PAX5-ELN oncoprotein promotes multistep B-cell acute lymphoblastic leukemia in mice

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Supporting Information

Supplemental text

PAX5-ELN oncoprotein alters the functional activity of pro-B cells *in vitro*.

Our *in vivo* experiments bring evidence that PAX5-ELN oncoprotein expands pre-leukemic pro-B cells at the expense of subsequent stages of maturation (**SI Appendix, Fig. 3*A-B***), suggesting that PAX5-ELN perturbs their B-cell differentiation potential. To directly address this hypothesis at the functional level, we purified and expanded pro-B cells from 30 days old wt and pre-leukemic PE^{tg} mice (**SI Appendix, Fig. S5*A***). Expanded wt and PE^{tg} pro-B cells were then cultured in undifferentiated (IL7+) or under B-cell differentiation (IL7-) conditions as described previously (1) (**SI Appendix, Fig. S5*A***). Our results demonstrated that IL7 withdrawal allowed for an efficient differentiation of wt pro-B cells in pre-B ($CD2^+Igk^-$) and immature-B ($CD2^+Igk^+$) cells (**SI Appendix, Fig. S5*B***). In contrast, PE^{tg} pro-B cells were not able to differentiate in the absence of IL7 (**SI Appendix, Fig. S5*B***), further corroborating the partial blockade of differentiation observed *in vivo* (**SI Appendix, Fig. 3*A-B***).

Pax5 is a critical B cell lineage commitment factor that restricts the developmental program of lymphoid progenitors to the B cell pathway. Precisely, Pax5 controls definitive B-cell commitment at the transition to pro-B cell stage (2, 3) and it is known that the loss of Pax5 affects the onset of fetal B cell development. Indeed, B lymphopoiesis is arrested prior to the appearance of $CD19^+$ progenitors in the fetal liver of *Pax5*-deficient embryos (4). We therefore compared the ability of PAX5 and PAX5-ELN to rescue the blockade of B-cell commitment in $Pax5^{-/-}$ pro-B cells. To this end, fetal liver cells from $Pax5^{-/-}$ mice were retrovirally transduced with either MIE-*PAX5*, MIE-*PAX5-ELN* (MIE-PE) or with the empty (MIE) vectors and were cultured in undifferentiated (IL7+) or under B-cell differentiation (IL7-) conditions (**SI Appendix, Fig. S5*C***). As expected, MIE-PAX5 induced CD19 expression in $Pax5^{-/-}$ pro-B cells in the presence of IL7 (**SI Appendix, Fig. S5*D***). Interestingly, this induction was not associated with a downregulation of the Kit marker (**SI Appendix, Fig. S5*D***), indicating that PAX5 activated its target gene *CD19* without inducing B-cell differentiation in the presence of IL7.

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However, the withdrawal of IL7 revealed the ability of PAX5 to rescue the differentiation of *Pax5*$^{-/-}$ pro-B cells as shown by the down-regulation of Kit (**SI Appendix, Fig. S5*D***). In stark contrast, PAX5-ELN modified neither CD19 nor Kit expression in the absence or presence of IL7 (**SI Appendix, Fig. S5*D***).

The data demonstrate that PAX5-ELN fails to rescue B-cell development of *Pax5*-deficient pro-B cells.

PAX5-ELN oncoprotein has a restricted effect on the B-cell lineage

B-cells are generated from HSCs and develop in the BM through several differentiation stages before they migrate to peripheral lymphoid organs. B-cell development can be divided into five cell compartments according to the expression of stage-specific cell-surface markers and the rearrangement status of their *Ig* loci (**SI Appendix, Fig. S3*C***). We first quantified *PAX5-ELN* transcript levels in the five B-cell compartments and observed a significant induction of *PAX5-ELN* expression from the pro-B stage onwards (**SI Appendix, Fig. S6*A***), which correlated with the Eμ-mediated activation of the ectopic PV_H promoter used to drive transgene expression.

To address the potential B cell-restricted expression and effect of PAX-ELN, we quantified *PAX5-ELN* transcript levels in thymocytes and analyzed T-cell development in the thymus of *PE*^{tg} mice (**SI Appendix, Fig. S6*B*-*D***). While *PAX5-ELN* transcripts were detected in *PE*^{tg} thymocytes, their levels were manifold lower than those in *PE*^{tg} BM B-cells (**SI Appendix, Fig. S6*B***). In addition, the T-cell compartment was unaffected in mutant mice (**SI Appendix, Fig. S6*C*-*D***), confirming the highly restricted effect of PAX5-ELN expression on the B-cell lineage.

PAX5 is also involved in V_H to D_HJ_H recombination step. Indeed, distal V_H to D_HJ_H recombination involving V_HJ558 genes are 100-fold reduced in *Pax5*-deficient pro-B cells (4). Here, our results indicated that pro-B cells expressing PAX5-ELN oncoprotein efficiently rearranged both their distal and proximal $V_HD_HJ_H$ genes (**Fig. 2*A***). Moreover, allelic competition between wt and mutant *IgH* loci enabled us to show that IgM expression almost exclusively originated from the wt allele in *PE*^{tg} mice (**SI Appendix, Fig. S6*E***), excluding that PAX5-ELN perturbs B-cell development by interfering with the Pax5-dependent regulation of *IgH* rearrangements.

Combined, the above data allow us to conclude that PAX5-ELN oncoprotein perturbs pro-B cell functional activity *in vitro* essentially by preventing their cell differentiation.

Effect of PAX5-ELN on B-cell progenitor turnover *in vivo*.

To evaluate the effect of PAX5-ELN oncoprotein on B-cell progenitor turnover *in vivo*, BM cells from 30 days old wt and pre-leukemic PE^{tg} mice were transplanted into myeloablated recipient mice and B cell reconstitution was assessed after one month (**SI Appendix, Fig. S7*A***). Analysis of BM showed similar engraftment efficiencies between wt and pre-leukemic PE^{tg} donor cells (**SI Appendix, Fig. S7*B***). However, analysis of B-cell lineage development in donor-derived wt and PE^{tg} BM cells revealed that PAX5-ELN induced an aberrant reconstitution of pro-B and pre-B cell compartments (**SI Appendix, Fig. S7*C***). Indeed, we observed a 4 to 5-fold expansion of donor-derived PE^{tg} pro-B and pre-B cells as compared to wt controls, while the other B-cell compartments were not affected (**SI Appendix, Fig. S7*C***). In addition, we verified that T-cell reconstitution of donor-derived PE^{tg} thymocytes was not affected (**SI Appendix, Fig. S7*D***), confirming the restricted effect of PAX5-ELN on the early B-cell compartment.

Together, these observations suggest that PAX5-ELN oncoprotein induce an aberrant expansion potential to a pro-B/pre-B progenitor before B-ALL transformation.

Supplemental experimental procedures

Generation of the PAX5-ELN knock-in mouse model

The vector (pAp-μ) used to insert the human PAX5-ELN cDNA has been previously described in detail (5). The human cDNA was first inserted between a mouse PV_H promoter and SV40 polyA signal in a modified pBluescript vector, and the whole unit (PVH-cDNA-SV40 polyA) was eluted as a ClaI fragment and inserted between the pause/polyA signal and Neo^R cassette of pAp-μ. An HSV *tk* gene was inserted into SalI site for negative selection. The ES cell line CK35 (kindly provided by C. Kress, Institut Pasteur, Paris) was transfected by electroporation, and selected using G418 (300 μg/ml) and gancyclovir (2 μM). Recombinant clones were identified by PCR using a forward primer in the Neo^R cassette and a reverse primer downstream of the 3'arm of the targeting vector. Two ES clones showing homologous recombination were injected into C57Bl/6 blastocysts, the male chimeras were then mated with C57Bl/6 females. Germ-line transmission of the mutation was checked by PCR. Homozygous mutant mice were mated with EIIa-cre transgenic mice. The progeny was checked by PCR for CRE-mediated deletion of Neo^R cassette. The experiments on mice have been carried out according to the CNRS and Inserm Ethical guidelines and approved by the Regional Ethical Committee.

FACS analysis, antibodies and cell sorting

Single cell suspensions were prepared from bone marrow (BM), spleen and lymph node (LN) of wt and PE^{tg} mice of the indicated ages. Immunostaining was performed using antibodies used for flow cytometry obtained from Pharmingen (BD Biosciences) : V500-labelled anti-B220 (RA3-6B2), AF700-labelled anti-CD19 (1D3), FITC-labelled anti-Igκ (187.1), FITC-labelled anti-Igλ (R26-46), PeCy7-labelled anti-CD23 (B3B4), BV711-labelled anti-c-Kit (2B8), PE-labelled anti-CD2 (RM2-5), APC-Cy7-labelled anti-CD45.1 (A20) and V450-labelled anti-CD45.2 (104). Dead cells were excluded by 7-AAD staining. FACS analysis was performed on a Fortessa cytometer (BD Biosciences) using DIVA (BD Biosciences) and FlowJo softwares.

BM cells from wt or pre-leukemic PE^{tg} mice were flushed and B-cell fraction were enriched by B220 magnetic microbead sorting (Miltenyi Biotech). B-cell fraction was subsequently incubated with CD19, B220, CD23, Igκ, Igλ and c-Kit antibodies. Cell sorting of B cell populations was performed on a MoFlo Astrios sorter (Beckman Coulter).

Immunoglobulin rearrangement assay

Pro-B cells were enriched from the BM of 30 days old wt or pre-leukemic *PE*^{tg} mice by using B220 magnetic microbeads and LS columns (Miltenyi) and purified by sorting the B220^{+}Igκλ^{-} CD23^{-} Kit^{+} fraction. B-ALL cells were obtained from the BM of leukemic *PE*^{tg} mice. Genomic DNAs were prepared by standard techniques and subjected to an *IgH* V(D)J recombination assay by quantitative PCR using forward primers binding to the distal V_{HJ558}, the proximal V_{H7183} or the D_H segments and reverse primers that pair 3' of each J_H segment. PCR of HS5 element downstream of the 3' regulatory region was performed for normalization of the DNA input. Primers are listed in **SI Appendix, Table S5**.

Whole-exome sequencing and specific resequencing

Whole-exome sequencing was performed using 50 ng genomic DNA extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, USA). Enrichment for coding exons was performed using a transposon-based method (SureSelect QXT Clinical research Exome, Agilent Technologies). The library was quantified by quantitative PCR (Kapa Biosystems, Wilmington, USA) and normalized at 4 nM. Sequencing was performed using NextSeq500 High Output kit V2 flowcell on a NextSeq 500 (Illumina) with a 150-bp paired-end mode. Sequence analysis was performed using NextGene software (SoftGenetics, State College, USA). For mutations resequencing, a library was generated using PCR from 7 specific mouse regions identified by whole exome sequencing. The library was sequenced on a MiSeq sequencer (Illumina, San Diego, USA) using a Miseq Reagent kit V2 (paired-end sequencing 2 x 150 cycles). Alignment and variant calling were performed using NextGene software (SoftGenetics, State College, USA). Targets and primers are listed in **SI Appendix, Table S5**.

Human B-ALL mutation analysis

101 bone marrow samples (99 pediatric and 2 additional adult cases with a PAX5 rearrangement) treated for de novo B-ALL in the Toulouse hospital were analyzed regarding the mutations identified in the whole exome analysis of leukemic PAX5-ELN mice. Cytogenetic analysis was performed using R- or G-banding and classified according to WHO 2016 classification. JAK2 (exon 16), JAK3 (exons 13 and 15), KRAS (exons 2 and 3), NRAS (exons 2 and 3), PAX5 (exons 1 to 10) and PTPN11 (exons 3 and 11) were amplified by multiplex PCR to generate

specific libraries using primers listed in the supplementary data. The library was sequenced using a MiSeq sequencer (Illumina, San Diego, USA) and Miseq Reagent kit V2 (paired-end sequencing 2 x 150 cycles). Alignment and variant calling were performed using NextGene software (SoftGenetics, State College, USA). The study on human B-ALL patients was conducted in accordance with the Declaration of Helsinski and approved by the national institutional review board and ethical committee. Written informed consent was obtained from the parents. Targets and primers are listed in **SI Appendix, Table S5**.

Transplantation assay

Total BM cells and total purified B-cells from wt, pre-leukemic and B-ALL *PE*^{tg} mice (CD45.2^{+}) were transplanted intravenously into 6-8 weeks old recipient mice (CD45.2^{+}) pre-treated, 24h before transplantation, with 30 mg/kg of Busulfan (Busilvex, Pierre Fabre). Chimerism was analyzed by flow cytometry (FACS) and was illustrated by the percentage of donor-derived cells (% CD45.2^{+}) found in the recipient BM and thymus.

Microarray experiments

Pax5$^{-/-}$ embryonic liver (E17.5) pro-B cells were transduced with either MIE or MIE-PAX5 as described previously. Transduced cells were purified according to their positivity for GFP fluorescence with a FACSAria II (BD Biosciences) cell sorter. For each condition, three independent transductions were performed. Total RNAs were extracted using the Trizol method according to manufacturer's instruction (Invitrogen). RNA quality was assessed using the RNA 6000 Nano Assay on the Agilent 2400 Bioanalyser (Agilent Technologies, Massy, France). RNA samples were purified and prepared according to the manufacturer's protocol with the Affymetrix's GeneChip Whole Transcript Sense Target Labeling Assay Kit (Affymetrix, UK) and hybridized on Affymetrix GeneChip Mouse Gene 2.0 ST arrays. Pro-B cells were purified from the BM of 30 days old wt (n=3) and pre-leukemic *PE*^{tg} (n=3) mice by cell sorting. RNAs extraction and preparation were performed as described previously and hybridized on Clariom D GeneChip Mouse Transcriptome array 1.0.

Bioinformatics analysis

Probe-signal intensities were normalized and summarized by the robust multi-array average

(RMA) method by using Affymetrix's Expression Console software. Data were then processed with AltAnalyzer software (6). Folds changes of genes were calculated as the ratio of the signal values of the experimental groups compared with the control groups and principal component analysis (PCA) was calculated considering the varying genes of each sample. Gene expression between *in vivo* purified wt and PE^{tg} pre-leukemic pro-B cells and between *ex vivo* E17.5 fetal liver $Pax5^{-/-}$ pro-B cells transduced with either MIE-PAX5 or MIE retroviral vectors are considered significantly modified when expression difference is upper 1.5 and 3 fold changes, respectively, and an adjusted *P*-value of < 0.05. Heatmap was established by log^2 genes expression confrontation for each gene of different groups, and clusturised hierarchically. The biological processes were represented as GO Term annotations provided by the data libraries from AltAnalyzer software. Gene enrichment analysis was performed using GSEA software from the Broad institute (7). Our *ex vivo* PAX5-modified genes were ranked based on log^2 ratio of classes and compared to a well-established gene set of *in vivo* PAX5-regulated genes in pro-B cells extracted from Revilla IDR*, et al.* (2012), Table S5 (8). PAX5-ELN-modified genes in pre-leukemic pro-B cells were overlapped with gene sets of PAX5-ETV6- and PAX5-FOXP1-regulated genes in pre-leukemic pro-B cells and in B-ALL cells extracted from Smeenk L*, et al.* (2017), Tables EV1 and EV4 (9).

Retroviral production and transduction

Full-length human *PAX5* and *PAX5-ELN* cDNA were amplified, sequenced and inserted into the retroviral vector pMSCV-IRES-EGFP (MIE). Retroviral supernatants were produced using the Phoenix retrovirus producer line (Orbigen) and Lipofectamine 2000 (Invitrogen) reagent for transfection and proceeding according to the manufacturer's instructions. After overnight incubation of the transfection mix, cells were treated with 10 mM sodium butyrate (Millipore) and washed with PBS before pro-B-cell medium was added. Viral supernatants were harvested after 24 h of incubation at 32°C, passed on a 0.45 µm filter, aliquoted and frozen at -80°C. *Pax5*-deficient pro-B-cells were harvested from $Pax5^{-/-}$ embryonic liver at E17.5, amplified on irradiated OP9 stromal cells in the presence of 2 ng/mL IL7 and infected during exponential growth. Retroviral supernatant was diluted at 1:3, supplemented with 4µg/ml polybrene (Sigma) and 2 ng/mL of IL7. Spinoculation was performed at 1,000 g at 32°C for 90 min. After centrifugation, 1mL of fresh medium supplemented with IL7 is added and the cells are incubated

overnight at 37°C. After infected pro-B-cell expansion for few days, cells are washed out of IL-7 and plated in IMDM, 5% FCS, 0.05 mM β-mercaptoethanol and 2 mM L-glutamine on irradiated OP9-derived stromal cells with or without IL7. After 3 days, single-cell suspensions were stained using standard protocols and subjected to flow cytometry.

Cytology and immunohistochemistry

BM cells from wt, pre-leukemic and leukemic *PE*^{tg} mice were cytospined and stained with May Grünwald-Giemsa coloration. Spleens from wt or leukemic *PE*^{tg} mice were fixed with PBS and 4% paraformaldehyde overnight at 4°C, processed and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed on 5µm paraffin sections according to common methods. 5µm sections were immunostained with biotinylated anti-B220 (Serotech) antibody and secondary antibody conjugated with the streptavidin, and visualized with the video-microscope Cell Observer (Zeiss).

RT-qPCR

RNA was isolated using Trizol method and cDNA was synthesized using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™) according to the manufacturer's instructions. Quantitative SYBR Green PCR was performed on a LightCycler®480 II System (Roche) to quantify human *PAX5-ELN* and murine *Pax5*, *CD19* and *CD79a* cDNAs expression in purified pre-proB, pro-B, pre-B and immature-B cells from the BM of 30 days old wt and pre-leukemic *PE*^{tg} mice using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH) according to the manufacturer's instructions. Targets and primers are listed in **SI Appendix, Table S5**. All PCR were carried out as follows in a 20 µl volume: 5 min at 95°C, followed by 45 cycles of 10 s at 95°C, 10 s at annealing temperature of 60°C and 10 s at 72°C. Quantification was performed using the Δ*Ct* method with normalization to the *Abl1* gene expression levels. Data were analyzed using the LC480 software (Roche Diagnostics).

Western blot

Protein extraction from splenocytes of 30 days old wt and pre-leukemic *PE*^{tg} mice was performed using RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Sodium Desoxycholate). The concentrations of protein extracts were determined using Bicinchroninic

Acid Kit (Sigma). Proteins were then separated on sodium dodecyl sulfate–PAGE (SDS-PAGE) and transferred on poly-vinylidene difluoride (PVDF) membranes (Millipore Corporation). Immunodetection was revealed using ECL Plus (Amersham Biosciences). The membranes were blocked and the antibodies were diluted in 5% dried milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20). The goat anti-PAX5 (N19, sc-1975) and the rabbit anti-ELN (H300, sc25736) antibodies were obtained from Santa Cruz and diluted 1/500. The secondary antibodies against rabbit IgG(H+L)-HRP conjugate and goat IgG(H+L)-HRP conjugate were obtained from Cell Signaling (Danvers, MA, USA) and diluted at 1/5000.

Culture of BM pro-B cells

Purified pro-B cells were amplified on 30 Gy irradiated OP9 stromal cells in Iscove's Modified Dulbecco's Medium (IMDM; Gibco), supplemented with 5 % Fetal Bovine Serum (FBS) (Stemcell technologies), 2 ng/mL murine IL7 (Peprotech), 0.05 mM β-mercaptoethanol (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), penicillin (100 units/ml) and streptomycin (100 units/ml). Experiments were performed after an initial pro-B-cell expansion on irradiated OP9 cells in the presence of 2 ng/ml IL7. Every 3 days, pro-B-cells were harvested and propagated on fresh OP9 stromal cells.

Statistical analysis

Student's *t* test was used for comparison of quantitative variable (***$p<0.0005$, **$p<0.005$, *$p<0.05$), assuming normality and equal distribution of variance between the different groups analyzed. Survival in mouse experiments was represented with Kaplan-Meier curves (Prism GraphPad).

Supplemental Figure legends

Fig. S1. (**A-B**) The germline transmission of the *PAX5-ELN* insertion and the genotype of mice were determined by PCR using primers that specifically amplify the wild type or the mutant allele.

Fig. S2. Representative immunophenoytpe of B-ALL induced by PAX5-ELN in mouse #83. Total cells from BM, spleen and LN of wt and leukemic PE^{tg} mice were immunophenotyped using the B220, CD19, CD23 and Igκ/λ markers.

Fig. S3. (**A-B**) PAX5-ELN does not affect the hematopoietic compartment distribution during the pre-leukemic phase. Representative pictures of May Grünwald-Giemsa-stained cytospin of BM cells from 30 days old wt and pre-leukemic PE^{tg} mice (A). Cells from lymphoid, erythroid and myeloid lineages were counted (n=300) and the proportion of cell lineages was compared between 30 days old wt and pre-leukemic PE^{tg} BM (B). (**C**) Schematic diagram of B-cell differentiation with representation of cell surface markers and rearrangement status of *Ig* genes that correlate with cell differentiation stages.

Fig. S4. (**A-C**) *PAX5-ELN* allows for normal B-cell maturation but reduces the number of immature-B and mature-B cell population. The size of the wt and PE^{tg} spleens was compared from 30 and 90 days old mice (A). Immunophenotype of splenocytes from 30 days old wt and PE^{tg} mice was performed using B220, CD19, CD23 and Igκ/λ markers (B) and absolute numbers of immature-B and mature-B cells were calculated (C, n=5 to 8 per condition).

Fig. S5. (**A-B**) PAX5-ELN prevents B-cell differentiation of pre-leukemic pro-B cells. Pro-B cells were purified from the BM of 30 days old wt and PE^{tg} mice and expanded *in vitro* in undifferentiated condition on OP9 stromal cells in the presence of IL7 (OP9 IL7+). B-cell differentiation was induced or not (OP9 IL7+) by culturing expanded pro-B cells in the absence of IL7 (OP9 IL7-) during 3 days (A). Igκ and CD2 expression was assessed by flow cytometry. Representative FACS profiles are shown (B) (n=4). (**C-D**) PAX5-ELN does not rescue the B-cell commitment blockade induced by the absence of Pax5. E17.5 Fetal liver cells from $Pax5^{-/-}$ embryos were transduced with either MIE-PAX5 or MIE-PAX5-ELN (MIE-PE) or MIE

retroviral vectors. Transduced cells were then cultured in B-cell differentiation condition (OP9 IL7-) or not (OP9 IL7+) during 3 days (C). The proportion of Kit and CD19 positive cells was calculated by FACS for each condition (D) (n=3).

Fig. S6. (**A**) Pre-pro-B, pre-B, proB and immature-B cells were purified from the BM of 30 days old pre-leukemic *PE*^{tg} mice by cell sorting and mRNA levels of human *PAX5-ELN* in each population were determined by qRT-PCR and normalized to murine *Abl1*. (**B**) mRNA levels of human *PAX5-ELN* (*left panel*) and murine *Pax5* (*right panel*) were determined by qRT-PCR in thymocytes and purified total B-cells from two 30 days old wt and pre-leukemic *PE*^{tg} mice and were normalized to murine *Abl1*. (**C**-**D**) *PAX5-ELN* does not affect T cell development at the pre-leukemic stage. Immunophenotype of thymocytes from 30 days old wt and *PE*^{tg} mice was performed using CD4 and CD8 markers and a gating strategy was applied to discriminate double negative (DN), double positive (DP), simple positive SP4 and SP8 subpopulations (C). Absolute numbers of DN, DP, SP4 and SP8 thymocytes were calculated for each genotype (D, n=4 mice per condition). (**E**) Allelic competition assay showing that the insertion of *PAX5-ELN* cDNA in the *IgH* locus does not impair $V_HD_HJ_H$ gene recombination in *trans*, and leads to normal B cell development driven essentially by IgM from the wt allele. The mutant allele originates from 129Sv and the wt allele from C57BL/6 following breeding of *PE*^{tg} mice with C57BL/6 wt mice. IgM allotypes were determined by FACS using monoclonal antibodies specific for IgMa (129S2/Sv) and IgMb (C57BL/6) allotypes.

Fig. S7. (**A**-**D**) BM transplantation reveals pro-B and pre-B cell expansion induced by PAX5-ELN but does not perturb thymic reconstitution. Pre-leukemic total BM *PE*^{tg} cells (CD45.2$^+$) were intravenously transplanted in recipient mice (CD45.1$^+$, n=5, 5.10^6 cells/mouse) pre-treated with 30mg/kg Busulfan (A). The same number of wt BM cells were transplanted in parallel as control (A, n=2). The percentage of donor-derived cells (CD45.2$^+$) from the BM of recipient mice were analysed by FACS 1 month after transplantation (B). Absolute numbers of donor-derived (CD45.2$^+$) pre-pro-B, pre-B, pro-B, immature-B and mature-B cells in the recipient BM were calculated (C). Absolute number of donor-derived DN, DP, SP4 and SP8 thymocytes were calculated in the thymus of recipient mice (D). (**E**) Fold expansions of donor-derived (CD45.2$^+$) pre-pro-B, pre-B, pro-B, immature-B and mature-B cells in the BM of recipient mice were

calculated after transplantation of total B-cells from wt and pre-leukemic PE^{tg} mice described in Fig. 3*C*. (**F**) The presence of *Ptpn11*, *Kras*, *Pax5* and *Jak3* mutations were analysed by targeted NSG in the BM of recipient mice transplanted with total B-cells from wt and pre-leukemic and B-ALL PE^{tg} mice (n=3 for each condition). Each colum represents a leukemia sample and each raw a genetic alteration. (**G-H**) Immunophenotype of engrafted B-cells ($CD45.2^{+}B220^{+}CD19^{+}$) in the spleen was monitored after transplantation of total B-cells from wt and pre-leukemic PE^{tg} mice described in Fig. 3*C* (G). Absolute number of engrafted immature-B and mature-B cells in the spleen of recipient mice were calculated (H).

Fig. S8. (**A**) Principal component analysis of varying genes of wt (blue, n=3) and PE^{tg} (red, n=3) pro-B cells. Only the varying genes of each sample were considered to calculate the principal components. (**B**) Gene set enrichment analysis (GSEA) of the well-established list of *in vivo* PAX5 regulated genes (8) with our new *ex vivo* PAX5 gene signature in pro-B cells revealing that many *in vivo* PAX5 target genes were similarly regulated in *ex vivo* PAX5 transduced pro-B cells. (**C**) Absence of a general dominant-negative effect of PAX5-ELN on established *in vivo* PAX5 regulated genes in pro-B cells (8). Comparison of PAX5-activated and PAX5-ELN-repressed genes (*left panel*) and of PAX5-repressed and PAX5-ELN-activated genes (*right panel*) in pre-leukemic pro-B cells. Overlap indicates that two genes were activated by PAX5 and repressed by PAX5-ELN and 13 genes of the PAX5-repressed genes were activated by PAX5-ELN as represented by colored bars. (**D**) Pre-leukemic pro-B cells were purified from the BM of three 30 days old wt and three pre-leukemic PE^{tg} mice and mRNA levels of human *PAX5-ELN,* murine *Pax5* and the Pax5 target genes *CD19* and *CD79a* were determined by qRT-PCR and normalized to murine *Abl1*. (**E-F**) PAX5-ETV6 and PAX5-FOXP1 do not antagonize normal PAX5 gene signature. Comparison of PAX5-activated and PAX5-ETV6-repressed genes (E, *left panel*) and of PAX5-repressed and PAX5-ETV6-activated genes (E, *right panel*) in pro-B cells (9). Overlap indicates that 11 genes were activated by PAX5 and repressed by PAX5-ETV6 and 16 genes of the PAX5-repressed genes were activated by PAX5-ETV6 as represented by colored bars (E). Comparison of PAX5-activated and PAX5-FOXP1-repressed genes (F, *left panel*) and of PAX5-repressed and PAX5-ETV6-activated genes (F, *right panel*) in pro-B cells (9). Overlap indicates that 19 genes were activated by PAX5 and repressed by PAX5-FOXP1 and 17 genes of the PAX5-repressed genes were activated by PAX5-FOXP1 as represented by colored bars (F).

Fig. S9. (**A**) PAX5-ELN-modified genes that were included in PAX5-ETV6 and in PAX5-FOXP1 signatures were listed according to their biological functions as signal transducers, secreting proteins and surface receptors. (**B**) Venn diagram indicating the overlap between PAX5-ETV6-modified genes in B-ALL (fold change >5) and in pre-leukemic pro-B (fold change >3) cells and with PAX5-ELN-modified genes in pre-leukemic pro-B cells (fold change >2) (*left panel*). PAX5-ELN-modified genes that were included in B-ALL but not in pre-leukemic PAX5-ETV6 signatures (9) were listed (*right panel*).

Table S1. Targeted next generation sequencing (NGS) of the indicated exons of *PTPN11*, *KRAS*, *NRAS*, *JAK3*, *JAK2* and *PAX5* genes were performed on 101 B-ALL patient samples. Pink boxes identify the mutation found and its corresponding variant allele frequency (VAF). NM indicates the absence of mutation.

Table S2. List of genes differentially expressed between purified wt and PE^{tg} pre-leukemic pro-B cells based on three independent microarray experiments. Genes with an expression difference of > 1.5 fold and adjusted *P*-value of < 0.05 are listed in the table.

Table S3. List of genes differentially expressed between E17.5 fetal liver $Pax5^{-/-}$ pro-B cells transduced with either MIE-PAX5 or MIE retroviral vectors based on three independent microarray experiments. Genes with an expression difference of > 3 fold, and adjusted *P*-value of < 0.05 are listed in the table.

Table S4. List of genes overlapping between PAX5-activated and PAX5-ELN-repressed genes and between PAX5-repressed and PAX5-ELN-activated genes using our *ex vivo* PAX5 regulated genes (**A**) ; between PAX5-activated and PAX5-ELN-repressed genes and between PAX5-repressed and PAX5-ELN-activated genes using the well-established *in vivo* PAX5 gene signature (8) (**B**) ; between PAX5-activated and PAX5-ETV6-repressed genes and between PAX5-repressed and PAX5-ETV6-activated genes using our *ex vivo* PAX5 regulated genes (**C**); between PAX5-activated and PAX5-FOXP1-repressed genes and between PAX5-repressed and

PAX5-FOXP1-activated genes using our *ex vivo* PAX5 regulated genes (**D**) in pre-leukemic pro-B cells.

Table S5. Sequences of oligonucleotide primers used for qPCR to detect V(D)J gene rearrangement of the *IgH* locus ; for the resequencing of *Jak3*, *Kras*, *Pax5* and *Ptpn11* regions by next generation sequencing in murine and *PAX5*, *PTPN11*, *KRAS*, *NRAS*, *JAK3* and *JAK2* regions in human B-ALL samples ; for gene expression in RT-qPCR.

References associated with supplementary information

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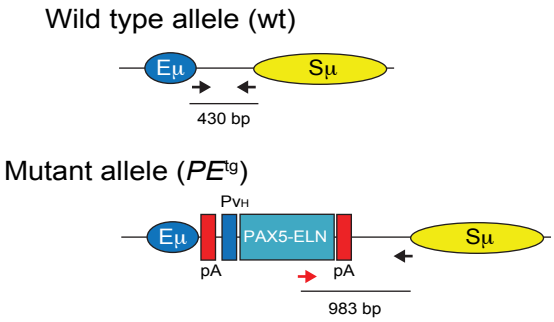
Supplemental Figure 1

A



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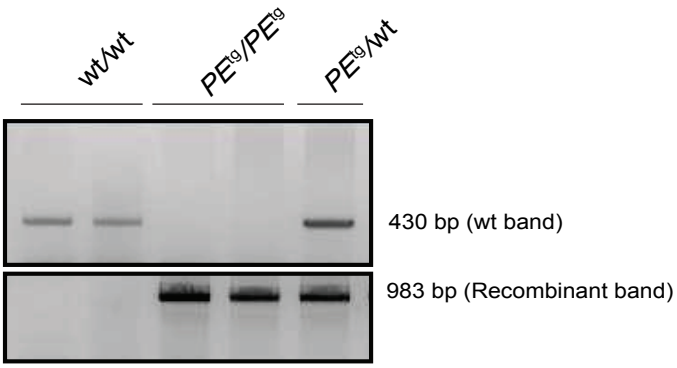

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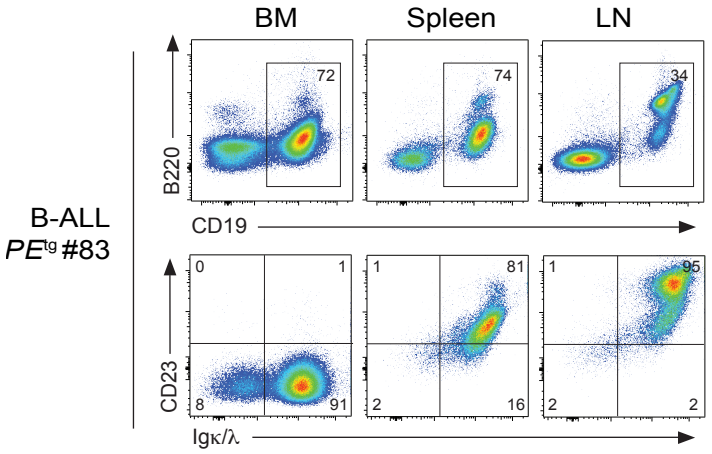

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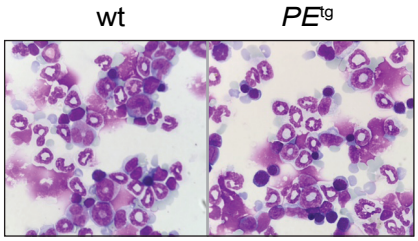

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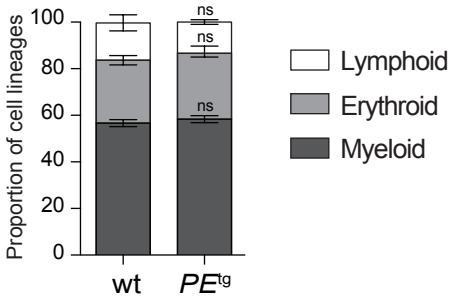

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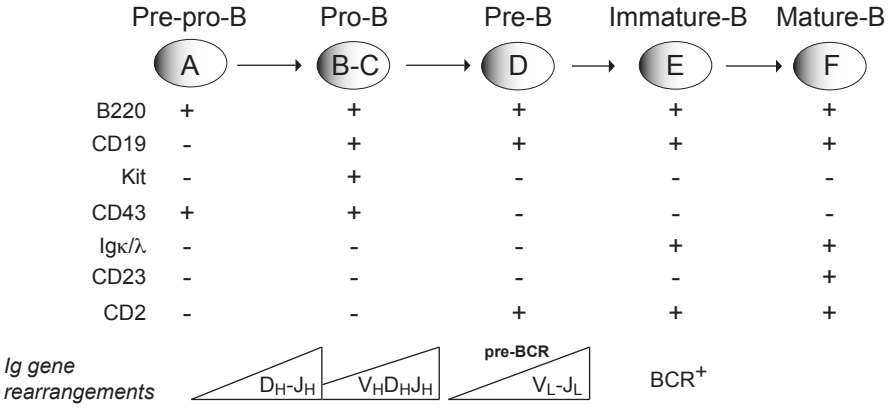

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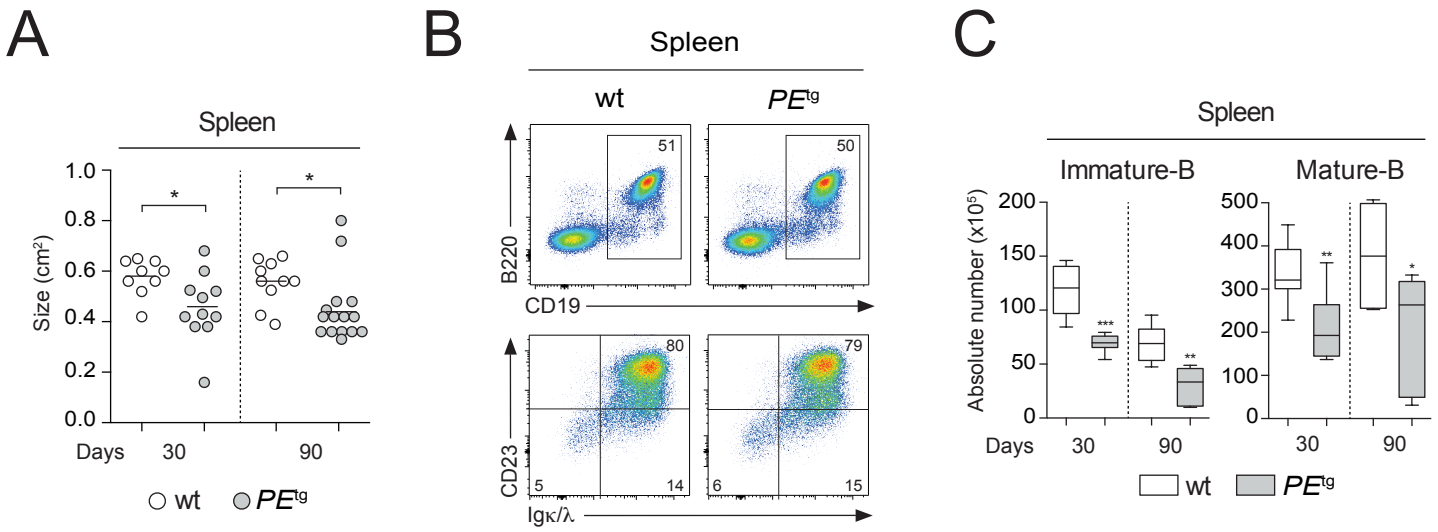

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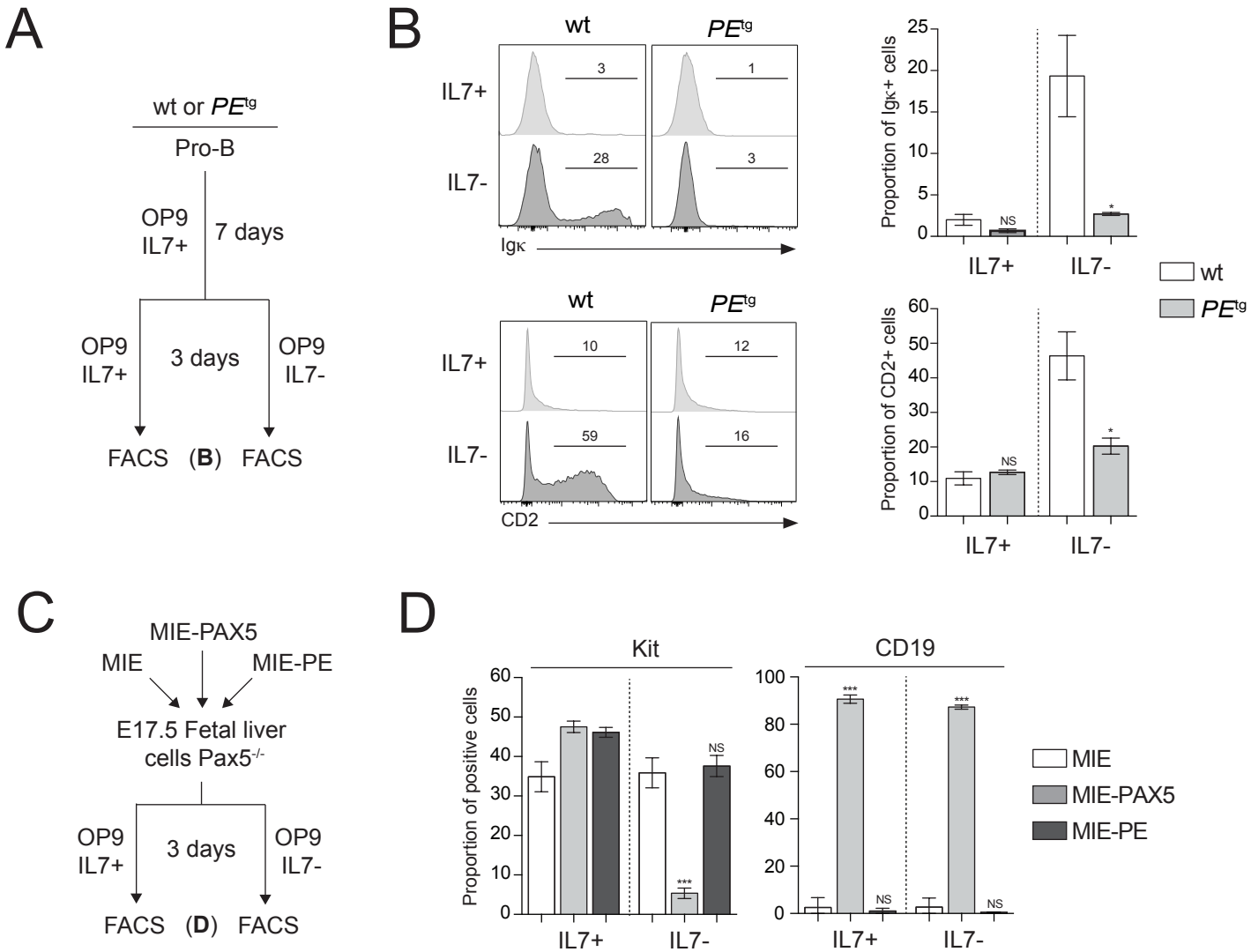

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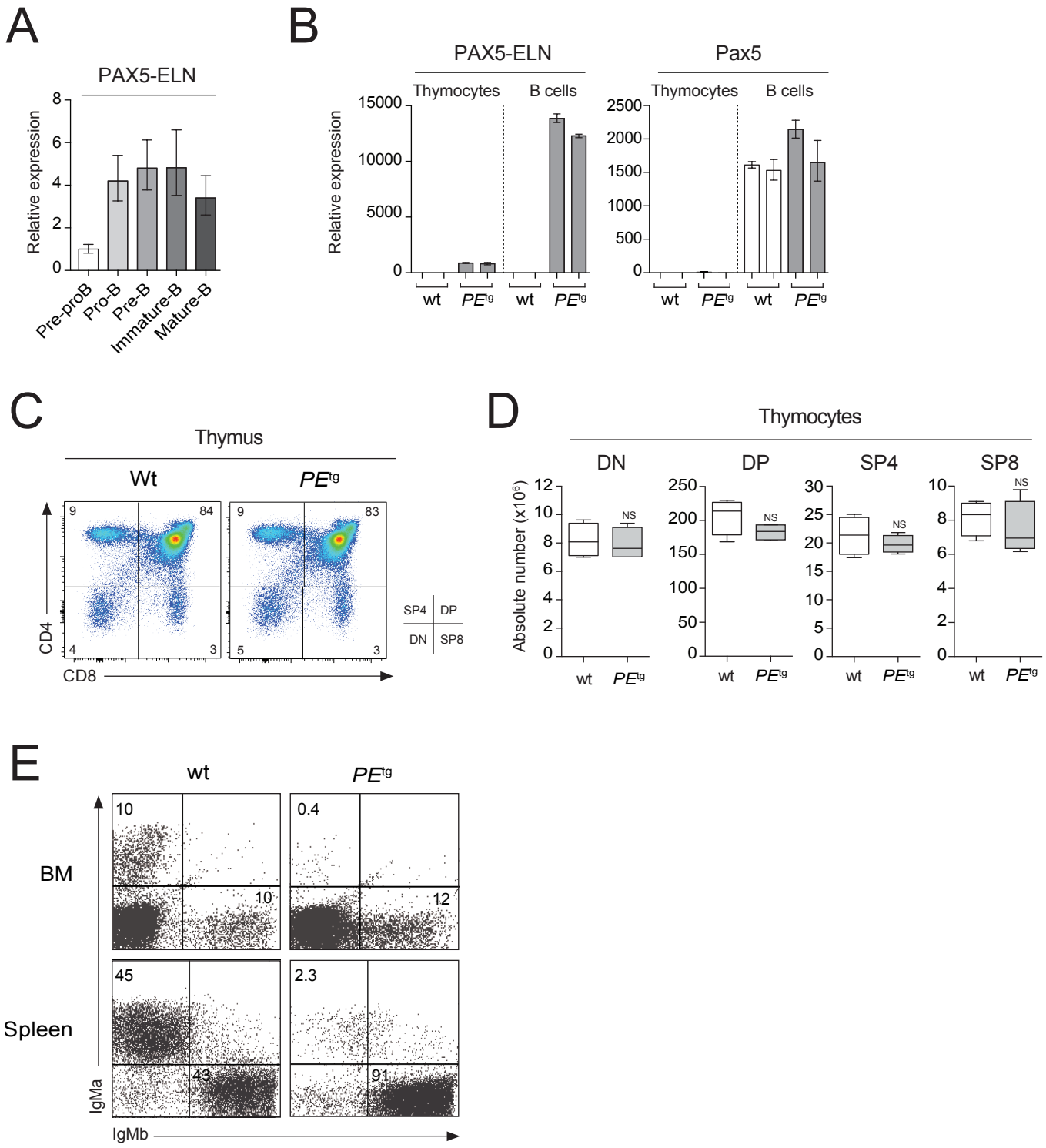

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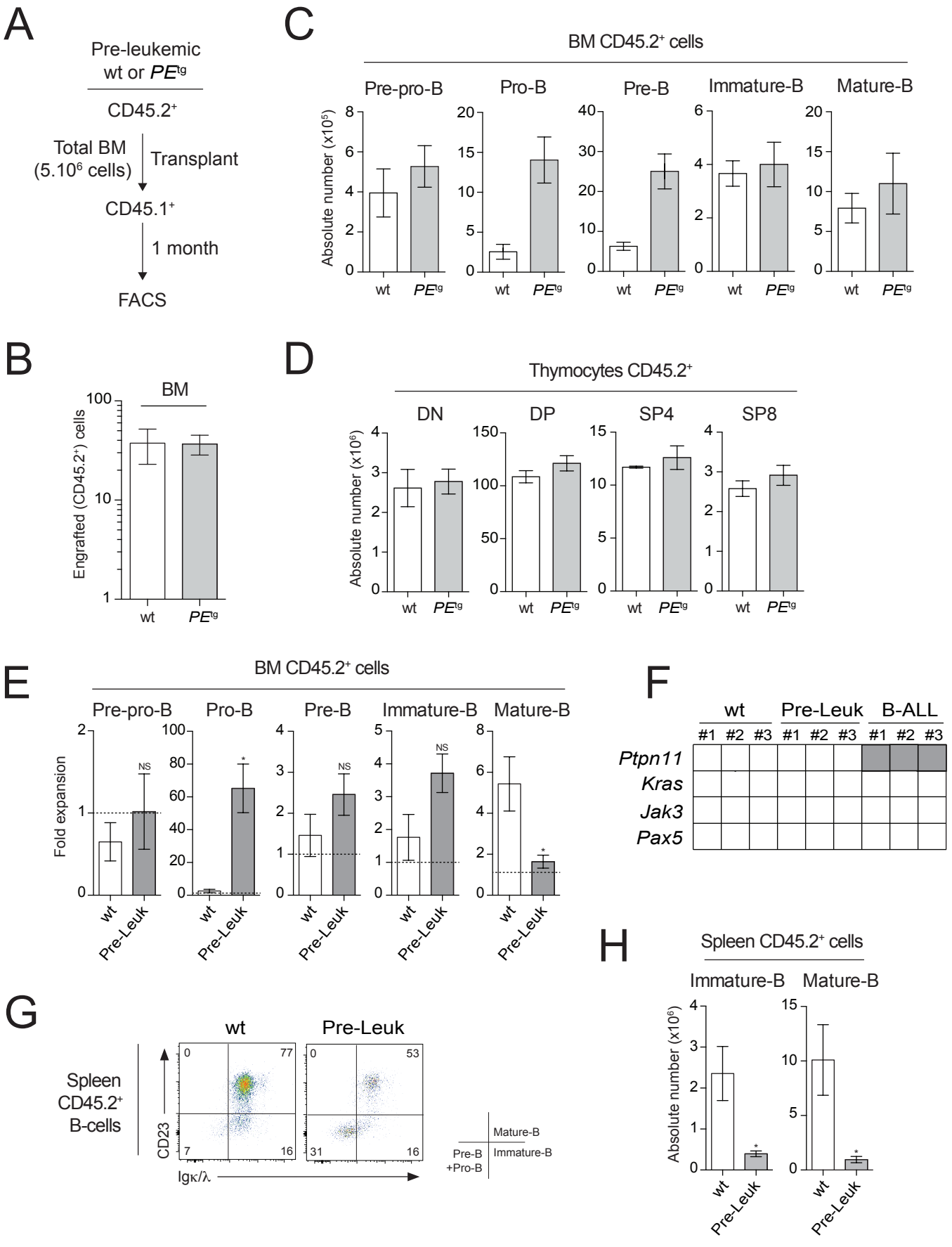

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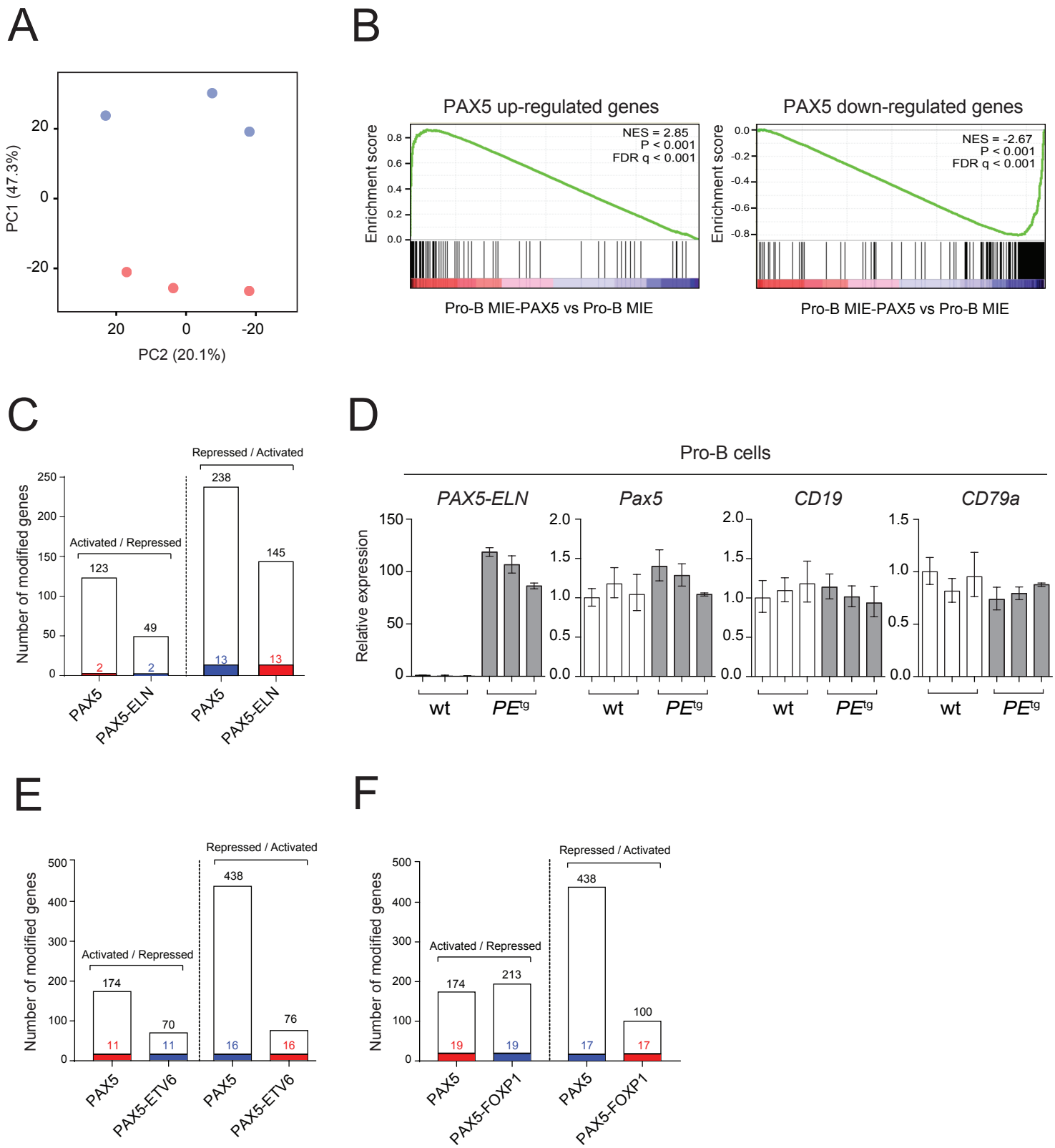

Supplemental Figure 9

A

Signal transducers	Secreted proteins	Surface receptors	Other functions
Gimap3	Sema3g	Il2ra Trem1	Pcdh9 Ly6d

B