A Flt3- and Ras-Dependent Pathway Primes B Cell Development by Inducing a State of IL-7 Responsiveness

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A Flt3- and Ras-Dependent Pathway Primes B Cell Development by Inducing a State of IL-7 Responsiveness

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Ras plays an important role in B cell development. However, the stage at which Ras governs B cell development remains unclear. Moreover, the upstream receptors and downstream effectors of Ras that govern B cell differentiation remain undefined. Using mice that express a dominant-negative form of Ras, we demonstrate that Ras-mediated signaling plays a critical role in the development of common lymphoid progenitors. This developmental block parallels that found in flt3−/− mice, suggesting that Flt3 is an important upstream activator of Ras in early B cell progenitors. Ras inhibition impaired proliferation of common lymphoid progenitors and pre-pro-B cells but not pre-B cells. Rather, Ras promotes STAT5-dependent pro-B cell differentiation by enhancing IL-7Ra levels and suppressing socs2 and socs3 expression. Our results suggest a model in which Flt3/Ras-dependent signals play a critical role in B cell development by priming early B cell progenitors for subsequent STAT5-dependent B cell differentiation. The Journal of Immunology, 2010, 184: 000–000.

The ubiquitously expressed Ras proteins play important roles in mammalian development. Three isoforms of Ras, K-, N-, and H-Ras, are expressed in a tissue-specific manner and are involved in proliferation and differentiation of various cell lineages in mammals. To elucidate the role of Ras in early B cell development, we made use of transgenic mice expressing a dominant-negative form of human H-Ras (dnRas) throughout the B and T cell lineage (1). These dnRas mice have been previously shown to have a profound block at the pre–pro-B to pro-B transition in B cell development (1). However, whether Ras signaling plays a role in the earlier steps of B lymphopoiesis remains to be elucidated. Moreover, the upstream activators and key downstream targets of the Ras/MAPK pathway in B cell development remain unknown.

Early lymphopoiesis in adult bone marrow and fetal liver relies on cytokine signaling involving predominantly two receptors: FLT3 (also known as fetal liver kinase-2) and IL-7R (2–4). Mice deficient in both IL-7Ra and Flt3 ligand (Flt3L) (il7ra−/− × flt3L−/−) lack all B lineage cells (5). IL-7− and IL-7Ra−deficient mice exhibit profound defects at the pro-B cell stage of early B cell development (2, 3). We and others have previously reported that IL-7R governs early B cell development through a Jak/Stat5-dependent pathway (6, 7) but could not provide any evidence for a role of Ras signaling in that process (6). In contrast to il7ra−/− mice, flt3−/− mice exhibit only a mild reduction in the numbers of pro-B and pre-B cells (4). Bone marrow chimeras generated by mixing wild-type (WT) and flt3−/− bone marrow cells demonstrated that Flt3 is required for mature B cell generation under competitive circumstances (4). However, the reagents and tools available when this initial analysis was done did not allow for precise identification of the stages of B cell development that were affected by Flt3 deficiency. Moreover, the signaling pathways activated by Flt3 in vivo that drive B cell development have not been characterized.

In this study, we report that early B cell development in dnRas mice is blocked at the common lymphoid progenitor (CLP) stage in both adult and fetal mice, a much earlier stage than that previously reported. Likewise, using mixed bone marrow chimeras, we observed an identical block at the CLP stage in flt3−/− cells. This developmental block is due to two distinct effects. First, Flt3/Ras-dependent signals govern CLP and pre–pro-B cell proliferation. Second, Flt3/Ras signals upregulate expression of the IL-7Rα-chain and suppress expression of socs2 and socs3. Our findings suggest a model in which Flt3-dependent Ras activation primes B cell development by inducing a state of STAT5 responsiveness, a key event required for subsequent B cell lineage commitment and differentiation (7).

Materials and Methods

Mice

C57BL/6 (B6) and B6.CD45.1 (B6.Ly5SJL) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). dnRas, Raf1-GFP, and Bcl-xL transgenic mice, mice expressing a constitutively activated form of Raf (Raf-CAAX), and mice expressing a constitutively active form of the key IL-7 effector STAT5 (STAT5b-CA) have been previously described (1, 6, 8, 9). flt3−/− mice were kindly provided by Dr. Rachel Gerstein. Mice used for analysis were 4–12 wk old, unless otherwise noted. All of the animal experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Flow cytometry

Bone marrow cells isolated from both femurs and tibias were treated with ACK lysis buffer (Lonza Walkersville, Walkersville, MD) to remove RBCs. To enrich for early progenitors, cells were stained with a mixture of
lineage-specific Abs described below, and lineage-positive cells were removed via magnetic depletion using an LS MACS column and magnetic beads (Miltenyi Biotec, Auburn, CA). Cell preparations were taken with a panel of Abs (listed below) and analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, CA). Abs used to define lineage-positive cells included biotin-B220, CD3, CD8, CD11b/Mac-1, DX5, Gr-1, and Ter-119. Additional Abs used included FITC-CD43, PE-CD45R/B220, PerCP-Cy5.5-CD19, PE-CD71, allopheocyanin-CD93/AA4.1, allopheocyanin-CD117/Kit, PE-Cy5.5-Sca-1, PE-IL-7R, Alexa Fluor 700-CD45.2, and Pacific blue-CD45.1. All Abs used were obtained from eBioscience (San Diego, CA) except for FITC-CD43 and PE-CD71, which were obtained from BD Pharmingen (San Diego, CA), and Pacific blue-CD45.1, which was obtained from BioLegend (San Diego, CA).

Generation of bone marrow chimeras
Recipient mice were given 1000 rad irradiation 4–6 h before injection. Bone marrow from WT (CD45.1), dnRas (CD45.2), and flt3−/− (CD45.2) mice was depleted of CD3+, CD8−, CD11b−, B220−, DX5−, Gr-1−, and Ter-119− positive cells. Following depletion, WT (CD45.1) bone marrow was mixed with bone marrow from dnRas (CD45.2) or flt3−/− (CD45.2) mice at a ratio of 1:2. One million cells were then injected i.v. via the tail vein. Host mice were analyzed 6–8 wk after reconstitution.

BrdU labeling assays
BrdU assays were conducted according to the manufacturer’s instructions (BD Biosciences). Briefly, mice were injected i.p. with 2 mg BrdU 24 and 12 h prior to analysis. Bone marrow cells were isolated and stained with surface markers to identify B cell progenitor populations. Intracellular staining was then conducted for BrdU. Cells were fixed with BD Cytofix/Cytoperm buffer, washed, and permeabilized with BD Cytoperm Plus buffer. Cells were refixed with BD Cytofix/Cytoperm buffer, resuspended in DNase (20 μg per ml) and incubated for 1 h at 37°C. Intracellular staining for BrdU was conducted for 20 min at room temperature. Flow cytometry was conducted on an LSR II flow cytometer to identify BdU+ cells.

In vitro B cell differentiation
Bone marrow from WT and dnRas mice was isolated and depleted of B220−, Mac-1−, Gr-1−, and Ter-119− positive cells. CLP cells (d amp− c-kit+IL-7R−) were sorted on a FACSVantage (BD Biosciences) and plated in X-VIVO medium (BioWhittaker, Walkersville, MD) supplemented with 5% BSA, 10 ng/ml IL-7, 40 ng/ml stem cell factor, and 80 ng/ml Flt3L. Cells were harvested at the time points indicated in the figures and analyzed by flow cytometry.

Intracellular phospho-STAT5 staining
Bone marrow from WT, dnRas, STAT5b−/−, and dnRas × STAT5b−/− mice were isolated and depleted of CD3−, CD8−, CD11b−, CD25−, IgM−, DX5−, Gr-1−, and Ter-119− positive cells. Depleted cells were surface stained with allopheocyanin-AAA4.1 and streptavidin-Cascade blue red, reconstituted in 20 ng/ml at 37°C, and stimulated with IL-7 (PeproTech, Rocky Hill, NJ). Samples were then stained for phospho-STAT5 as previously described (10, 11).

Real-time RT-PCR
RNA was prepared from sorted populations using an RNasey kit (Qiagen, Valencia, CA). A two-step method was then conducted for real-time PCR assays (Invitrogen, Carlsbad, CA) using a Cepheid SmartCycler (Sunnyvale, Valencia, CA). A two-step procedure was then conducted for real-time PCR assays (Invitrogen, Carlsbad, CA) using a Cepheid SmartCycler (Sunnyvale, Valencia, CA). A two-step procedure was then conducted for real-time PCR assays (Invitrogen, Carlsbad, CA) using a Cepheid SmartCycler (Sunnyvale, Valencia, CA).

Results
Expression of a dnRas transgene impairs early B cell development in both fetal and adult mice
To study the role that the Ras signaling pathway plays in the earliest steps of B cell development, we crossed dnRas transgenic mice with Rag1-GFP reporter mice. The dnRas mouse model has been previously described (1). Briefly, a human H-Ras transgene with an N17 point mutation was fused to the lek proximal promoter and the immunoglobulin intronic H chain (Eμ) enhancer. This results in expression of dnRas throughout B and T cell development. Consistent with this expression pattern, we observed decreased levels of phospho-ERK in lymphocyte progenitor cells (lin− c-kithi Flt3+) from dnRas mice (data not shown). Likewise, expression of CD71, a downstream PI3K target gene (12), was significantly reduced (p = 0.015) in early B cell progenitors (data not shown).

Thus, dnRas mice have reduced activation of at least two of the primary downstream effectors of the Ras signaling pathway in early B cell progenitors.

The Rag1-GFP knock-in model facilitates the precise identification of early lymphocyte progenitors by flow cytometry (8, 13). We used this system to analyze the precise developmental stages at which the dnRas transgene affects B cell development. Consistent with previous findings, we observed a significant reduction in the percentage and total cell numbers of pro-B and pre-B cells (32-fold and 38-fold, respectively; p < 0.001) in dnRas × Rag1-GFP−/− mice compared with those of WT littermate controls (Fig. 1A, 1B). Interestingly, we also observed reduced percentages and total numbers of CLP (lin−Rag1-GFP+c-kit+ Sca-1+IL-7R− B220−, 2.5-fold↓, p < 0.002) and pre-pro-B cells (lin−Rag1-GFP c-kit+ Sca-1+IL-7R− B220+, 6.2-fold↓, p < 0.001) in dnRas × Rag1-GFP−/− mice, whereas hematopoietic stem cell (HSC) frequency and total cell number remained unchanged (Fig. 1A, 1B). These results demonstrate that the actual effect of the dnRas transgene occurs at a much earlier stage than previously appreciated, namely, the CLP stage.

B cell development in adult versus fetal mice has been shown to differ in many ways, including their dependence on cytokine signaling (14). To test whether Ras signaling also plays a role in fetal B cell development, we examined fetal liver from dnRas × Rag1-GFP−/− mice at 18.5 d of gestation. Consistent with our observations in adult bone marrow, we found a decrease in both the percentages and the total cell numbers of fetal liver CLP, pre-pro-B, pre-B, and pre-B cells (6.9-fold↓, 9.6-fold↓, 11.2-fold↓, and 66-fold↓, respectively; p < 0.01; Fig. 1C, 1D). Taken together, our data demonstrate that Ras signaling plays important roles in the very earliest steps of B cell development in both fetal and adult mice.

flt3−/− and dnRas mice exhibit similar defects in B cell development
To identify the potential upstream activator of the Ras signaling pathway in developing B cells, we focused on the receptor tyrosine kinase Flt3. Flt3L has been previously reported to induce Erk phosphorylation in the Ba/F3 cell line transfected with Flt3 in vitro (15). Likewise, we observed that ex vivo stimulation of lineage-negative bone marrow cells with Flt3L leads to elevated phospho-ERK levels in B cell progenitors (data not shown). Mice deficient for either flt3L or flt3 exhibit relatively subtle defects in early B cell development (4, 16). This most likely reflects compensatory responses mediated by other receptor tyrosine kinases, such as c-Kit, that are also capable of activating the Ras/MAPK pathway (17). Therefore, to more robustly test the effect of Flt3 on B cell development and to determine whether flt3−/− and dnRas mice had similar defects in B cell development, we generated mixed bone marrow chimeras. Bone marrow cells from either flt3−/− or dnRas mice were mixed with WT bone marrow at a ratio of 2:1 and injected into lethally irradiated B6 host mice. Eight weeks after injection, the bone marrow of these hosts was analyzed for donor-derived cells. Although the ratio of flt3−/− to WT cells in the HSC compartment remained unchanged (2:1), flt3−/− CLP, pre-pro-B, and pro-B compartments exhibited a profound competitive disadvantage compared with WT cells (6.5-fold↓, 23-fold↓, and 686-
FIGURE 1. dnRas transgene expression blocks B cell development at the CLP stage. A. The percentages of bone marrow HSCs and CLP and pre–pro-B cells in dnRas and WT mice. Bone marrow cells were depleted for CD19 and non-B lineage markers. HSCs were identified as Rag1-GFP<sup>+</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> (upper panels). CLP and pre–pro-B cells were identified by first gating on c-kit<sup>+</sup>IL-7R<sup>+</sup> cells (middle panels), and then further characterized as Rag1-GFP<sup>+</sup>B220<sup>+</sup> CLP and Rag1-GFP<sup>+</sup>B220<sup>+</sup> pre–pro-B cells (bottom panels). B. Total B cell progenitors recovered from the bone marrow of WT and dnRas × Rag1-GFP<sup>+</sup> mice. Cell numbers are plotted on a log scale. These results are derived from four independent experiments (11 WT mice and 11 dnRas mice). Error bars represent the mean ± SD; **p < 0.01. C. The percentages of fetal liver HSCs and CLP and pre–pro-B cells in dnRas and WT mice. Fetal mice were analyzed at embryonic day 18.5. The same depletion and staining protocols were used as in A. D. Total B cell progenitors recovered from the livers of WT and dnRas fetal mice. Cell numbers are plotted on a log scale. These results are representative of three independent experiments (11 WT mice and 15 dnRas mice). Error bars represent the mean ± SD; **p < 0.01.

Ras regulates early B cell proliferation but not survival

To test whether the decrease in B cell progenitors in dnRas mice was due to reduced cell survival, we first crossed dnRas donors when compared with that by WT donors in similar mixed bone marrow chimera experiments (2.7-foldâ†’1, 19-foldâ†’1, and 313-foldâ†’1, respectively; Fig. 2A, lower panels, and Fig. 2B, C). These data clearly demonstrate that flt3<sup>1/2</sup> and dnRas mice have a similar block at the CLP stage of B cell development, suggesting that Flt3-dependent Ras activation is essential for early B cell differentiation.

Decreased IL-7Rα expression in dnRas and flt3<sup>1/2</sup> B cell progenitors

Although the proliferative defect that we observed in the BrdU labeling studies provides an explanation for the diminished numbers of CLP and pre–pro-B cells, it is unlikely that this accounts entirely for the defect in pro-B cells, which is the most dramatically affected population in dnRas mice. Importantly, the pre–pro-B to pro-B transition requires signals sent by IL-7R (2). Thus, one potential explanation for the defect in pre-B cell generation in flt3<sup>1/2</sup> and dnRas mice would be if Flt3/Ras-dependent signals regulate expression of IL-7R. To test this hypothesis, we measured the mean fluorescence intensity (MFI) for IL-7R<sub>α</sub> staining at various stages of B cell development. We observed modestly reduced levels of IL-7R<sub>α</sub> expression on CLP and pre–pro-B cells in dnRas mice. More importantly, IL-7R<sub>α</sub> expression is markedly upregulated during the pre–pro-B to pro B cell transition in WT mice; this marked upregulation of the IL-7R<sub>α</sub>–chain did not occur in dnRas pro-B cells (Fig. 4B). In contrast, we observed no difference in the MFI for Flt3 when comparing B cell
progenitors from WT and dnRas mice (Fig. 4B). Similar results were seen when comparing IL-7Rα expression levels on dnRas and WT B cell progenitors in the mixed bone marrow chimeras described earlier (data not shown). Supporting these findings, Raf-CAAX mice showed the opposite result when examining IL-7Rα expression on B cell progenitors. Specifically, IL-7Rα expression was prematurely elevated in both CLP and pre–pro-B cells in Raf-CAAX mice relative to those in WT littermate controls (Fig. 4C). Finally, to test whether IL-7Rα levels are also decreased in $flt^3$−/− mice, we measured the MFI for IL-7Rα expression in $flt^3$−/− and WT mixed bone marrow chimeras (previously described; see Fig. 2). We found that IL-7Rα levels on pro-B cells derived from $flt^3$−/− donor cells were clearly reduced when compared with those seen on pro-B cells derived from WT donor cells (Fig. 4D). Taken together, our observation that $dnRas$ and $flt^3$−/− B cell progenitors fail to upregulate IL-7Rα demonstrates that Flt3/Ras-dependent signals play an important role in regulating IL-7Rα expression, which in turn regulates pro-B cell development.

**IL-7 fails to induce the STAT5-dependent pre–pro-B to pro-B transition in $dnRas$ mice**

A key question is whether the reduced level of IL-7Rα expression on B cell progenitors in $dnRas$ mice is biologically relevant. To directly address this question, we first examined the activation of STAT5 in WT and $dnRas$ mice. IL-7 stimulation induced phosphorylation of STAT5 in early B cell progenitors in both WT and $dnRas$ mice (Fig. 5A). Phosphorylation levels peaked 20 min after IL-7 addition (Fig. 5B). However, the percentage of phospho-STAT5+ cells was significantly lower in $dnRas$ mice compared with those in WT littermate controls at all of the time points measured (Fig. 5B). Similar results were observed when we varied the dose of IL-7 used (Fig. 5C). Importantly, at low IL-7 concentrations (100 pg/ml), which are more likely to mimic physiological IL-7 levels in vivo, WT but not $dnRas$ progenitor B cells responded to IL-7 by inducing STAT5 phosphorylation. Finally, we also examined the expression of ebf1 in CLP and pro-B cells of $dnRas$ and WT littermate control mice. Ebf1 is induced by IL-7R–dependent signals and is required for the generation of pro-B cells. We found that ebf1 levels were reduced ∼3-fold in CLP cells of $dnRas$ mice. In contrast, ebf1 levels in the few pro-B cells that we
in culture, the WT pre–pro-B cells differentiated into B220+ (39% versus 85%, respectively) (Fig. 5B).

In contrast, B cell development in the dnRas mice is not sufficient to entrain IL-7R signaling because BrdU incorporation studies demonstrated that dnRas and dnRas × STAT5b-CA pro-B cells incorporated BrdU at the same rate (data not shown). Finally, although IL-7Rα levels remained low in dnRas × STAT5b-CA mice, STAT5 activation was restored to levels seen in WT controls (Fig. 6D).

Taken together, we conclude that Flt3/Ras regulates IL-7R signaling either by governing IL-7Rα expression alone or via additional effects on IL-7R–dependent STAT5 activation.

GABPα and PU.1 expression levels are not altered in dnRas mice

A potential mechanism by which Flt3/Ras signaling could induce IL-7Rα expression would be if they upregulated expression of the transcription factors GA-binding protein α (GABPα) or purine-rich box binding protein-1 (PU.1), or both. Both of these factors have been shown to bind to the il7ra promoter and are required for IL-7Rα expression in early pro-B cells (18–20).
of socs2 and socs3, we measured the mRNA levels of these two genes in early B cell progenitors. socs2 expression is upregulated 5-fold in CLP and pre-pro-B cells and 3-fold in pro-B cells in dnRas mice (Fig. 7A, 7B). Similarly, socs3 expression is increased

### Table I. Real-time RT-PCR results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
<th>CLP and Pre-pro-B</th>
<th>Pro-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>sfpi1</td>
<td>1.16 ± 0.11</td>
<td>1.00 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>gapba</td>
<td>1.10 ± 0.16</td>
<td>1.11 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>tyns</td>
<td>0.88 ± 0.11</td>
<td>1.09 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>pola</td>
<td>1.03 ± 0.09</td>
<td>1.17 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>skp2</td>
<td>0.76 ± 0.14**</td>
<td>1.04 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>il7ra</td>
<td>0.86 ± 0.29</td>
<td>0.95 ± 0.17</td>
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</tr>
<tr>
<td>ebf1</td>
<td>0.31 ± 0.39**</td>
<td>1.27 ± 0.20</td>
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Fold changes of sfpi1, gapba, tyns, pola, skp2, il7ra, and ebf1 mRNA levels in dnRas mice. Bone marrow cells from WT and dnRas mice were isolated and pooled. A mixture of CLP and pre-pro-B cells, or pro-B cells, was isolated by sorting on a FACSAria. Real-time RT-PCR was performed as described in Materials and Methods. Each reaction was conducted in triplicate. The fold changes in mRNA levels in dnRas mice compared with WT littermate controls was calculated by ΔΔ cycle threshold. These results are derived from three or four independent experiments.

**p < 0.01.
2-fold in CLP and pre–pro-B cells and 8-fold in pro-B cells in dnRas versus WT mice (Fig. 7A, 7B). Thus, increased socs2 and socs3 expression may contribute to the reduced ability of B cell progenitors from dnRas mice to induce STAT5 activation following stimulation with IL-7.

Discussion
Although B cell progenitors express a variety of receptor tyrosine kinases, only IL-7R has been shown to be absolutely essential for B cell development (2). Flt3 is known to act synergistically with the IL-7R to promote B cell differentiation (5). However, Flt3 deficiency by itself results in only mild defects in B cell differentiation in vivo (4). One potential explanation for the relatively mild phenotype in flt3−/− mice is that other receptors, such as c-Kit, for example, may be able to substitute for Flt3 function in vivo. Herein, we have clearly demonstrated, using mixed bone marrow chimeras, that Flt3 is essential for B cell development under competitive circumstances. In other words, when B cell progenitors are given the choice of using a Flt3-dependent versus a Flt3-independent pathway for generating pro-B cells, the Flt3-dependent pathway is essentially the only one used. Thus, under physiological circumstances (i.e., in WT Flt3+ mice), Flt3 signals play a critical role in normal B cell differentiation.

Although Flt3 has been demonstrated to activate numerous signaling pathways using in vitro cell lines, the downstream signaling pathways that drive Flt3-dependent B cell differentiation in vivo have not been identified. Using mice expressing a dominant-negative Ras transgene, we observed a developmental block at the CLP stage, which exactly parallels that observed in flt3−/− mice. In fact, the developmental block observed in dnRas mice is much stronger than that seen in flt3−/− mice. This finding suggests that although there may be some redundancy with regard to the upstream receptors involved in driving early B cell differentiation, they all function via induction of Ras activity.
A key remaining question is how Ras signals entrain early B cell development. Ras has been shown to play an important role in regulating cell proliferation in a number of systems (23). Thus, one potential mechanism by which Ras could govern B cell differentiation is by regulating cell proliferation. As shown in Fig. 3, this is in fact one of the key functions of Ras in both CLP and pre–pro-B cells. The exact mechanism by which Ras regulates the cell cycle in CLP/pre–pro-B cells remains to be precisely determined. However, a potential mechanism may involve Skp2, an E3 ligase that mediates p27 degradation and thereby regulates S phase entry (24). Consistent with this hypothesis, Skp2 expression is reduced in CLP/pre–pro-B cells in dnRas mice. Thus, Fli3/Ras signals play an important role in expanding the pool of progenitor cells that are poised for B cell differentiation.

A second question involves which Ras effectors are required to drive B cell development. The Ras pathway activates a number of downstream signaling pathways, including the Raf/Mek/Erk signaling cascade and the PI3K signaling pathway. The dnRas mice show reduced activation of both of those pathways. Our observation that mice expressing Raf-CAAX have elevated IL-7Rα expression in CLP and pre–pro-B cells suggested that Raf-dependent signals might be sufficient to drive Fli3-dependent B cell development. However, when we mixed bone marrow from WT and Raf-CAAX × flt3−/− mice and generated mixed bone marrow chimeras, we observed that Raf activation alone was completely unable to reverse the fitness of flt3−/− cells to compete with WT progenitors (data not shown). Thus, Fli3 and Ras likely act via multiple downstream effectors to promote early B cell development.

Pro-B cells are the most dramatically affected population in dnRas mice. However, unlike CLP and pre–pro-B cells, they do not exhibit a defect in proliferation in vivo. Pro-B cell differentiation requires signaling through an IL-7R/STAT5-dependent pathway (2, 6, 7). Importantly, we demonstrate that B cell progenitors in dnRas mice show defects in IL-7–dependent STAT5 activation and IL-7–dependent pro-B cell differentiation. At least two potential mechanisms account for this Ras-dependent defect in B cell differentiation. First, dnRas mice fail to upregulate the IL-7Rα–chain during the pre–pro-B to pro-B transition. Ras appears to govern IL-7Rα expression via a Raf-dependent pathway because Raf-CAAX mice show prematurely elevated levels of IL-7Rα on CLP and pre–pro-B cells. A potential caveat to this explanation is that IL-7R levels are lower in pro-B cells from Raf-CAAX mice versus those from WT mice. It is unclear why that should happen, although one potential explanation is that Ras/Raf signals may enhance the ability of pro-B cells to respond to IL-7, thereby allowing cells with lower IL-7Rα expression levels to emerge. The effect of Ras does not involve alterations in the expression of PU.1 or GABPa, two previously characterized regulators of il7ra transcription (18–20). Moreover, the fact that il7ra mRNA levels remain unchanged indicates that IL-7Rα is regulated via a posttranscriptional mechanism in dnRas B cell progenitors. Second, dnRas mice also show dramatically increased expression of two negative regulators of STAT5 activation, socs2 and socs3. Consistent with these findings, we observed that expression of a constitutively active form of STAT5, which restores lymphocyte development in both il7ra−/− and socs1 over-expressing mice (6, 26), largely rescues pro-B cell differentiation in dnRas mice. Thus, a Fli3/Ras-dependent pathway governs the ability of early pro-B cell progenitors to respond to IL-7. These findings suggest that IL-7R signaling during pro-B cell differentiation is both limiting and tightly regulated. In addition, the finding that Ras regulates pre–pro-B to pro-B cell differentiation and not proliferation is consistent with the linear dose-response curve initially described between dnRas expression and pro-B cell numbers (1); if the defect at this stage was predominately due to an effect on cell proliferation, then we would have predicted to see a logarithmic relationship between these two parameters instead. Thus, our results suggest a model in which a Fli3/Ras-dependent pathway regulates B cell development via two distinct mechanisms. First, Fli3/Ras-dependent signals promote proliferation of CLP and pre–pro-B cells, thereby expanding the pool of early B cell progenitors. Second, Fli3/Ras-dependent signals prime these B cell progenitors for subsequent IL-7/STAT5-dependent pro-B cell differentiation by enhancing IL-7Rα expression and suppressing inhibitors of STAT5 signaling, socs2 and socs3 (Fig. 8). Our data clearly demonstrate that Fli3/Ras signals govern B cell development via effects on IL-7R/STAT5 signaling. An important question is whether this is the only mechanism by which Fli3/Ras signals regulate B cell development. Our finding that Ras regulates socs3 expression in early B cell progenitors suggests that this may not be the case. Specifically, SOCS3 was shown to negatively regulate CXCR4-dependent retention of B cell progenitors in the bone marrow (27). socs2 is expressed at low levels in early B cell progenitors and is upregulated ∼8- to 9-fold at the immature B cell stage. Furthermore, this study demonstrated that SOCS3 negatively regulates the ability of CXCR4 to induce focal adhesion kinase-dependent adhesion of B cell progenitors to VCAM. The conclusion of this study was that SOCS3 levels must be low in early B cell progenitors to allow for CXCR4-dependent retention of these cells in the bone marrow and that the subsequent increase in SOCS3 expression at the immature B cell stage allows for their emigration from the bone marrow. Interestingly, the increase in socs3 expression associated with immature B cell emigration and defective CXCR4 activation of B cell adhesion is virtually identical to the increase in socs3 expression that we observed in dnRas pro-B cells (∼8-fold). Thus, Ras may regulate early B cell differentiation not just via effects on IL-7R/STAT5-dependent B cell differentiation but possibly via CXCR4-dependent retention of B cell progenitors in the bone marrow environment required for appropriate B cell development.

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