decay of normally spliced PTC-bearing mRNA triggers the increase in alt-mRNA, is more plausible. This could be achieved in many ways; one possibility is that there is massive decay of the normally spliced TCR mRNA at the site of transcription (as a result of PTC recognition there), which consequently alters the concentration of splicing factors and the rate of splicing reactions specifically in that local milieu of the nucleus. Splicing factor levels could either increase or decrease, depending on whether they are prematurely released from degraded PTC-bearing transcripts or are instead degraded along with the TCR transcripts harboring nonsense codons.

Two other well-established examples of nonsense codon–associated altered splicing (*4*) are the up-regulation of exon-skipped transcripts from the fibrillin gene (*5*, *26*) and a splicing enhancer–debilitated version of minute virus of mice (MVM) (*19*). In both cases, frameshifting experiments showed that recognition of in-frame stop codons was responsible for increased levels of alternatively spliced transcript, just as we showed here for TCRβ. However, only nonsense mutations at a single codon position adjacent to exonic splicing enhancers in fibrillin and MVM have been shown to trigger this event (*5*, *19*, *27*). In contrast, we found that TCRβ alt-mRNA was up-regulated in response to nonsense mutations and frameshifts at many different positions in the VDJ exon by a mechanism apparently independent of splicing enhancers. The discovery that classic translation signals (nonsense codons) act through a translation-like pathway to up-regulate alternatively spliced mRNA in the nuclear fraction of cells suggests that a novel mode of regulating gene expression exists in eukaryotic cells. It is important to follow up on initial indications that this regulatory response reduces the severity of some genetic diseases by generating alternative proteins that retain at least partial function (*5*–*7*, *28*).

Note added in proof: Recent further evidence for a relationship between nonsense codons and RNA splicing is the finding that mutation of the stop codon between natural and latent 5′ splice sites can induce alternatively spliced transcripts derived from the latent site (*29*).

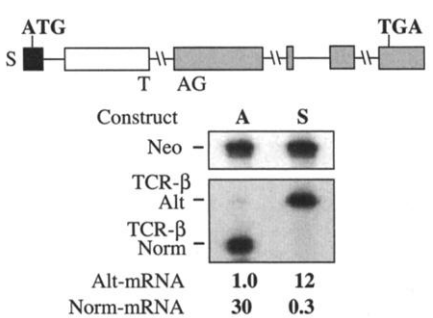

Fig. 4. Alt-mRNA up-regulation is triggered by nonsense codon recognition after normal splicing. RPA of total cellular RNA (5 μg) from HeLa cells transiently transfected with construct S [pAC/NS1 (9)] and analyzed in the same manner as in Fig. 1. Previous analysis (*10*) showed that the normally spliced mRNA from construct S contains the TAG nonsense codon. Similar results were obtained in at least two independent transfection experiments.

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Supporting Online Material

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Reversion of B Cell Commitment upon Loss of *Pax5* Expression

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The transcription factor Pax5 is essential for initiating B cell lineage commitment, but its role in maintaining commitment is unknown. Using conditional *Pax5* inactivation in committed pro-B cells, we demonstrate that Pax5 is required not only to initiate its B lymphoid transcription program, but also to maintain it in early B cell development. As a consequence of *Pax5* inactivation, previously committed pro-B cells regained the capacity to differentiate into macrophages in vitro and to reconstitute T cell development in vivo in *RAG2*$^{-/-}$ mice. Hence, Pax5 expression is continuously required to maintain B cell lineage commitment, because its loss converts committed pro-B cells into hematopoietic progenitors with multilineage potential.

The hematopoietic stem cell gives rise to all lymphoid lineages by differentiating through the intermediary stage of the common lymphoid progenitor (*1*). Entry of this progenitor into the B cell pathway depends on the transcription factors E2A, EBF, and Pax5 (BSAP), which control two separate transcriptional programs (*2*). The regulators E2A and EBF coordinately activate the expression of B cell–specific genes (*3*) and are essential for the generation of the earliest B cell progenitors (pro-B cells) (*4*, *5*). However, mere activation of the B lymphoid expression program is not sufficient for commitment to the B cell lineage, because Pax5-deficient pro-B cells still retain the broad developmental potential of early hematopoietic progenitors (*6*, *7*) despite normal expression of E2A and EBF (*8*). Instead, Pax5 restricts the multilineage potential of early progenitors to the B cell pathway by repressing lineage-inappropriate genes and further activating B cell–specific genes (*6*, *7*).

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A fundamental aspect of organogenesis concerns whether the commitment of tissue-specific stem cells to different developmental pathways is a reversible or irreversible process. This question has recently been investigated for the neural crest stem cell that generates neurons or glial cells during peripheral nervous system development. In this case, transient Notch activation was sufficient to irreversibly commit neural crest stem cells to the glial cell fate (*9*). Similarly, we were interested in determining whether B cell lineage commitment requires only the transient activation or continuous expression of *Pax5* during early B cell development. To address this issue, we took advantage of the Cre-*loxP* system for conditional inactivation of a floxed (F) $Pax5^{F}$ allele carrying *loxP* sequences on either side of the paired domain exon 2 (*10*).

B cell development is normal in $Pax5^{F/F}$ mice, whereas $Pax5^{\Delta/\Delta}$ mice, generated by germ line deletion (Δ) of exon 2 (*10*), are phenotypically indistinguishable from $Pax5^{-/-}$ mice (*11*). For conditional *Pax5* inactivation in pro-B cells, we crossed $Pax5^{F/F}$ mice with the transgenic line *CreED-30*, which expresses the Cre-EBD(G521R) fusion protein from the simian virus 40 promoter and Eμ enhancer in B lymphocytes (*12*). The Cre-EBD(G521R) protein consists of the Cre recombinase linked to a mutant estrogen receptor ligand-binding domain (EBD containing a glycine to arginine substitution at position 521) that is responsive to the synthetic antagonist 4-hydroxytamoxifen (OHT) instead of β-estradiol. The *CreED-30* transgene therefore facilitates OHT-mediated induction of Cre activity in B lymphocytes (*12*). Pro-B cells were sorted from the bone marrow of $Pax5^{F/F}$ *CreED-30* mice and cultured in vitro on stromal cells in the presence of interleukin-7 (IL-7), which promotes the expansion of pro-B cells while preventing their further differentiation (*13*). Flow cytometric analysis revealed normal expression of the B cell markers B220 and CD19 on these pro-B cells (Fig. 1A, F/F). Because *CD19* transcription is strictly dependent on Pax5 function (*8*, *14*), these data demonstrate that the $CD19^{+}$ $Pax5^{F/F}$ pro-B cells express normal levels of Pax5 and have undergone commitment to the B lymphoid lineage. OHT treatment of these pro-B cells (*13*) resulted in loss of CD19 expression and thus in efficient *Pax5* inactivation under the in vitro culture conditions used (Fig. 1A, Δ/Δ). The proliferation of pro-B cells remained largely unaffected by OHT treatment (*15*), indicating that *Pax5* inactivation did not result in an appreciable cell loss or in outgrowth of a minor subpopulation of $Pax5^{\Delta/\Delta}$ pro-B cells.

Time-course analyses revealed that the $Pax5^{F/F}$ *CreED-30* pro-B cells deleted *Pax5* exon 2 (Fig. 1B) and expressed the exon 2–truncated *Pax5* transcript (Fig. 1D) within 1 day of OHT treatment. Concomitant with the loss of Pax5 protein (Fig. 1C), the expression of activated Pax5 target genes started to decline at day 4, as revealed by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis (*13*) (Fig. 1D). These target genes encode the cell surface proteins CD19 and Igα (mb-1) (*14*), the transcription factor CIITA (*10*), and the alternatively spliced δ_m transcript of the *IgH* gene (*10*). Conversely, *Pax5* inactivation led to reexpression of the repressed Pax5 target genes encoding the secreted J chain (*16*), the macrophage colony-stimulating factor (M-CSF) receptor (*6*), and transcripts of unknown function (rEST1-3) (*15*) (Fig. 1D). However, loss of Pax5 did not affect the expression of control genes encoding hypoxanthine phosphoribosyltransferase and the B cell–specific *EBF*, *B29* (*Ig*β), and μ_m transcripts (Fig. 1D). In summary, the expression pattern of $Pax5^{F/F}$ pro-B cells was indistinguishable from that of $Pax5^{+/+}$ pro-B cells and could be converted by *Pax5* inactivation into that of $Pax5^{-/-}$ pro-B cells. Hence, the Pax5-dependent gene expression program is reversible in pro-B cells undergoing *Pax5* inactivation.

Because the $Pax5^{\Delta/\Delta}$ pro-B cells were derived from committed $Pax5^{F/F}$ pro-B cells, we next investigated whether the $Pax5^{\Delta/\Delta}$ pro-B cells remained committed or had again adopted a broad developmental potential characteristic of early hematopoietic progenitors. We assessed the potential of the $Pax5^{\Delta/\Delta}$ pro-B cells to differentiate into macrophages by culturing them in the absence of IL-7 on stromal ST2 cells (*13*), which produce the myeloid cytokine M-CSF (*6*). Within 3 weeks, the $Pax5^{\Delta/\Delta}$ cells downregulated the B cell surface protein B220, expressed the macrophage markers Mac-1 and F4/80 (Fig. 2A), displayed the morphology of enlarged vacuolar macrophages (Fig. 2B), and

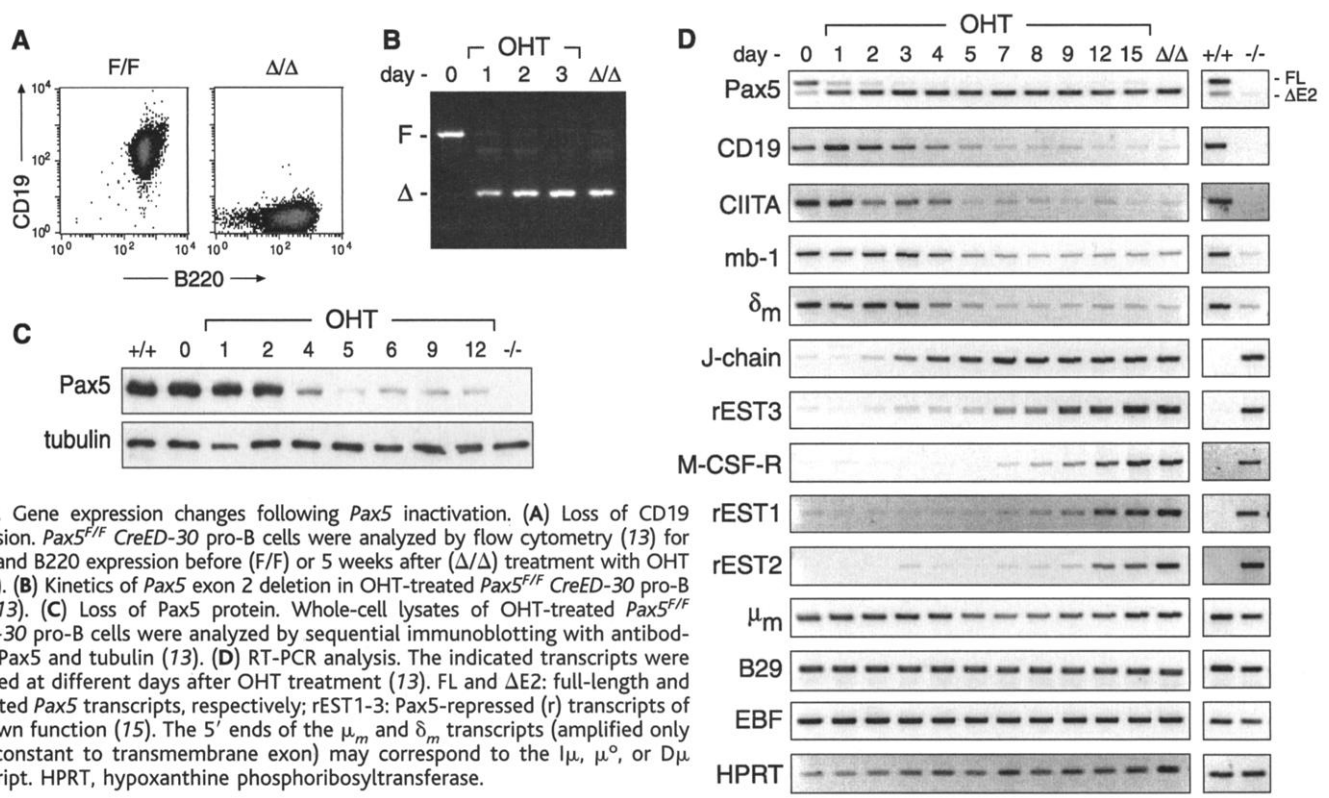

Fig. 1. Gene expression changes following *Pax5* inactivation. (**A**) Loss of CD19 expression. $Pax5^{F/F}$ *CreED-30* pro-B cells were analyzed by flow cytometry (*13*) for CD19 and B220 expression before (F/F) or 5 weeks after (Δ/Δ) treatment with OHT (1 μM). (**B**) Kinetics of *Pax5* exon 2 deletion in OHT-treated $Pax5^{F/F}$ *CreED-30* pro-B cells (*13*). (**C**) Loss of Pax5 protein. Whole-cell lysates of OHT-treated $Pax5^{F/F}$ *CreED-30* pro-B cells were analyzed by sequential immunoblotting with antibodies to Pax5 and tubulin (*13*). (**D**) RT-PCR analysis. The indicated transcripts were analyzed at different days after OHT treatment (*13*). FL and ΔE2: full-length and truncated *Pax5* transcripts, respectively; rEST1-3: Pax5-repressed (r) transcripts of unknown function (*15*). The 5′ ends of the μ_m and δ_m transcripts (amplified only from constant to transmembrane exon) may correspond to the Iμ, μ°, or Dμ transcript. HPRT, hypoxanthine phosphoribosyltransferase.

phagocytosed fluorescently labeled *Escherichia coli* (Fig. 2C). Hence, the $Pax5^{\Delta/\Delta}$ pro-B cells were able to differentiate into mature macrophages, as observed with $Pax5^{-/-}$ pro-B cells (*6*).

We next examined the potential of $Pax5^{\Delta/\Delta}$ pro-B cells to develop into T lymphocytes after intravenous injection into sublethally irradiated, T cell–deficient $RAG2^{-/-}$ mice (*13*, *17*). Three weeks after cell transfer, the cellularity of the thymus was massively increased owing to the presence of $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD8^+CD4^-$ thymocytes, in which expression of the T cell receptor (TCR) β was similar to that in wild-type thymocytes (Fig. 2D). Hence, the $Pax5^{\Delta/\Delta}$ pro-B cells could fully reconstitute T cell development but were unable to generate immunoglobulin M–positive (IgM^+) B cells in the spleens of $RAG2^{-/-}$ mice (Fig. 2E), in analogy to $Pax5^{-/-}$ pro-B cells (*7*). This result was obtained regardless of whether the pro-B cells were cultured after Cre recombinase activation for only 4 days or for up to 5 weeks before injection into $RAG2^{-/-}$ mice (Fig. 2D; figs. S1 and S2) (*13*). However, $Pax5^{F/F}$ pro-B cells did not give rise to $TCR\beta^+$ thymocytes; instead, they developed into IgM^+ B cells in the spleens of injected $RAG2^{-/-}$ mice (Fig. 2F). The $Pax5^{F/F}$ pro-B cells are therefore fully committed to the B cell pathway. Hence, the inactivation of *Pax5* alone is sufficient to revert the narrow B lymphoid potential of $Pax5^{F/F}$ pro-B cells to the broad multilineage potential characteristic of early hematopoietic progenitors.

The heterozygous $Pax5^{+/-}$ mouse provided a second opportunity to investigate the reversibility of Pax5-dependent B cell lineage commitment, because the pro-B cells of this mouse preferentially express only one of the two *Pax5* alleles (*18*). During in vitro culture, the heterozygous $CD19^+$ pro-B cells consistently switch expression from the wild-type *Pax5* gene to the mutant allele, which carries an in-frame *lacZ* gene insertion (*18*, *19*). This allele switching results in loss of expression of the Pax5 target gene *CD19* and simultaneous activation of the *lacZ* gene of the targeted *Pax5* locus (*18*, *19*) (Fig. 3, A and B). Therefore, we derived $CD19^-$ β-galactosidase–positive ($\beta\text{-Gal}^+$) pro-B cell clones (*13*) from $Pax5^{+/-}$ bone marrow (Fig. 3A) and demonstrated by RT-PCR analysis (*13*) that these pro-B cells switched off the expression of activated Pax5 target genes and derepressed genes normally suppressed by Pax5 (Fig. 3B) (*15*). Hence, the $CD19^-$ $\beta\text{-Gal}^+$ pro-B cells acquired the gene expression pattern of Pax5-deficient pro-B cells, although, as genotypically heterozygous cells, they retained the potential to reactivate the wild-type *Pax5* allele. These $CD19^-$ $Pax5^{+/-}$ pro-B cells were also decommitted, because they differentiated, upon IL-7 withdrawal, into functional $Mac\text{-}1^+F4/80^+$ macrophages with phagocytic activity (Fig. 3, C and D) (*15*). To study the T lymphoid potential of $CD19^-$ $Pax5^{+/-}$ pro-B cells, we first infected these cells with a human (h) CD2-expressing retrovirus before injecting them into sublethally irradiated $RAG2^{-/-}$ mice (*13*). Three weeks after cell transfer, the thymus contained $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD8^+CD4^-$ T cells of donor origin ($hCD2^+$), indicating that the $CD19^-$ $Pax5^{+/-}$ pro-B cells could reconstitute T cell development (Fig. 3E). Thymic reconstitution was still observed 11 weeks after cell transfer owing to long-term engraftment of the $CD19^-B220^+$ $Pax5^{+/-}$ pro-B cells in the bone marrow, from where these progenitors continuously seed the thymus (Fig. 3F). The bone marrow additionally contained $CD19^+B220^+$ B lymphocytes of donor origin ($hCD2^+$), which had reentered the B cell pathway through activation of the wild-type *Pax5* allele (Fig. 3F). Finally, a third population of $hCD2^+CD19^-B220^-$ cells was present (Fig. 3F), which consisted of mature $CD4^+$ and $CD8^+$ T lymphocytes (*15*). In conclusion, heterozygous $Pax5^{+/-}$ pro-B cells not only regained the broad developmental potential of early hematopoietic progenitors upon switching to

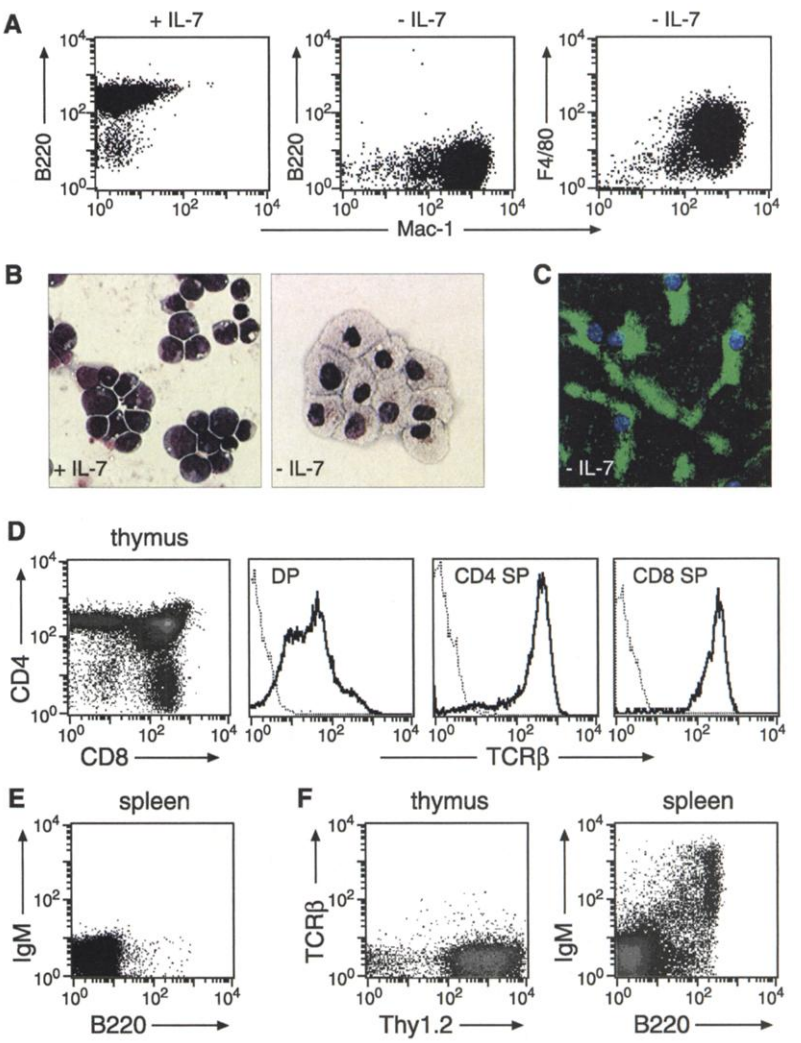

Fig. 2. Reacquisition of multilineage potential upon *Pax5* inactivation. (**A**) Macrophage differentiation. $Pax5^{\Delta/\Delta}$ pro-B cells were cultured for 18 days on ST2 cells with or without IL-7 before flow cytometric analysis (*13*). (**B**) May-Grünwald-Giemsa staining of $Pax5^{\Delta/\Delta}$ pro-B cells grown for 3 weeks on ST2 cells with or without IL-7. (**C**) Phagocytosis. In vitro–differentiated $Pax5^{\Delta/\Delta}$ macrophages were incubated with fluorescein isothiocyanate–labeled *E. coli*, followed by DAPI (4′,6-diamidino-2-phenylindole) staining of the nuclei (*13*). (**D**) Thymus reconstitution by $Pax5^{\Delta/\Delta}$ pro-B cells (10^6) that were injected into 5.5 Gy–irradiated $RAG2^{-/-}$ mice (*13*). Three weeks after transfer, the thymus consisted of 80×10^6 cells (compared with 5×10^6 cells of uninjected $RAG2^{-/-}$ mice). The $CD4^+CD8^+$ double-positive (DP) and the $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes were analyzed by flow cytometry for TCRβ expression (*13*). Stippled lines indicate control $RAG2^{-/-}$ thymocytes. (**E**) Absence of IgM^+B220^+ B cells in the spleen of the same injected $RAG2^{-/-}$ mouse analyzed in (D). (**F**) B cell lineage commitment of $Pax5^{F/F}$ pro-B cells. $B220^+$ pro-B cells sorted from $Pax5^{F/F}$ *CreED-30* bone marrow were cultured for 2 weeks (without OHT treatment) before injection of 5×10^5 cells into 5.5 Gy–irradiated $RAG2^{-/-}$ mice. After 3 weeks, the thymus and spleen were analyzed by flow cytometry (*13*). The $Thy1.2^+TCR\beta^-$ thymocytes correspond to pro-T cells of the $RAG2^{-/-}$ host.

the mutant *Pax5* allele, but at the same time maintained their competence to undergo B cell development by reactivating the wild-type *Pax5* allele in vivo.

In summary, we have shown by conditional *Pax5* inactivation and allele switching experiments that Pax5 is continuously required to maintain its B lymphoid transcription program and B cell lineage commitment during early B cell development. These findings raise the intriguing possibility that wild-type pro-B cells may differentiate into other hematopoietic cell types by down-regulating *Pax5* expression, thus reversing B cell commitment in response to external signals. Because the target gene *CD19* is totally dependent on Pax5 for its transcription (*8*, *14*), we argued that *CD19* expression is not only an indicator of Pax5 activity (*18*), but is also a decisive marker of B cell lineage commitment (*6*, *19*). Unexpectedly, however, $CD19^+$ progenitors of the bone marrow were shown to differentiate upon exposure to appropriate cytokines, into dendritic cells (*20*), macrophages (*21*), or osteoclasts (*22*), which was accompanied by down-regulation of CD19 expression (*20*). These observations, together with our data, suggest that committed B cell progenitors can again broaden their developmental potential by repressing *Pax5* transcription under physiological conditions.

Finally, the generation of multipotent hematopoietic progenitors from wild-type pro-B cells is also of medical relevance. Ribozyme, antisense RNA, or RNA interference strategies could be used to inactivate or down-regulate *PAX5* expression in wild-type pro-B cells isolated from human leukocyte antigen (HLA)–matched donors (*23*). In analogy to the mouse model (*7*), these human Pax5-deficient pro-B cells should have regained the potential to restore T cell development in patients with AIDS or inherited immunodeficiency syndromes (*24*).

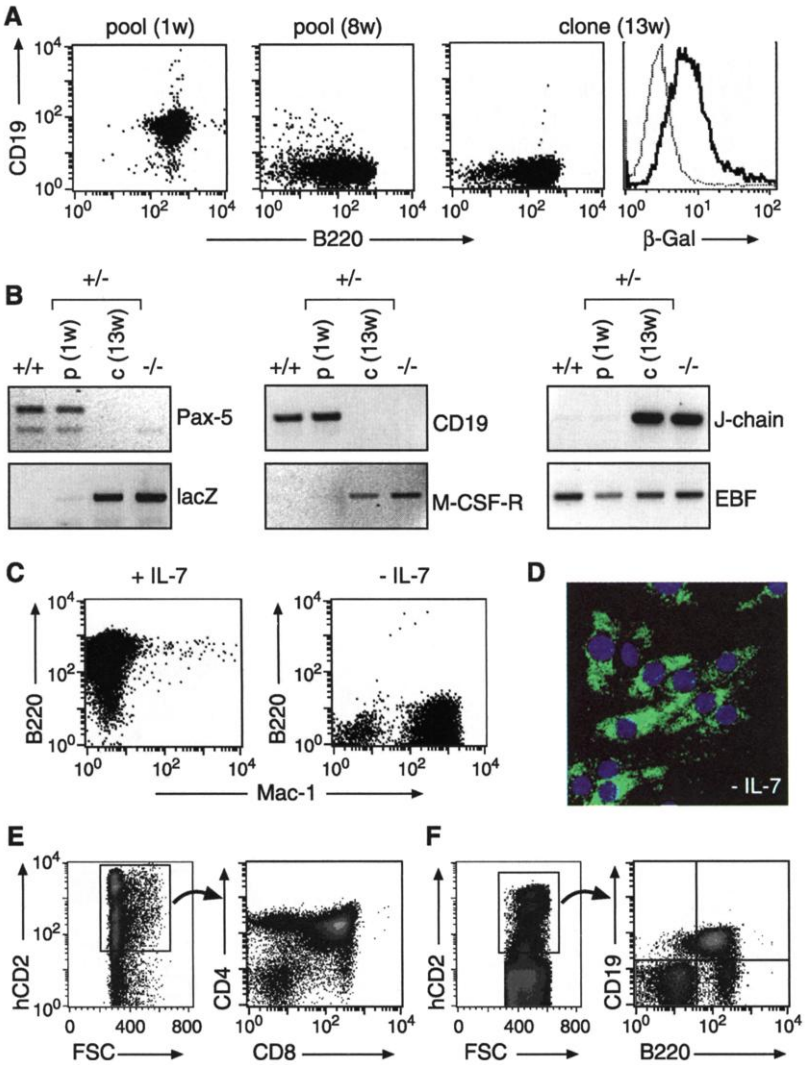

Fig. 3. Decommitment of $Pax5^{+/-}$ pro-B cells upon switching expression from the wild-type to the targeted *Pax5* allele. (**A**) Generation of $CD19^-$ $Pax5^{+/-}$ pro-B cell clones. $CD19^+$ pro-B cells from $Pax5^{+/-}$ bone marrow were cultured with ST2 cells and IL-7 for 8 weeks (w) before single-cell cloning (*13*). The pro-B cell pool and a derived clone were analyzed by flow cytometry for CD19, B220, and β-Gal expression at the indicated times after the start of in vitro culture (*13*). The stippled line denotes wild-type pro-B cells. (**B**) RT-PCR analysis of the indicated transcripts in wild-type (+/+), homozygous (−/−), and heterozygous (+/−) *Pax5* mutant pro-B cells. The pool (p) and a clone (c) of $Pax5^{+/-}$ pro-B cells were analyzed after 1 and 13 weeks of in vitro culture, respectively. (**C** and **D**) Macrophage differentiation. Cloned $CD19^-$ $Pax5^{+/-}$ pro-B cells were cultured for 3 weeks on ST2 cells with or without IL-7 and analyzed for B220 and Mac-1 expression (C) and phagocytic activity (D). (**E**) Thymus reconstitution. The cells of a $CD19^-$ $Pax5^{+/-}$ pro-B clone were infected with a retrovirus expressing a truncated human (h) CD2 protein, injected into 5.5 Gy–irradiated $RAG2^{-/-}$ mice, and analyzed by flow cytometry at 3 weeks after transfer (*13*). CD4 and CD8 expression is shown for the $hCD2^+$ thymocytes of donor origin. (**F**) Long-term engraftment. Eleven weeks after cell transfer, the bone marrow of reconstituted $RAG2^{-/-}$ mice was analyzed by flow cytometry; expression of CD19 and B220 is displayed for $hCD2^+$ donor cells.

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Supporting Online Material

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Materials and Methods
Figs. S1 and S2
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Table S1

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