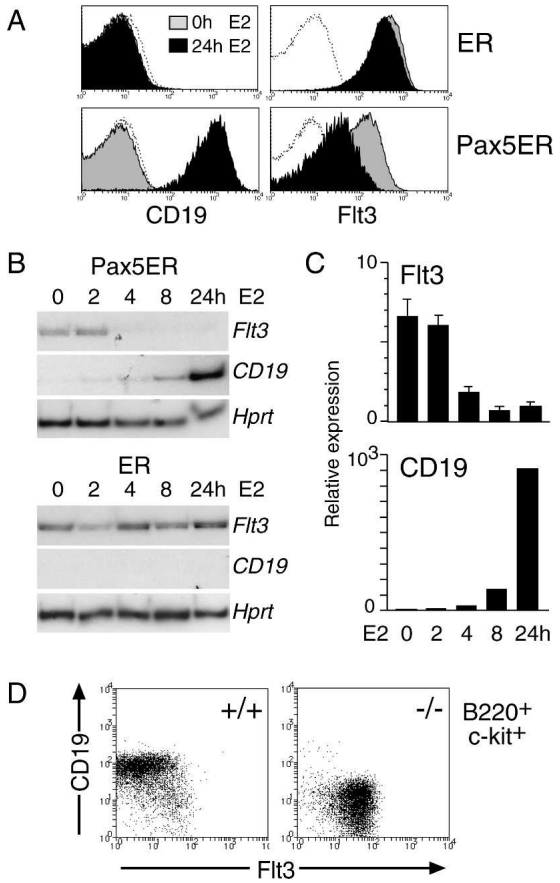

Figure 2. *Flt3* is down-regulated by Pax5. (*A*) FACS analysis for CD19 and Flt3 on *Pax5*$^{-/-}$ pro-B cell lines stably expressing an inducible Pax5ER or the estrogen receptor alone (ER) before and after 24-h treatment with 1 μM E2. (*B*) RT–PCR analysis of *CD19* and *Flt3* regulation in the Pax5ER and ER cell lines at the indicated time points after E2 addition. (*C*) Real-time PCR analysis of PaxER induction time course. cDNAs were normalized using *Hprt*. (*D*) Analysis of CD19 and Flt3 levels on bone marrow pro-B cells from *Pax5*$^{-/-}$ (–/–) and wild-type (+/+) mice.

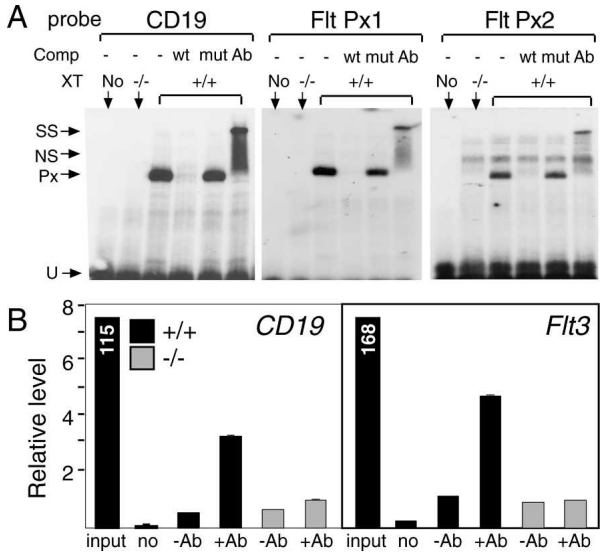

Figure 3. Pax5 binds to the *Flt3* promoter. (*A*) EMSA of nuclear extracts (XT) from pro-B cell lines. The probes were ^{32}P-labeled oligonucleotides corresponding to a known Pax5-binding site from the *CD19* promoter (CD19) and the two putative sites in the *Flt3* promoter (Flt Px1 and Px2). Preincubation of nuclear extracts with either wild-type (wt) or a mutated (mut) CD19 oligonucleotide was used in competition assays (Comp). Pax5–DNA complexes were supershifted with a Pax5 antibody (Ab). Pax5-specific binding complexes (Px), nonspecific interactions (NS), complexes supershifted with Pax5 antibody (SS), or unbound probes (U) are indicated. (*B*) Determination of in vivo Pax5 binding to the *Flt3* and *CD19* promoter regions in pro-B cell lines using ChIP and real-time PCR analysis. Assay was performed ±Pax5 polyclonal antibody (Ab). Input is the equivalent amount of cross-linked chromatin prior to ChIP (the number in the column indicates the actual value for the input). DNA was quantified by real-time PCR using *Flt3* and *CD19* specific primers (see Supplemental Material) and calculated relative to *Pax5*$^{-/-}$ cells + Ab.

site (Fig. 3A). Preincubation of nuclear extracts with a Pax5-specific antibody resulted in a supershifted band, confirming that Pax5 bound to the sites identified in the *Flt3* promoter.

We used chromatin immunoprecipitation (ChIP) assays to investigate whether the Pax5 sites in the *Flt3* promoter are occupied in vivo. Pax5–chromatin complexes were precipitated from wild-type pro-B cells using a polyclonal antibody against Pax5, before PCR amplification with gene-specific primers. There was a fourfold enrichment of Pax5 binding to the known *CD19*-binding site in the sample immunoprecipitated with a Pax5 antibody compared with samples either from the *Pax5*$^{-/-}$ cells or in the absence of antibody (Fig. 3B). Most importantly, the *Flt3* promoter region spanning both Pax5-binding sites was similarly enriched in the sample derived from wild-type cells, providing compelling evidence that Pax5 bound to this region in vivo.

Enforced expression of Flt3 on hematopoietic cells impairs B lymphopoiesis

To address the importance of *Flt3* down-regulation by Pax5 to B-cell development, we expressed *Flt3* throughout hematopoiesis using retroviral transduction. The MigR1 retroviral vector (Pear et al. 1998) was engineered to coexpress *Flt3* and *GFP* (MigR1–Flt3), allowing transduced cells to be identified by flow cytometry. C57BL/6-Ly5.2 bone marrow stem cell cultures were transduced with the MigR1–Flt3 or MigR1 constructs and injected into lethally irradiated C57BL/6-Ly5.1 congenic recipients. Reconstitution was assessed at 7 wk by analyzing Ly5.2 and GFP expression in hematopoietic organs (Fig. 4A).

Flt3 expression was readily detected on GFP^{hi} cells from MigR1–Flt3 HSC reconstituted mice in both bone marrow and thymus (Fig. 4A,B). However splenocytes showed few GFP^{hi} cells, which did not express Flt3, suggesting Flt3 negatively impacted on the ability of predominantly mature lymphocytes to repopulate the spleen. In contrast, cells transduced with the MigR1 vector, stably expressed GFP in all hematopoietic organs, and lacked endogenous Flt3 (Fig. 4A,B).

A comparison of the percentages of total B cells (B220$^+$CD19$^+$) in bone marrow GFP^{hi} fractions from control and Flt3-expressing mice revealed a significant reduction in B cells in the presence of constitutive Flt3 expression (Fig. 4C,D). B-cell developmental stages can be followed by the expression of surface markers, progressing from B220$^+$IL-7R$^+$ stage encompassing pro-B/pre-B cells to B220$^+$CD25$^+$ pre-BII cells and finally to IgM$^+$IgD$^+$ mature B cells. MigR1–Flt3-transduced stem cells gave rise to fewer B cells in each bone marrow compartment (Fig. 4C). The combination of reduced GFP$^+$Flt3$^+$ cells in early B-cell differentiation and the selection against Flt3 expression in peripheral B cells highlights the importance of the Pax5-mediated repression of *Flt3*.

Interestingly, within the non-B-cell compartment of the bone marrow, the majority of GFP^{hi} cells were myeloid (Mac1$^+$Gr1$^+$), while NK cells (NK1.1$^+$CD49b$^+$) were strongly reduced in numbers (Fig. 4C,D). In contrast, Flt3 appeared to have no effect on thymocyte differentiation with similar numbers of CD4$^+$CD8$^+$ cells compared with the MigR1 control, even though Flt3 was readily detectable on CD4$^+$ cells (Fig. 4B). Thus, the developing B- and NK-cell lineages appeared to be particularly susceptible to Flt3 overexpression.

To determine whether ectopic Flt3 reduced the frequency at which progenitors commit to the B-cell lineage or whether it inhibited maturing B cells directly, we examined the in vitro differentiation of B cells from stem cells transduced with the MigR1–Flt3 or control viruses. Lineage-negative (Lin$^-$) GFP$^+$ cells were cultured for 7 d on OP9 stromal cells in B-cell conditions. Limiting dilution assays indicated that the MigR1-transduced cells very efficiently produced B220$^+$CD19$^+$ B cells, whereas the MigR1–Flt3-transduced cells consistently produced clones at fourfold lower dilutions (Fig. 5A). A similar analysis of the cloning frequency of myeloid progenitors revealed no effect of Flt3 expression (Fig. 5A). Importantly, proliferation assays performed on B220$^+$CD19$^+$ committed B cells from the MigR1–Flt3 cultures showed a similar dose-dependent response to Flt3L compared with that shown for *Pax5*$^{-/-}$ pro-B cells, indicating that the Flt3–Flt3L interaction did not inhibit, and actually promoted, the proliferation of committed B-cell progenitors (Fig. 5B). Taken together, these data indicated that the reduced B-lymphopoiesis in Flt3 overexpressing mice resulted from lower B-cell precursor frequency and not a pronounced effect of Flt3 on committed B cells.

The data presented here demonstrate that the repression of *Flt3* by Pax5 is an essential step in the process of

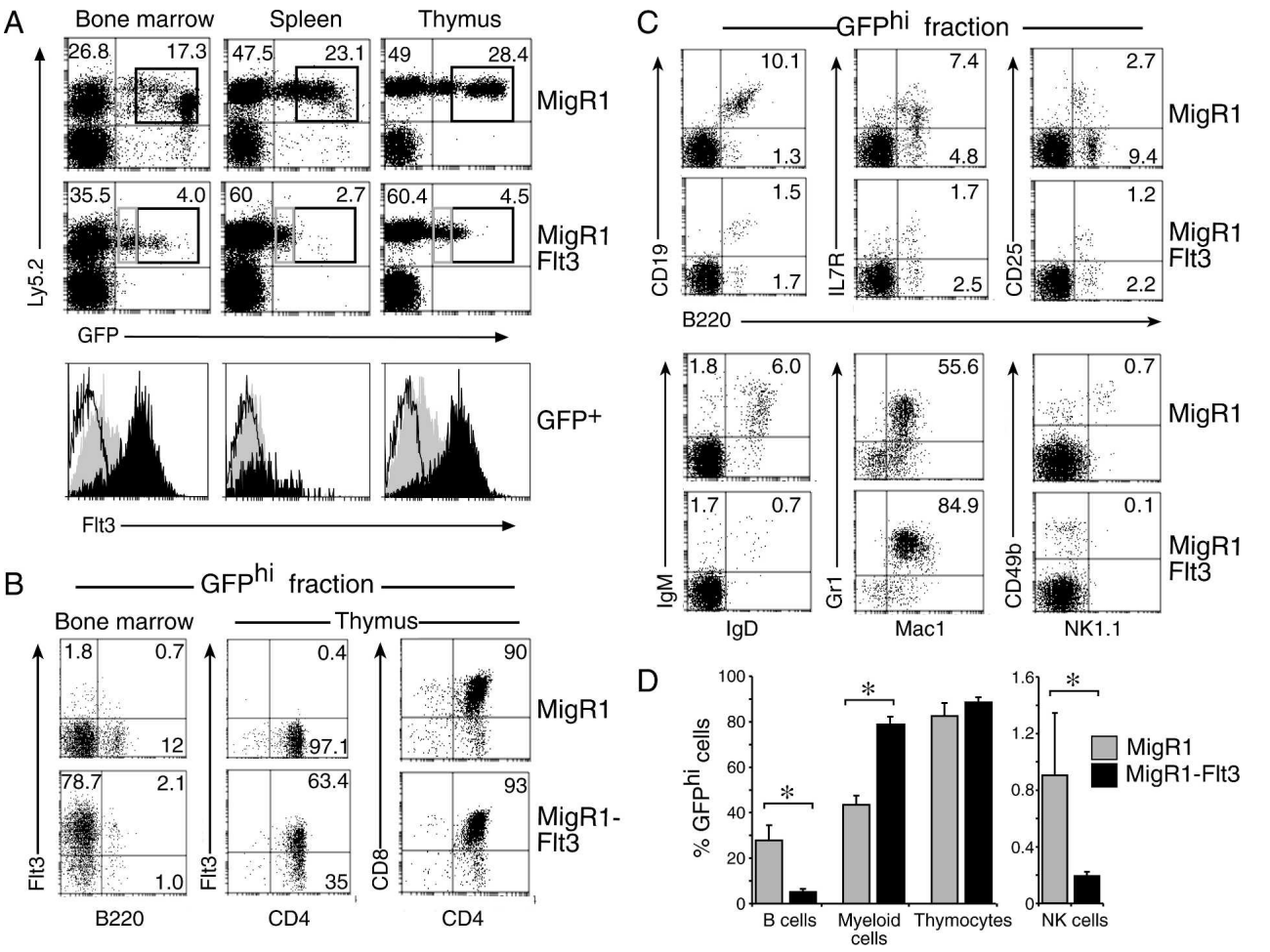

Figure 4. Enforced *Flt3* expression impairs B-cell development. (*A*) C57BL/6-Ly5.2 bone marrow stem cell cultures were transduced with GFP expressing MigR1 or MigR1–Flt3 retroviral vectors and used to reconstitute lethally irradiated C57BL/6-Ly5.1 recipients. Flt3 expression in donor Ly5.2$^+$ GFP^{lo} (gray box) and GFP^{hi} (black box) cells was determined after 7 wk by FACS. (*B*) FACS analysis of Flt3 GFP^{hi} cells. (*C*) GFP^{hi} cells in the bone marrow compartment were identified using the indicated markers. Data are representative of five experiments. (*D*) Percentage of bone marrow B cells (B220$^+$CD19$^+$), myeloid cells (Gr1$^+$Mac1$^+$), NK cells (NK1.1$^+$CD49b$^+$), and thymocytes (CD4$^+$CD8$^+$) derived from GFP^{hi} cells. Mean of five experiments ± SEM is shown. (*) $P < 0.05$ paired Student's *t*-test.

B-cell lineage commitment. Initial studies of Flt3- and Flt3L-deficient mice reported reduced frequencies of B-cell progenitors, suggesting that Flt3 signaling was important for early B cells (Mackarehtschian et al. 1995; McKenna et al. 2000). Later studies, however, showed that the reduced B-lymphopoiesis in these strains reflects the requirement of the Flt3–Flt3L interaction for the maintenance of normal CLP numbers (Adolfsson et al. 2001; Sitnicka et al. 2002). These data support our expression analysis to indicate that Flt3 is specifically important at the CLP stage, before the induction of *Pax5*. As well as displaying synergy with cytokines such as IL-7 to support the survival and/or proliferation of lymphocytes, Flt3L also induces DC differentiation (Saunders et al. 1996), demonstrating that its role in early progenitors affects all CLP-derived lineages. The reduced B- and NK-cell differentiation, but normal thymopoiesis, we observed after ectopic expression of *Flt3* suggests the inhibitory function of prolonged Flt3 occurs after the CLP stage of differentiation. Moreover, as our in vitro assays showed committed B220$^+$CD19$^+$ cells ectopically expressing *Flt3* did not appear impaired in their proliferative response, we propose that the inhibitory effect of continued *Flt3* expression occurs at the transition between the CLP and the committed B-cell progenitor. We postulate that enforced Flt3 in this population allows these multipotent progenitors to be influenced by other stimuli, such as those favoring DC or T-cell differentiation, thereby impairing the capacity of CLP to produce the normal complement of B-cell precursors.

The transcriptional regulation of the commitment to the B-cell lineage has been a subject of intense interest in recent years, and a model has emerged where factors including Ikaros and PU.1 are required for the specification of the CLP (Fig. 5C; for review, see Busslinger 2004; Singh et al. 2005). These proteins induce the expression of two essential growth factor receptors, *IL-7Rα* and *Flt3*, in lymphoid progenitors (Nichogiannopoulou et al. 1999; DeKoter et al. 2002). Once these progenitors are specified, EBF and E2A are required for the initiation of the B-cell expression program. An important consequence of E2A-EBF activity is the expression of *Pax5* (O'Riordan and Grosschedl 1999), which mediates exclusive B-cell commitment by coordinately activating B-cell-specific

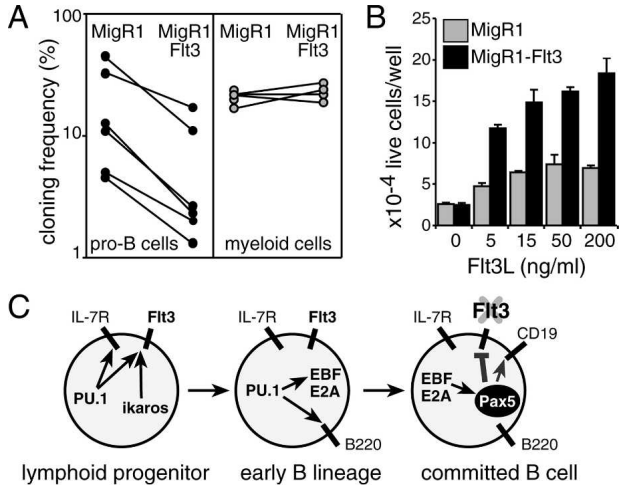

Figure 5. Flt3 impairs pro-B cell formation in vitro. (*A*) Cloning frequency of B and myeloid lineage progenitors transduced with MigR1 or MigR1–Flt3. Data from the same experiment are linked by a line. (*B*) GFP$^+$ pro-B cells were cultured in IL-7 plus the indicated amounts of Flt3L for 72 h. The mean live cell number of triplicate wells ± SD is shown. (*C*) Multistep model of B-cell lineage commitment. Key transcriptional regulators and cell surface molecules are shown.

transcripts, such as *CD19*, and repressing genes important for the adoption of alternative cell fates, such as *MCSF-R* (for macrophages) and *Notch1* (for T cells) (Nutt et al. 1999; Souabni et al. 2002). While this model satisfactorily explains how Pax5 represses the ability to differentiate along the macrophage or T-cell pathway, it has not revealed how the E2A–EBF–Pax5 axis results in the loss of the more immature multipotent state. We now propose an extension to the lineage commitment model where in early B-cell differentiation, Pax5 represses a crucial growth factor receptor, *Flt3*, which is required for the multipotency of early lymphoid progenitors, and thereby facilitates unilineage differentiation by a negative feedback mechanism (Fig. 5C).

Materials and methods

Mice

Pax5$^{-/-}$ (Urbanek et al. 1994) mice were maintained on a C57BL/6 background and genotyped as described (Nutt et al. 1997).

Flow cytometry

The mAbs against CD4, CD8, Ly5.2, Ter119, NK1.1, Mac1, Gr1, CD19, B220, IgM, and Flt3 were purified and conjugated in our laboratory. Labeled mAbs against CD25, IL-7R, TCRβ, c-kit, CD49b, and IgD were from BD Biosciences. For flow cytometry, single-cell suspensions were stained with the appropriate mAb in PBS containing 2% FCS. Biotinylated mAbs were revealed by Cy5- or PE-streptavidin (Southern Biotech). Cells were analyzed on a LSR (BD Biosciences) and sorted using a FacsDIVA (BD Biosciences).

Cell culture

Pro-B-cell lines were derived and propagated as described (Nutt et al. 1997). Recombinant Flt3L was added to some cultures. The Pax5ER fusion protein and control ER retroviral vectors were introduced into *Pax5*$^{-/-}$ pro-B cells and activated by 1 µM E2 as described (Nutt et al. 1998). Bone marrow progenitors were cultured in HSC media (DMEM, 15% FCS, 10% IL-6 supernatant, 100 ng/mL SCF, 120 ng/mL Flt3L, 50 ng/mL TPO, 1 mM L-glutamine, 50 µM 2-mercaptoethanol). OP9 stromal cells were cultured as described (Schmitt et al. 2004).

RT–PCR and real-time PCR

Total RNA was isolated from cells using Trizol (Invitrogen) and was reverse-transcribed with M-MLV RT (Promega) according to the manufacturer's instructions. Quantitative real-time RT–PCR was performed using SYBR Green PCR (Qiagen) on an ABI PRISM 7900HT cycler (ABI). cDNA input was normalized to *Hprt*. Primers are in the Supplemental Material.

EMSA

Nuclear extracts (Andrews and Faller 1991) and EMSAs were performed as described (Barberis et al. 1990). End-labeled double-stranded oligonucleotides containing a Pax5-binding site from human *CD19* (Kozmik et al. 1992), *Flt3 Px1*, and *Px2* were assayed. Sequences are in the Supplemental Material. A mutated *CD19* site was used as a nonspecific competitor and complexes supershifted by incubation with a Pax5 mAb made in our laboratory.

ChIP assay

ChIPs were performed as described previously with minor modifications (Forsberg et al. 2000). A Pax5 polyclonal antibody (2 µg, sc-1974; Santa Cruz) was immunoprecipitated with Protein G-Sepharose (100 µL) preblocked with dIdC (10 µg), BSA (10 µg), and sheared salmon sperm DNA (10 µg). PCR was performed using the SYBR Green PCR kit on an ABI PRISM 7900HT cycler. Products were amplified with similar efficiency, and absolute quantitation was performed using a standard curve. Primer sequences are in the Supplemental Material.

Retroviral expression of Flt3 in bone marrow stem cells

The mouse *Flt3* cDNA was cloned into the MigR1 vector upstream of an IRES-GFP cassette (Pear et al. 1998). See Supplemental Material for a detailed description of the protocol for retroviral transduction and stem cell transplantation. For B-cell and myeloid differentiation studies, transduced progenitors were sorted as GFP$^+$Lin$^-$ (Gr1$^-$CD19$^-$NK1.1$^-$Ter119$^-$TCRβ$^-$) and cocultured on OP9 (+1% IL7/Flt3L [5 ng/mL], B cells) or ST2 (+SCF [100 ng/mL], myeloid cells) stromal cells for 7 d. Progenitor frequencies were determined by limiting dilution assays. Proliferation assays with the Flt3$^+$B220$^+$CD19$^+$ cells differentiated in vitro were performed in OP9 B-cell media containing IL-7 and a titration of Flt3L (0–200 ng/mL). Total live cell number was determined by FACS.

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