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Early B cell progenitors deficient for GON4L fail to differentiate due to a block in mitotic cell division

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Abstract

B cell development in *Justy* mutant mice is blocked due to a pre-mRNA splicing defect that depletes the protein GON4-like (GON4L) in B cell progenitors. Genetic and biochemical studies have suggested GON4L is a transcriptional regulator that coordinates cell division with differentiation, but its role in B cell development is unknown. To understand the function of GON4L, we characterized B cell differentiation, cell cycle control, and mitotic gene expression in GON4L-deficient B cell progenitors from *Justy* mice. We found that these cells established key aspects of the transcription factor network that guides B cell development and proliferation and rearranged the immunoglobulin heavy chain gene locus. However, despite intact IL-7 signaling, GON4L-deficient pro-B cell stage precursors failed to undergo a characteristic IL-7-dependent proliferative burst. These cells also failed to upregulate genes required for mitotic division, including those encoding the G1/S cyclin D3 and E2F transcription factors and their targets. Additionally, GON4L-deficient B cell progenitors displayed defects in DNA synthesis and passage through the G1/S transition, contained fragmented DNA and underwent apoptosis. These phenotypes were not suppressed by transgenic expression of pro-survival factors. However, transgenic expression of cyclin D3 or other regulators of the G1/S transition restored pro-B cell development from *Justy* progenitor cells, suggesting GON4L acts at the beginning of the cell cycle. Together, our findings indicate GON4L is essential for cell cycle progression and division during the early stages of B cell development.

Introduction

B cell development sustains a pool of peripheral B cells that support antibody-mediated immunity. During the earliest stages of this process, a network of transcription factors and signaling pathways guide B cell progenitors through alternating phases of differentiation and proliferation (1–4). Differentiation requires the DNA-binding proteins E2A, EBF1, PAX5 and STAT5 (among others) (5–9), which form a transcriptional regulatory network that directs the formation of early B cell precursors. In the most primitive progenitors, E2A and

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EBF1 activate B-lineage genes (10–13), promoting specification towards a B cell fate (1, 2, 14, 15). EBF1 and PAX5 then activate additional B-lineage genes and repress others that promote alternative developmental programs, sealing commitment to a B cell fate (16–20). Additionally, the receptors c-Kit, FLT3 and that for IL-7 provide signals that are essential for the formation of early B cell progenitors (4).

The B cell transcription factor network and signaling pathways also control the proliferation of early-stage B cell precursors. A main driver of this process is IL-7 signaling, which activates the transcription factor STAT5 and the MAPK/ERK and PI3K signaling pathways (21), thereby promoting expression of proteins essential for survival and mitotic division. These include cyclin D3, which controls the G1/S transition of the cell cycle and is essential for B cell development (22–24). Further, IL-7 signaling sustains expression of EBF1, which also activates mitotic genes (25–28). The roles of STAT5 and EBF1 in B cell development are well established (29–31), but less is known about pathways downstream of these proteins that control cell division by B cell progenitors.

In *Justy* mice, B cell development is blocked at an early stage due to a recessive point mutation in the *Gon4-like (Gon4l*) gene (32). This lesion disrupts splicing of *Gon4l* pre-mRNA in B cell progenitors, greatly reducing expression of full-length *Gon4l* transcript and protein. The function of GON4L is not understood, but studies in organisms ranging from plants to invertebrates to zebrafish have implicated this protein in pathways that control differentiation and cell division within developmental programs (33–37). For example, GON4L deficiency in zebrafish embryos blocks erythropoiesis, somite formation, and tail extension, which was correlated with cell cycle arrest and apoptosis (34, 37). Validating a role in cell division, other studies identified GON4L as important for the growth of cultured human cancer cells (38–40).

GON4L is a nuclear protein predicted to form domains characteristic of transcriptional regulators, including a highly acidic region, 2 paired amphipathic helix repeats and a SANT-L domain (41). Further, molecular analysis showed GON4L forms complexes with the transcriptional regulators YY1, SIN3A and HDAC1, which have all been implicated in the regulation of cell division (41–45). Additionally, GON4L binds to NPAT, a transcriptional coactivator that regulates histone gene expression during DNA replication (46, 47), and to MCM3 and 4, components of the mini-chromosome maintenance complex required for DNA replication (37, 48). However, the importance of these interactions for GON4L function is poorly understood.

The findings outlined above suggest GON4L is important for cell division during B cell development. Therefore, we determined how GON4L deficiency in B cell progenitors from *Justy* mice affected cell cycle progression, proliferation and mitotic gene expression. In *Justy* B cell progenitors, the critical B-lineage transcription factor PAX5 was expressed normally and the IL-7 signaling pathway was functional, but these cells nevertheless failed to proliferate. This proliferative arrest correlated with impaired cell cycle progression and DNA synthesis, and induction of apoptosis. Also, *Justy* B cell progenitors failed to activate genes needed for mitotic division. Enforced expression of proteins that regulate the G1/S transition augmented B cell development from *Justy* cells, suggesting GON4L is critical at

this stage of the cell cycle. Together, our data indicate GON4L regulates pathways that guide proliferation by early-stage B cell progenitors.

Materials and Methods

Mice

Mice were housed in a specific pathogen-free facility. *Justy* mice were described previously (32). *p53*-deficient mice (49) were from Jackson Labs (Bar Harbor, ME). All procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Antibodies and streptavidin conjugates

Antibodies were from BD Biosciences (San Jose, CA), Biolegend (San Diego, CA), eBioscience (San Diego, CA) and Cell Signaling Technology (Danvers, MA). Antibody clones used were: B220 (RA3-6B2), BP-1 (6C3), BrdU (BU20A), CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD11b/Mac-1 (M1/70), CD11c (N418), CD16/32 (2.4G2), CD19 (1D3), CD43 (S7), CD49b (DX5), CD127 (A7R34), CD135 (A2F10), Gr-1 (RB6-8C5), IgM (B76), Ly6C (AL-21), PAX5 (1H9), pSTAT5 (47), pERK (197G2), pAKT (D9E) and Ter-119. Biotinylated IgH-specific antibody was obtained from Vector Laboratories (Burlingame, CA). Streptavidin Brilliant Violet 605 was obtained from BD Biosciences.

Flow cytometric analysis and cell sorting

Flow cytometric analysis was performed as described previously (32). For sort purifications, target cells were enriched by negative selection by labeling cells with rat anti-mouse antibodies specific for Gr-1, IgM, Ly6C and Ter119 and selecting out antibody-bound cells using sheep anti-rat Dynabeads (Life Technologies, Carlsbad, CA). Recovered cells were stained for non-B lineage (Lin) markers (CD3, CD4, CD8, CD11c, CD49b, Ly6C) and B-lineage markers (B220, BP-1, CD19, CD43, CD127, FLT3). Pre-pro-B cells (defined as $Lin^{-}B220^{+}CD43^{+}CD19^{-}FLT3^{+}CD127^{+}$) and pro-B cells (defined as $Lin^{-}B220^{+}CD43^{+}CD19^{+}BP\text{-}1^{-}FLT3^{+/-}CD127^{+}$) were isolated by 2 consecutive rounds of sort purification using a FACSAria. A representative gating strategy used for sort purification is shown in supplemental figure 2.

Analysis of BrdU incorporation and DNA content

Mice were injected with 1mg of BrdU and bone marrow harvested 6 hours later. Cultured cells were incubated for 2 hours in 10 μM BrdU and harvested. Cells were stained for surface markers and analyzed as described previously (50). For DNA content analysis, cells were stained for surface markers and processed using buffers from BD Biosciences. Cells were incubated on ice for 15 min in Cytofix/Cytoperm, washed with Perm Wash and then incubated on ice for 10 min in Cytoperm Plus. After washing with Perm Wash, cells were incubated at room temperature for 5 min in Cyofix/Cytoperm and then stained with FxCycle Violet dye (Life Technologies) immediately prior to analysis.

RT-PCR analysis

RNA from 2 independent sort purifications was pooled and reverse-transcribed using SuperScript III (Life Technologies). Quantitative real-time PCR was performed as described previously (32). Analysis of some cell cycle-associated genes was performed using the Mouse Cell Cycle PCR Array (Qiagen, Germantown, MD).

Igh gene rearrangement analysis

Cells were digested with Proteinase K (Thermo Fisher Scientific, Pittsburgh, PA) and genomic DNA recovered by precipitation. PCR primers used were described previously (51). Primer sequences were as follows: J_HR3: GTTCTAATGTCACCACAGACCAG; V_H7183F: CGG-TACCAAGAA(C/G)A(A/C)CCTGT(A/T)CCTGCAAATGA(C/G)C; V_HJ558F: CGAGCTC-TCCA(A/G)CACAGCCT(A/T)CATGCA(A/G)CTCA(A/G)C.

Intracellular staining

All buffers were from BD Biosciences. Cells were stained for surface markers immediately prior to staining for intracellular proteins. To stain for PAX5, cells were incubated on ice for 15 min in Cytofix/Cytoperm, washed with Perm Wash, incubated on ice for 10 min in Cytoperm Plus, washed with Perm Wash and then incubated again in Cytofix/Cytoperm at room temperature for 5 min. Cells were then washed with Perm Wash, incubated on ice for 30 min in Perm Wash containing PAX5 antibody and washed again with Perm Wash prior to analysis. To stain for pSTAT5, pERK and pAKT, cells were incubated at 37°C for 10 min in Phosflow Fix Buffer 1, washed with stain buffer (PBS containing 3% FBS) and then incubated at −20°C for 30 min in Perm Buffer II. Cells were then washed with stain buffer, incubated on ice for 30 min in stain buffer containing antibodies and washed again with stain buffer prior to analysis.

IL-7 Stimulation

Cells were stained for surface markers, resuspended in ice-cold PBS and incubated on ice for 15 min. IL-7 was added to a final concentration of 400 ng/ml followed by incubation at 37°C for 15 min. Cells were then fixed and processed for pSTAT5 staining as described above.

Stromal cell co-cultures and retroviral transduction

Cytokines were from PeproTech (Rocky Hill, NJ). Multipotent progenitors (MPPs) were isolated as described previously (32). Cells were added to 24-well plates containing OP9 stromal cells in RPMI 1640 supplemented with 10% FBS, FLT3 ligand (FLT3L), IL-7 and stem cell factor (SCF). Cells were cultured for 7 days and then analyzed. For retroviral transduction, progenitor cells were added to OP9 cells in StemSpan SFEM medium (Stemcell Technologies, Vancouver, BC) containing FLT3L, IL-3, IL-11, SCF and thrombopoietin and cultured for 24 hrs. Cells were then transferred to Retronectin-coated plates containing retroviral particles. After 24 hrs, cells were transferred to wells containing OP9 cells in Opti-MEM (Life Technologies) supplemented with 5% FBS, FLT3L, IL-7 and SCF. Cells were cultured for 11 days and then analyzed.

Retroviral particle production

Retroviral vectors were constructed using the plasmid pMIG (52) and cDNAs from GE Dharmacon (Lafayette, CO). pMIG vector and pCL-Eco (53) were transfected into 293T cells. After 24 hours, supernatants were collected, filtered and added to plates coated with Retronectin (Clontech, Mountainview, CA).

Analysis of caspase activity

Activated caspase levels were analyzed as described previously (50).

Statistical analysis

Data were analyzed using the unpaired, two-tailed Student's t test in Prism software (GraphPad, San Diego, CA).

Results

GON4L deficiency prevents the accumulation of Flt3^{-} pro-B cells

We previously showed the *Justy* mutation causes a deficiency for GON4L in B cell progenitors, arresting B cell development at the pre-pro-B to pro-B cell transition (32). To better understand how this transition is affected, B cell progenitors from wild-type (WT) and *Justy* mice were separated into pre-pro-B and pro-B cell fractions based on expression of FLT3 and CD19 (Fig. 1A). These markers were chosen because cells transitioning from the pre-pro-B to pro-B cell stage normally downregulate FLT3 and upregulate CD19 (54–57). As expected, FLT3^{+}/CD19^{-} pre-pro-B cells and FLT3^{-}/CD19^{+} pro-B cells were easily distinguishable in WT bone marrow. Additionally, some cells were detected that reached the pro-B stage as signified by CD19 and PAX5 expression (see Supplemental Fig. 1A) but still expressed FLT3. We named these FLT3^{+} pro-B cells, which we reasoned are a transient intermediate between pre-pro-B and pro-B cells. In WT mice, low numbers of pre-pro-B and FLT3^{+} pro-B cells numbers were observed, while those for FLT3^{-} pro-B cells were much greater (Fig. 1B). In *Justy* mice pre-pro-B and FLT3^{+} pro-B cell numbers were similar to WT, but the numbers of FLT3^{-} pro-B cells were greatly decreased (Fig. 1B). These data indicate GON4L deficiency impairs accumulation of B cell progenitors at the FLT3^{-} pro-B cell stage.

GON4L deficiency impairs DNA synthesis and cell-cycle progression by B cell progenitors

The accumulation of FLT3^{-} pro-B cells in WT mice implies this population is generated by a burst of proliferation. This idea is supported by previous studies, which suggested pro-B cells undergo division *in vivo* as judged by DNA content profiles (23, 58). To assess proliferation by the B cell progenitor fractions we defined based on FLT3 and CD19 expression, we analyzed DNA synthesis by injecting WT and *Justy* mice with the thymidine analog BrdU and measuring its incorporation into cells (Fig. 1C, 1D). In WT mice, FLT3^{+} and FLT3^{-} pro-B cells incorporated more BrdU than pre-pro-B cells, suggesting the former divide more rapidly. Compared to WT, *Justy* pre-pro-B and FLT3^{+} pro-B cells incorporated significantly less BrdU, indicating DNA synthesis was impaired by GON4L deficiency. Surprisingly, no difference in BrdU incorporation was observed between WT and *Justy*

FLT3$^-$ pro-B cells. This may be attributable to selection for mutant cells that express enough GON4L to reach the FLT3$^-$ pro-B cell stage but are still unable to accumulate or progress further in development.

We also analyzed the cell cycle distribution of B cell progenitors by staining for DNA content *ex vivo* (Fig. 1E, 1F). Less than 20% of WT pre-pro-B cells were in the S or G2M phases of the cell cycle, suggesting these cells divide at a low rate. Compared to WT, significantly more *Justy* pre-pro-B cells were in the G1 phase of the cell cycle, while fewer were in S phase. A trend toward fewer *Justy* pre-pro-B cells in G2M was also apparent. Consistent with high BrdU incorporation, most (~60%) WT FLT3$^+$ pro-B cells were in S or G2M, indicating these cells divide rapidly. As with pre-pro-B cells, significantly more *Justy* FLT3$^+$ pro-B cells were in G1 relative to WT and fewer were in S or G2M relative to WT. Analysis of WT and *Justy* FLT3$^-$ pro-B cells showed no differences in the frequencies of cells in G1 or S, but significantly fewer *Justy* cells were in G2M, indicating the mutation had impact on this stage of the cell cycle. Collectively, these results suggest GON4L deficiency disrupts the cell cycle in B cell progenitors as they make the transition from the pre-pro to pro-B cell stage, thus preventing these cells from undergoing a proliferative burst that normally drives the accumulation of FLT3$^-$ pro-B cells.

The IL-7 signaling pathway is functional in GON4L-deficient B cell progenitors

B cell progenitor proliferation requires IL-7 (21), which activates the STAT5, ERK, and PI3K signal transduction pathways. Therefore, we analyzed the levels of phosphorylated (activated) STAT5, ERK and AKT, the latter being a surrogate for PI3K pathway activity. All were either normal or elevated in cells from *Justy* mice that were directly analyzed *ex vivo*, indicating the IL-7 signaling pathway is functional (Fig. 2A–F). To further assess responsiveness to IL-7, bone marrow cells from WT and *Justy* mice were incubated in the absence or presence of IL-7, after which the levels of phosphorylated STAT5 in B cell progenitors were analyzed (Fig. 2G). We found that exposure to IL-7 increased phosphorylated STAT5 levels in both WT and *Justy* B cell progenitors, confirming the IL-7 signaling pathway is functional in *Justy* cells.

Justy B cell progenitors express PAX5 and undergo IgH rearrangement

Pro-B cell formation requires a transcription factor network that controls both B-lineage commitment and cell division (1, 2, 31). Our previous study indicated RNAs encoding key members of this transcription factor network (e.g. E2A, EBF1 and PAX5) are expressed at normal levels in *Justy* B cell progenitors (32). The DNA-binding protein PAX5 functions at the apex of the B-lineage transcription factor network, so we directly measured its expression in WT and *Justy* B cell progenitors (Supplemental Fig. 1A). In cells from WT mice, PAX5 expression was low in pre-pro-B cells, much higher in FLT3$^+$ pro-B cells, and maintained at high levels in FLT3$^-$ pro-B cells. *Justy* B cell progenitors showed the same expression pattern and the absolute PAX5 levels in these cells were similar to WT. These data agree with others that showed *Justy* B cell progenitors express normal levels of CD19 and other genes activated by the B-lineage transcription factor network (32).

PAX5 and other members of the B-lineage transcription factor network (e.g. E2A and STAT5) also promote *Igh* gene rearrangement in B cell progenitors (5, 59, 60), so this process was analyzed in WT and *Justy* pro-B cells. Similar to WT, *Justy* cells rearranged the *Igh* gene and expressed normal levels of IgH protein, which is only produced upon successful gene rearrangement (Supplemental Fig. 1B, 1C). These findings show GON4L deficiency does not impair the regulation of PAX5 expression or *Igh* gene rearrangement and suggest that critical aspects of the B-lineage transcription factor network are functional in GON4L-deficient cells.

Expression of RNAs encoding the major cyclins is decreased in GON4L-deficient B cell progenitors

Prior studies suggested GON4L coordinates cell division with cell differentiation (33–37), an idea in line with our data suggesting GON4L-deficient pro-B cells fail to undergo a proliferative burst. Further, other studies suggested GON4L drives proliferation by human cancer cells (38–40). Given these findings, we explored the possibility that GON4L deficiency due to the *Justy* mutation affects the expression of genes that control cell-cycle progression and division.

The cyclin proteins are key regulators of the cell cycle, so we measured expression of RNAs encoding these proteins in WT and *Justy* B cell progenitors using quantitative RT-PCR analysis. RNA was isolated from sort-purified pre-pro-B and pro-B cells using the strategy shown in supplemental figure 2. For pro-B cells, the $FLT3^-$ and $FLT3^+$ subsets were pooled to obtain enough cells from *Justy* mice. The D-type cyclins (cyclin D1, D2, and D3) were of particular interest because they control the G1 and G1/S phases of the cell cycle, although only cyclin D3 is essential for B cell development (23, 24). Correspondingly, *Ccnd3* RNA expression was much higher in WT and *Justy* B cell progenitors relative to that for *Ccnd1* or *Ccnd2* (Fig. 3A). Compared to those in WT, *Ccnd1* and *Ccnd2* RNA levels were significantly elevated in *Justy* pro-B cells (Fig. 3A). More notably, *Ccnd3* RNA levels were clearly and significantly decreased in *Justy* B cell progenitors and were not upregulated at the pro-B cell stage as seen in WT. The E-type cyclins and cyclin A2 regulate the G1/S and S phases of the cell cycle, while B-type cyclins and cyclin F control mitosis. Expression of RNAs for all these proteins was significantly reduced in *Justy* cells except those for *Ccne1* and *Ccne2*, which was normal (Fig. 3B–3E). Thus, expression of cyclins is reduced in GON4L-deficient B cell progenitors.

Expression of RNAs encoding E2F transcription factors and their target genes is impaired in GON4L-deficient B cell progenitors

We also analyzed expression of RNAs that encode E2F transcription factors, which are activated by cyclin D and E, and upregulate genes needed for DNA replication and mitosis. *E2f1* and *E2f3* RNA levels were normal in *Justy* cells, but those for *E2f2* and *Tfdp1* were significantly decreased (Fig. 4A, 4B). E2F-regulated genes include those encoding cyclin A2 and D3 as well as those for E2F2 and DP1 themselves (61, 62). Expression of all these genes was reduced in *Justy* cells (see Fig. 3A, 3C and 4B), so we analyzed expression of other E2F targets. RNA levels for some E2F targets were normal in *Justy* cells (data not shown), but those transcribed from the *Myb*, *Rbl1*, *Skp2* and *Rad21* genes (61–66) among several others

were significantly reduced (Fig. 4C). Further, in the case of Ki-67 both RNA and protein expression were significantly lower in *Justy* cells compared to WT (Fig. 4D–4F). These findings demonstrate that B cell progenitors deficient for GON4L express key cell-cycle regulators at abnormally low levels.

The developmental block caused by GON4L deficiency is reproduced in culture

The experiments described above analyzed WT and *Justy* B cell progenitors removed directly from bone marrow, an environment in which defective or dying B cell progenitors are likely rapidly eliminated. To assess the impact of GON4L deficiency in a simplified setting, we tracked pre-pro-B and pro-B cell development in a culture system. MPPs were isolated from bone marrow and cultured with stromal cells and cytokines for several days. Afterward, B cell progenitor development and accumulation were assessed by flow cytometry (Fig. 5A). WT cultures contained few pre-pro-B and $FLT3^+$ pro-B cells but had large numbers of $FLT3^-$ pro-B cells, reflecting normal differentiation from the pre-pro-B to pro-B cell stage (Fig. 5B, 5C). In comparison, *Justy* cultures generated abnormally high numbers of pre-pro-B cells, fewer $FLT3^+$ pro-B cells and dramatically less $FLT3^-$ pro-B cells, indicating the pre-pro-B to pro-B cell transition was disrupted. Similar to results from our *ex vivo* analysis, pSTAT5 levels were normal in *Justy* B cell progenitors generated in culture, confirming IL-7 signaling was functional (Fig. 5D). Additionally, cultured *Justy* pro-B cells expressed significantly less *Ccnd3* RNA relative to WT (Fig. 5E) as was observed in cells isolated directly from bone marrow. Thus, the *Justy* phenotype was faithfully reproduced in the culture system.

Cultured GON4L-deficient B cell progenitors show defects in DNA synthesis, DNA profiles, and survival

We next assessed BrdU incorporation by B cell progenitors generated in culture (Fig. 6A, 6B). Similar to findings from *in vivo* labeling studies (see Fig. 1C, 1D), *Justy* pre-pro-B cells incorporated significantly less BrdU relative to WT. This was also observed for $FLT3^+$ pro-B cells, but here the difference between WT and *Justy* cells was much more dramatic. BrdU incorporation by *Justy* $FLT3^-$ pro-B cells was modestly, but still significantly, lower as well. Therefore, the GON4L deficiency impairs DNA synthesis by B cell progenitors produced *in vivo* and *in vitro*.

The cell-cycle profiles of cultured B cell progenitors were visualized by staining for DNA content (Fig. 6C, 6D). Here, $FLT3^+$ and $FLT3^-$ pro-B cells were pooled for the analysis. Consistent with a decrease in BrdU incorporation, significantly fewer *Justy* pre-pro-B and pro-B cells were in S phase. More strikingly, both pre-pro-B and pro-B cells in *Justy* cultures had much greater numbers of cells with a sub G1 (< 2N) DNA content, indicating DNA fragmentation and apoptosis (Fig. 6C, 6E). Given these results, we analyzed levels of activated caspase 8, an initiator of pro-apoptotic pathways (Fig. 6F, 6G). WT and *Justy* pre-pro-B cells contained low levels of activated caspase 8, but the amount detected in *Justy* cells was significantly increased. $FLT3^+$ and $FLT3^-$ pro-B cells contained higher levels of activated caspases 8 and these were significantly elevated in *Justy* cells. Activated levels of the pro-apoptotic caspases 3 and 7 were also elevated in *Justy* cells (data not shown). Combined, these findings indicate that deficiency for GON4L in B cell progenitors leads to

the induction of pro-apoptotic pathways, which helps explain why *Justy* pro-B cells fail to accumulate in bone marrow or in culture.

Promoting survival fails to rescue pro-B cell development from GON4L-deficient cells

Given *Justy* B cell progenitors are prone toward apoptosis, we asked how B cell development from *Justy* cells was affected by promoting survival. In zebrafish, defects caused by GON4L deficiency were partially rescued by suppressing p53 expression (34, 37). In *Justy* mice, however, *Trp53* deficiency failed to restore B cell development (Supplemental Fig. 3A, 3B). We next asked whether enforced expression of pro-survival factors could rescue *Justy* pro-B cell development *in vitro*. WT and *Justy* MPPs were transduced with a control retroviral vector or that expressing BCL-2 or BCL-x_L and then cultured for several days. Afterward, the frequencies of pro-B cells within the Mac-1^-Gr-$1^-$$GFP^+$ fraction were determined, with GFP expression marking transduced cells. Enforced expression of BCL-2 or BCL-x_L had no effect on pro-B cell accumulation in WT cultures and suppressed, rather than augmented, pro-B cell formation in *Justy* cultures (Supplemental Fig. 3C–3E). Thus, the effects of GON4L deficiency in B cell progenitors cannot be overcome by promoting survival.

Enforced expression of G1/S regulators rescues pro-B cell development from GON4L-deficient cells

Our data showed *Ccnd3* RNA expression is decreased in *Justy* B cell progenitors and that events controlled by cyclin D3 (e.g. activation of E2F expression) are impaired (see Figs. 3 and 4). Therefore, we asked how pro-B cell development *in vitro* was affected by increasing cyclin D3 expression. MPPs were transduced with a control retroviral vector or that expressing cyclin D3 and several days later the frequencies of pro-B cells within the Mac-1^-Gr-$1^-$$GFP^+$ (transduced) populations were determined. For WT cultures, enforcing cyclin D3 expression significantly reduced pro-B cell development (Fig. 7A, 7B). In contrast, enforced expression of cyclin D3 in *Justy* cells greatly increased the frequency of pro-B cells (Fig. 7A, 7C). Determining total cell yields confirmed that the changes in pro-B cell frequencies caused by cyclin D3 overexpression correlated with differences in the absolute numbers of pro-B cells generated (Supplemental Fig. 4). We then asked how enforced expression of other cyclins affected pro-B cell development (Fig. 7A–7C). Retroviral-mediated expression of cyclin E1 had no effect on pro-B cell development from WT MPPs, but dramatically increased that from *Justy* cells. In contrast, enforced expression of cyclin A2 or cyclin B1 had no effect on pro-B cell formation from either WT or *Justy* MPPs.

Justy pro-B cells expressed significantly less *E2f2* RNA, so we asked how enforcing E2F2 expression affected pro-B cell development. Retroviral E2F2 expression slightly decreased pro-B cell development from WT cells but significantly increased that from *Justy* cells (Fig. 7D, 7E). Here, the effect was less dramatic than that caused by overexpression of cyclin D3 or E1. These data demonstrate that impairment of pro-B cell development caused by GON4L deficiency is overcome by increasing the expression of factors important for the G1/S and S phases of the cell cycle.

Discussion

We defined how GON4L deficiency affected the differentiation and proliferation of B cell progenitors. Our findings show GON4L-deficient cells express the critical B-lineage transcription factor PAX5, undergo *Igh* gene rearrangement and can signal through the IL-7 receptor. However, these cells had defects in cell cycle progression and DNA synthesis, and underwent apoptosis. Further, GON4L deficiency impaired activation of cell cycle-associated genes. Transgenic expression of proteins that promote the G1/S transition overcame the block in B cell development, allowing GON4L-deficient pro-B cells to accumulate. Combined, our data suggest GON4L regulates proliferation by B cell progenitors, likely by acting during the early phases of the cell cycle.

In zebrafish embryos, loss of GON4L disrupts erythropoiesis and the formation of somites, the latter giving rise to the vertebral column and other tissues (34, 37, 67). For both defects, GON4L deficiency prevented expression of transcription factors required for the respective developmental pathway (i.e., GATA-1 for erythropoiesis, MyoD for somite formation). These results suggest GON4L is required to activate the gene expression programs that drive erythropoiesis and somite formation. In contrast, our data suggest GON4L is not required to put central aspects of the B cell developmental program in motion, as *Justy* B cell progenitors express PAX5 at normal levels and undergo *Igh* gene rearrangement. This conclusion is also supported by data from our previous study, which showed that critical B-lineage genes (e.g. *Ebf1*, *Cd19*, *Cd79a* and *Cd79b*) are expressed at normal levels in *Justy* B cell progenitors (32). Thus, our findings argue that GON4L-deficient pro-B cells can establish specific parts of the B-lineage gene program but fail to further develop, potentially due to defects in downstream pathways that affect gene expression and cell division.

The B cell transcription factor network is critical for proliferation by B cell progenitors. Key players here are IL-7 signaling, STAT5 and EBF1 (21, 27). At the cellular level, the developmental arrest associated with GON4L deficiency closely resembles that caused by the absence of IL-7 signaling or EBF1 (7, 25). However, in *Justy* cells IL-7 signaling and *Ebf1* mRNA expression are normal (32). Of note, proliferation and development of B cell progenitors deficient for the IL-7 receptor can be partially rescued by enforced expression of EBF1 (25). However, overexpression of EBF1 in *Justy* progenitors had only a slight effect on B cell development (JYB and JDC, unpublished data), suggesting defects due to GON4L deficiency are distinct from those caused by disruption of IL-7 signaling. Together, these findings suggest GON4L acts downstream of IL-7 signaling, STAT5, and EBF1.

GON4L deficiency in zebrafish embryos causes a G2M arrest in primitive erythroid progenitors (34). A similar but more modest G2M arrest appears to occur in cells within the tail of the embryo, which fails to undergo extension in the absence of GON4L (37). Consistent with a defect at the G2M phase, apoptosis in specific tissues is dramatically increased in GON4L-deficient zebrafish embryos (37). These defects were attributed to disruption of cell cycle regulation and activation of p53-dependent pathways for G2M arrest or apoptosis, a conclusion supported by data showing p53 knockdown in zebrafish embryos partially rescues erythropoiesis, somite formation, and tail extension and also suppresses

apoptosis (34, 37). One implication of these findings is that the absence of GON4L causes DNA damage or some other genotoxic stress that induces p53-dependent pathways.

Consistent with the findings described above, our data show GON4L deficiency in B cell progenitors disrupts the cell cycle and causes apoptosis. However, our results suggest p53-dependent pathways are not an important factor in the defects caused by GON4L deficiency in B cell progenitors. It remains unclear why different effects were observed in our studies and those of zebrafish. One possible explanation is that GON4L-deficient B cell progenitors undergo apoptosis via p53-independent pathways that supersede p53 activation and G2M arrest, thus lessening the role of p53.

Our data suggest GON4L acts at the G1/S phase of the cell cycle. Particularly compelling are our results showing pro-B cell development from *Justy* cells is partially rescued by enforced expression of the G1/S regulators cyclin D3, E, and E2F2. Previous studies demonstrated cyclin D3 is essential for B cell development (23, 24) but the role of the E-type cyclins has not been addressed. We imagine enforced expression of cyclin D3 or cyclin E affects similar, or overlapping, pathways that promote progression through the G1/S transition. In support of this conclusion, defects caused by cyclin D1 deficiency are rescued by cyclin E (68), indicating functional overlap between the D- and E-type cyclins. Enforced E2F2 expression had a more modest effect relative to that caused by overexpressing cyclin D3 or E. This result implies the function of GON4L goes beyond acting as a cofactor for E2F2. More likely is the possibility that GON4L controls proliferation by functioning together with (or in parallel to) the G1/S cyclins. B-lymphoblastic leukemia (B-ALL) originates from early-stage B cell progenitors (69). The generation and growth of B-ALL cells likely relies on the proliferative capacity of B cell progenitors. Our data argue that GON4L is a key component of the machinery that controls B cell progenitor proliferation. Given this, it would be of interest to determine the importance of GON4L for the transformation and expansion of cells that give rise to B-ALL. A central role for GON4L in B-ALL, and a better understanding of how GON4L functions, could lead to novel therapeutic strategies for treating B-ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

FLT3L	FLT3 ligand
MPPs	multipotent progenitors
ppB	pre-pro-B
SCF	stem cell factor
WT	wild-type

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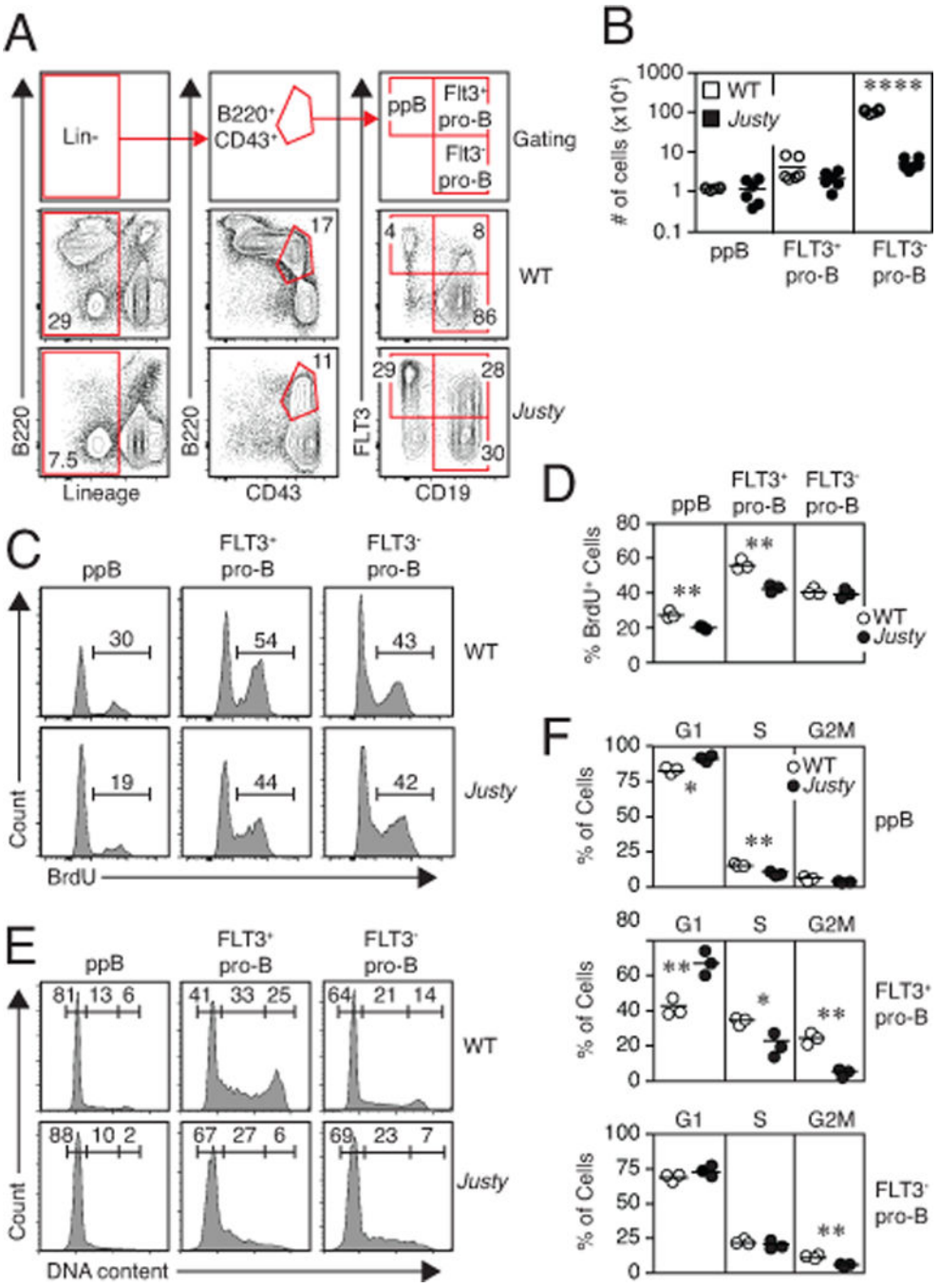

Figure 1. *Justy* B cell progenitors display impaired DNA synthesis and alterations in cell cycle distribution

(**A**) Analysis of early B cell progenitors in bone marrow. Lineage-marker negative, $B220^{+}CD43^{+}$ cells were subdivided into pre-pro-B (ppB) cells, $FLT3^{+}$ pro-B cells and $FLT3^{-}$ pro-B cells based on CD19 and FLT3 expression as shown at the top of the panel. (**B**) Yields of the indicated B cell progenitors from WT and *Justy* bone marrow. Circles represent values from 6 independent experiments in which a WT and a *Justy* mouse were analyzed; lines represent mean values. (**C**) DNA synthesis by WT and *Justy* B cell progenitors as

measured by BrdU incorporation. Mice were injected with BrdU and bone marrow harvested 6 hours later for analysis. Cells were identified as represented in panel A. The percentages of $BrdU^+$ cells are shown. (**D**) Frequencies of $BrdU^+$ cells within the indicated cell populations. (**E**) DNA profiles for the indicated cell populations. The percentages of cells in the G0/G1, S or G2M phases of the cell cycle are shown. (**F**) Frequencies of cells in the indicated stages of the cell cycle as determined from DNA content profiles. For panel D and F, circles represent values from 3 independent experiments in which a WT and a *Justy* mouse were analyzed; lines represent mean values. * $P < .05$; ** $P < .01$; **** $P < .0001$.

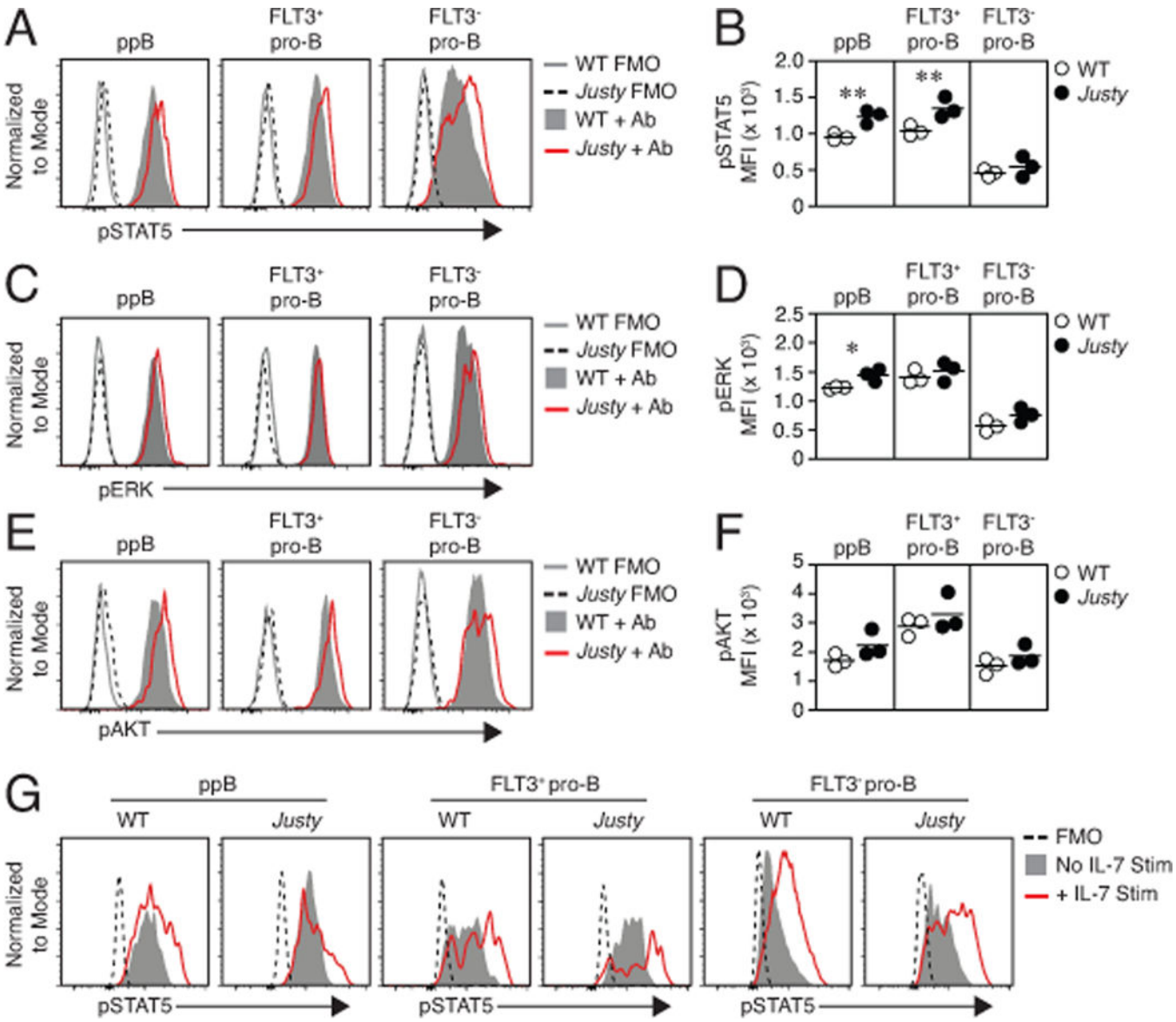

Figure 2. The IL-7 signaling pathway functions normally in *Justy* mutant B cell progenitors

Bone marrow cells were stained for surface markers and analyzed as shown in Figure 1A to identify pre-pro-B (ppB) cells, $FLT3^+$ pro-B cells and $FLT3^-$ pro-B cells. Cells were also stained and analyzed for intracellular levels of phosphorylated STAT5 (pSTAT5; panels A and B), phosphorylated ERK (pERK; panels C and D) and phosphorylated AKT (pAKT; panels E and F). (G) Bone marrow cells were stained for surface markers, rested on ice and then incubated at 37°C in the absence or presence of IL-7. Intracellular levels of pSTAT5 in $Flt3^-$ pro-B cells were then analyzed. Histograms in panels A, C, E and G represent data obtained from at least 3 independent experiments. Circles in bar graphs (panels B, D and F) each represent median fluorescence intensity (MFI) values obtained from 3 independent experiments. * $P < .05$; ** $P < .01$.

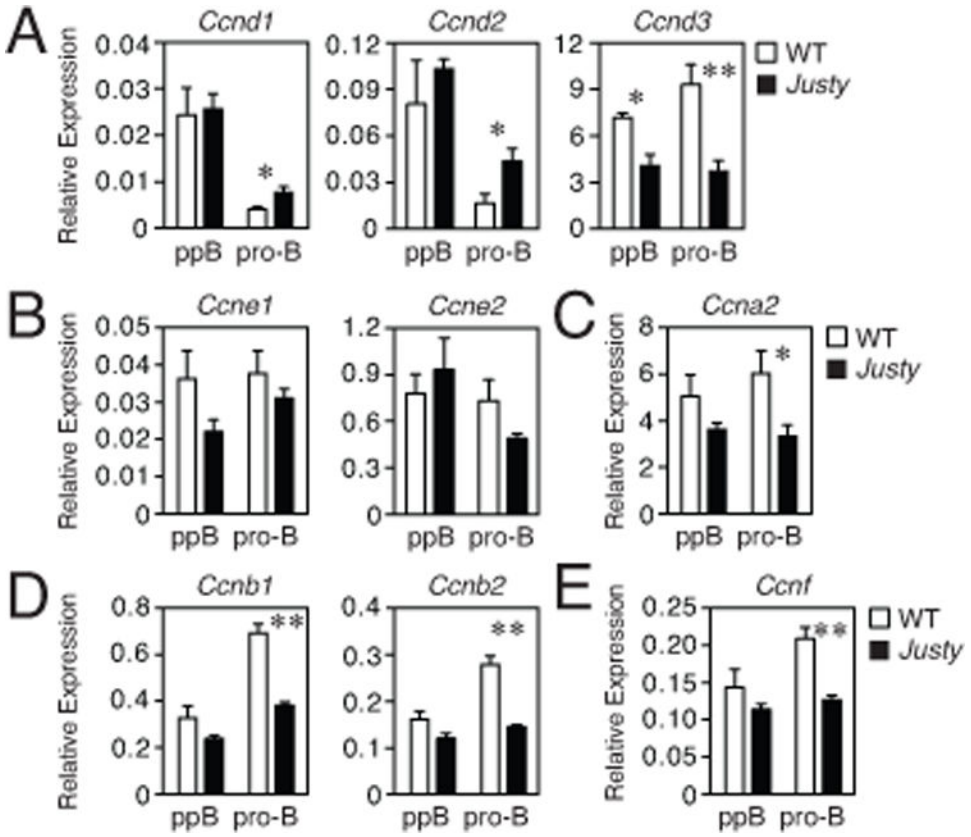

Figure 3. ***Justy*** **B cell progenitors display decreased expression of cyclin genes**

(**A**–**E**) Quantitative RT-PCR analysis measuring the levels of RNAs encoded by the indicated genes. RNA was isolated from sort-purified pre-pro-B (ppB) and pro-B cells. Values are relative to that for *Hprt* RNA, which was analyzed as an internal standard. Bars represent the mean and standard error of values from at least 3 independent experiments. * $P < .05$; ** $P < .01$.

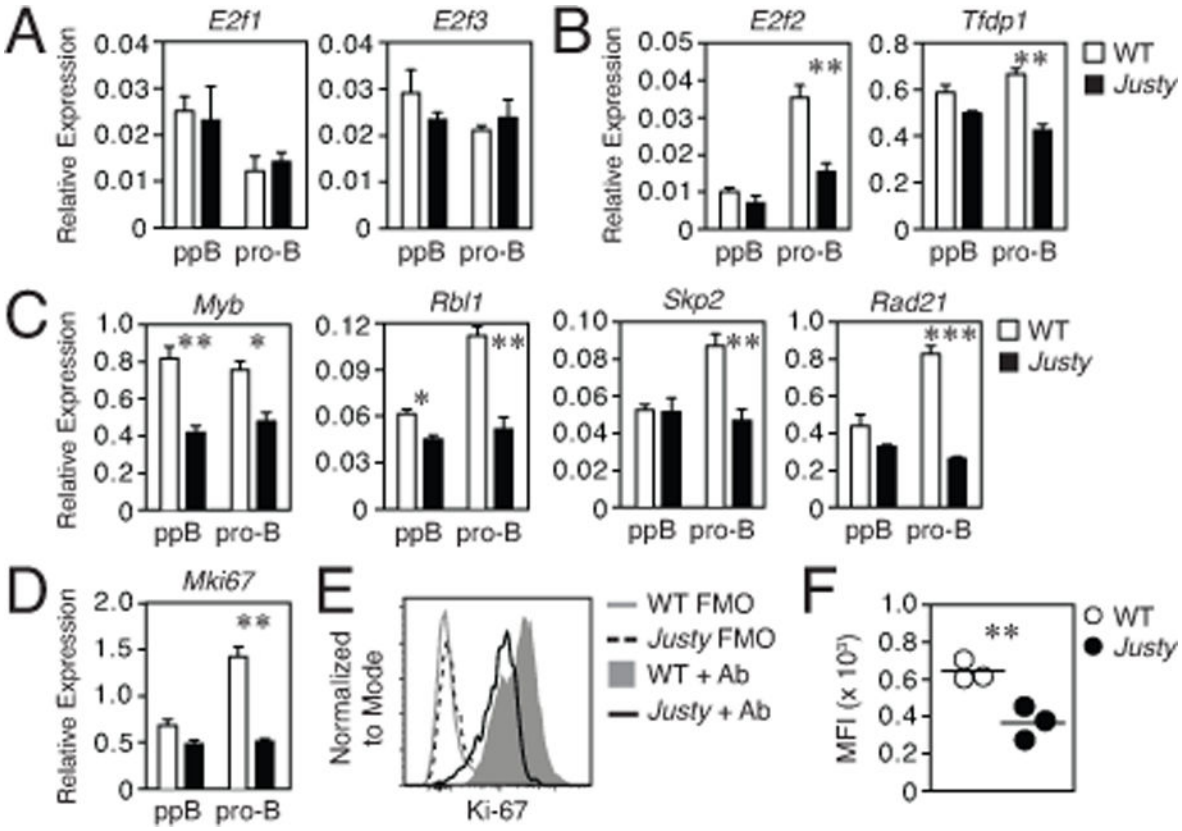

Figure 4. Decreased expression of genes encoding E2F transcription factors and their target genes in *Justy* B cell progenitors

(**A**–**D**) Quantitative RT-PCR analysis measuring the levels of RNAs encoded by the indicated genes. RNA was isolated from sort-purified pre-pro-B (ppB) and pro-B cells, which were isolated as shown in supplemental figure 2. Values are relative to that for *Hprt* RNA, which was analyzed as an internal standard. Bars represent the mean and standard error of values from at least 3 independent experiments. (**E**) Analysis of Ki-67 protein expression in pro-B cells (pool of $FLT3^+$ and $FLT3^-$ cells). Pro-B cells were identified as shown in Figure 1A. FMO samples represent pro-B cells that were not stained with anti-Ki-67 antibody. Data shown are representative of results from 3 independent experiments. (**F**) Median fluorescence intensities (MFI) for Ki-67 staining. Circles represent values from 3 independent experiments in which a WT and a *Justy* mouse were analyzed; lines represent mean values. * $P < .05$; ** $P < .01$; *** $P < .001$.

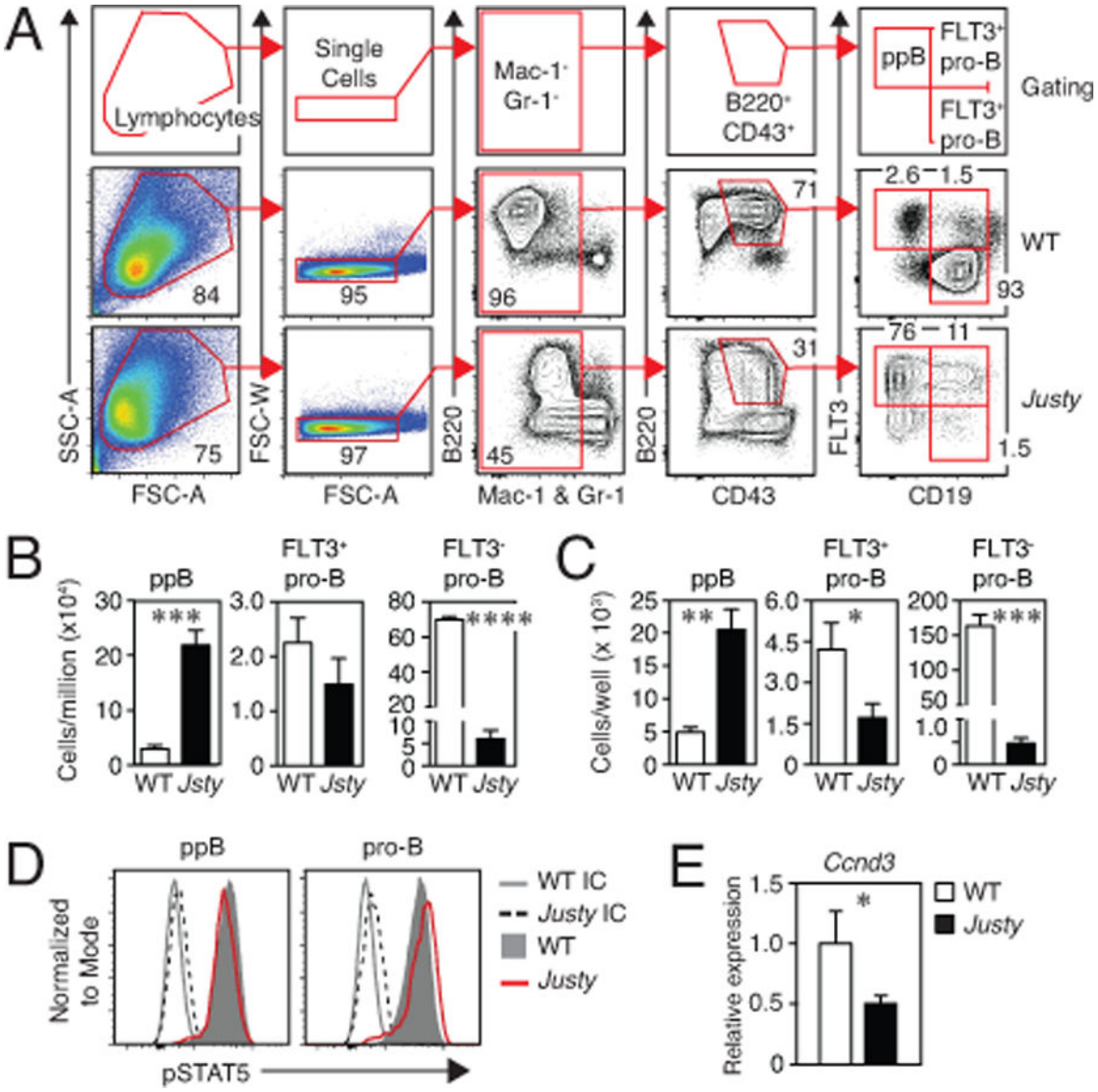

Figure 5. The ability of *Jsty* multipotent progenitors to generate pro-B cells *in vitro* is greatly impaired

(**A**) Analysis of B cell progenitors generated from WT and *Jsty* MPPs after culturing for 7 days on OP9 stromal cells in the presence of IL-7, SCF, and FLT3L. Pre-pro-B (ppB) cells, $FLT3^+$ pro-B cells, and $FLT3^-$ pro-B cells were identified as shown at the top of the panel. (**B, C**) Frequencies (**B**) and numbers (**C**) of the indicated of B cell progenitors generated in the cultures. (**D**) Analysis of phosphorylated STAT5 (pSTAT5) levels in pre-pro-B and pro-B cells (pool of $FLT3^+$ and $FLT3^-$ cells). Data are representative of results from 3 independent experiments. (**E**) quantitative RT-PCR analysis of cyclin D3 RNA levels in sort-purified pro-B cells (pool of $FLT3^+$ and $FLT3^-$ cells). For panels B, C, and E, bars represent the mean and standard error of values from at least 3 independent experiments. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

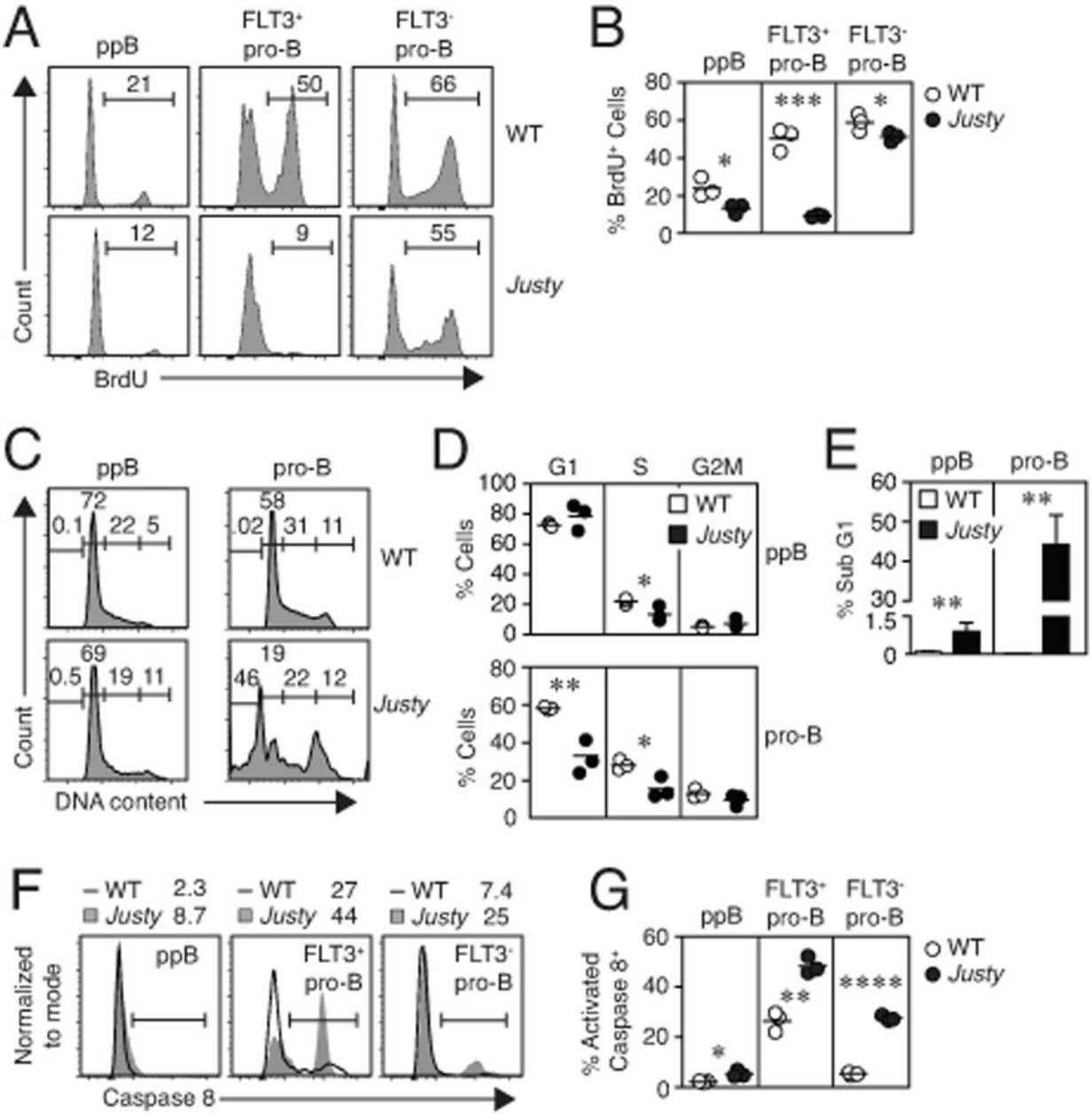

Figure 6. Cultured *Justy* B cell progenitors show cell-cycle defects and increased levels of apoptosis

MPPs from WT and *Justy* mice were cultured for 7 days on OP9 stromal cells in the presence of IL-7, SCF and FLT3L. B cell progenitors were identified as shown in Figure 5A. (**A**) DNA synthesis by WT and *Justy* B cell progenitors as measured by BrdU incorporation. BrdU was added to cultures, which were harvested 2 hours later and stained for surface markers and BrdU. The percentages of $BrdU^+$ cells are shown. (**B**) Frequencies of $BrdU^+$ cells within the indicated cell populations. (**C**) DNA content profiles for the indicated cell populations. The percentages of cells in the G0/G1, S and G2M phases of the cell cycle or in the sub G1 fraction are shown. (**D**) Frequencies of cells in the indicated stages of the cell cycle as determined from DNA content profiles. (**E**) Frequencies of pre-pro-B and pro-B

cells in the sub G1 fraction based on DNA content profiles. Bars represent the mean and standard error of values from 3 independent experiments. (**F**) Analysis of activated caspase 8 in the indicated cell populations. The frequencies of activated caspase 8^+ cells are shown. (**G**) Frequencies of activated caspase 8^+ cells in the indicated cell populations. For panel B, D and G, circles represent values from 3 independent experiments in which a WT and a *Justy* culture were analyzed; lines represent mean values. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

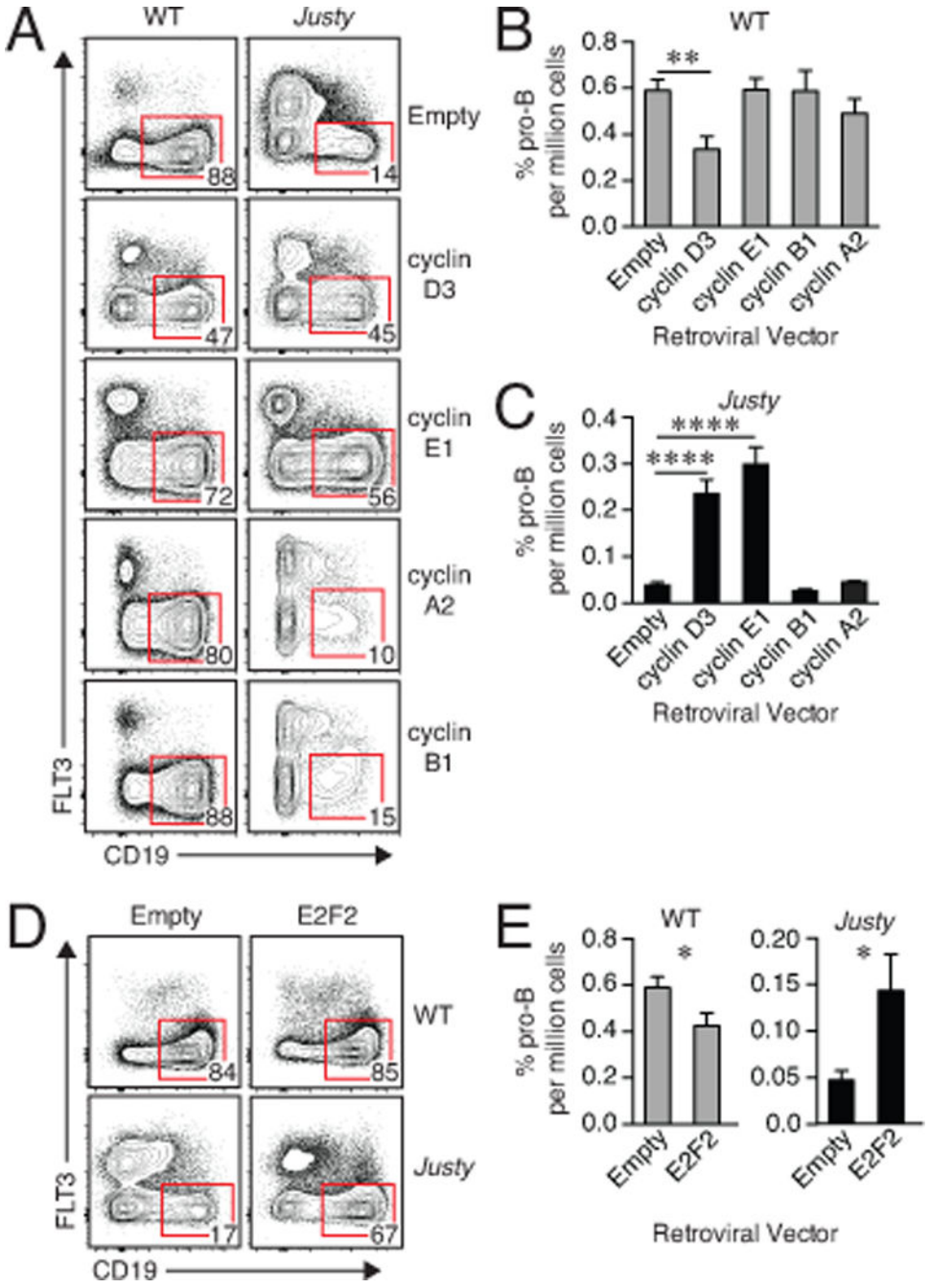

Figure 7. Enforced expression of cyclin D3, cyclin E or E2F2 promotes pro-B cell development from *Justy* multipotent progenitors

MPPs from WT and *Justy* mice were transduced with a GFP-expressing control retrovirus lacking a cDNA insert (Empty) or that expressing both GFP and the indicated protein. Cells were cultured on OP9 stromal cells in the presence of IL-7, SCF, and FLT3L for 11 days and analyzed as shown in supplemental figure 3C. (**A, D**) Analysis of Gr-1^{-}Mac-1^{-}B220^{+}CD43^{+} cells that were GFP^{+} following transduction with the indicated retroviral vectors. Gates in the flow plots identify pro-B cells; numbers represent the percentage of cells in the gates. Data are representative of results from at least 6 independent experiments. (**B, C and E**)

Frequencies of GFP^+ pro-B cells per million GFP^+ cells generated by WT or *Justy* MPPs following transduction with the indicated retroviral vectors. Bars represent the mean and standard error of values from at least 6 independent experiments. * $P < .05$; ** $P < .01$; **** $P < .0001$.