Supplementary Materials for

Pax5 regulates B cell immunity by promoting PI3K signaling via PTEN down-regulation

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- Tables S1 to S7
Materials and Methods

Mice
The following mice were maintained on the C57BL/6 genetic background: Pax5+/– (19), Pax5fl/fl (9), Pax5Bio/Bio (14), Cd19Cre/+ (59), Ptenfl/fl (70), Dicer1fl/fl (71), miR-29a/b-1−/− (46), transgenic Cd23-Cre (18), transgenic Aicda-Cre (18) and transgenic Vav-Bcl2 (21) mice. The Cd19Cre/+ genotype is also referred to as Cd19-Cre or Cd19+/– in this manuscript. As Cd19-Cre Pax5fl/– mice are known to develop tumors with a median survival of 8 months and an emergence of the first tumors at 4 months (15), we performed all experiments with Cd23-Cre Pax5fl/– mice at an age of up to 17 weeks, before these mice developed any tumor. All animal experiments were carried out according to valid project licenses, which were approved and regularly controlled by the Austrian Veterinary Authorities.

Antibodies
The following monoclonal antibodies were used for flow-cytometric analysis of lymphoid organs from 6-12 week-old mice: B220/CD45R (RA3-6B2), CD1d (1B1), CD4 (GK1.5), CD5 (53-7.3), CD8a (53-6.7), CD19 (1D3), CD21/CD35 (7G6), CD22 (Cy34.1), CD23 (B3B4), CD25/IL-2Rα (PC61), CD28 (37.51), CD38 (90), CD49b (DX5), CD95/Fas (Jo2), CD138 (281-2), CD169 (MOMA-1), CD267/TACI (8F10), F4/80 (CI:A3-1), Gr1 (RB6-8C5), GL7 (GL-7), IgD (11-26c), IgG1 (A85-1) and IgM (II/41) antibodies.

The anti-Pax5 antibody directed against amino acids 17-145 (80), monoclonal anti-Hsp90 antibody (68/Hsp90; BD Biosciences), PTEN (138G6, Cell Signaling Technology) and anti-GAPDH (14C10; Cell Signaling Technology) were used for immunoblot analysis (Figs. S1B and S7B). The following antibodies were used for intracellular staining and phospho-specific flow cytometry: Pax5 (D19F8; Cell Signaling Technology), IκBa (rabbit polyclonal Ab; Cell Signaling Technology), Myc (D84C12; Cell Signaling Technology), PTEN (138G6; Cell Signaling Technology), phospho-AKT (p-Ser473) (D9E; Cell Signaling Technology), phospho-AKT (p-Thr308) (D25E6, Cell Signaling Technology), phospho-BLNK (p-Tyr84) (J117-1278; BD Biosciences), phospho-PLCγ2 (p-Tyr759) (K86-689.37; BD Biosciences), phospho-SYK (p-Tyr525/526) (C87C1; Cell Signaling Technology), phospho-FOXO1 (p-Thr24)/phospho-FOXO3 (p-Thr32) (rabbit polyclonal Ab; Cell Signaling Technology), phospho-S6 ribosomal protein (p-Ser240/244) (D68F8, Cell Signaling Technology) and phospho-4E-BP1 (p-Thr37/46) (236B4, Cell Signaling Technology).

Definition of cell types by flow cytometry
The different hematopoietic cell types were identified by flow cytometry or sorted with a FACS Aria machine (Becton Dickinson) as follows: pre-B (CD25+Kit+B220+CD19+IgM−IgD−), splenic immature B (B220+CD19+CD21−CD23−), mature B (B220+CD19+IgMhiIgDlo), MZ B (B220+CD19+CD21hiCD23lo/− or B220+CD19+CD23lo/−CD1dhi[TACI+]), FO B (B220+CD19+CD21hiCD23hi), Pax5 mutant FO B (B220+CD19+CD21hiCD23hi), GC B (B220+CD19+Fas−PNA− or B220+CD19+Fas−GL7+), NP-IgG1+ GC B (NP+IgG1+CD38loCD19−
B220⁺Lin⁻), NP-IgG1⁺ memory B cells (NP⁺IgG1⁺CD38hiCD19⁺B220⁺Lin⁻), plasma cells (CD138hiCD28⁺Lin⁻), peritoneal B-1a cells (IgMhiCD5⁺) and total B cells (CD19⁺B220⁺). Lineage-positive cells (Lin⁺) cells were electronically gated away with the following lineage (Lin) marker antibodies: CD4, CD8a, CD21, CD49a, F4/80 (for plasma cells) and IgM, IgD, CD138, Gr1 (for NP-IgG1⁺ memory and GC B cells). NP⁺ B cells were stained with NP-tetraethylene glycol-biotin (Biosearch Technologies), followed by detection with PE/Cy5-streptavidin (BioLegend). PNA⁺ GC B cells were stained with biotinylated PNA (B-1075, Vector Laboratories), which was detected with FITC-streptavidin.

Intracellular staining and phospho-specific flow cytometry

Intracellular staining for detecting the Pax5, Myc and IκBα protein was performed after fixation-permeabilization with the Foxp3 Staining Buffer Set (eBioscience). Analysis of phosphorylated signaling components (Figs. 4B,C and 6A) was performed with mature CD43⁻ B cells from lymph nodes, which were enriched by immunomagnetic depletion with CD43-MicroBeads (Milteny Biotec), followed by resuspension in RPMI-1640 medium (Gibco), incubation for 15 min at 37 °C and subsequent stimulation for 5 min with 10 μg/ml goat anti-mouse IgM F(ab')₂ fragment (Jackson ImmunoResearch Laboratories) or 15 min with 1 μM CpG (ODN 1826, InvivoGen). The stimulated cells were mixed with an equal volume of Fixation buffer (BD Cytofix) and fixed for 15 min at 37 °C. Cells were washed with Perm/Wash Buffer I (BD Phosflow) and stained in this buffer with antibodies detecting phosphorylated signaling components or PTEN for at least 1 h at 23 °C at the dilution recommended by the manufacturer. The phosho-specific antibodies used are described under ‘Antibodies’.

For the phospho-specific flow-cytometric analysis shown in Figs. 7A and S6E, CD43⁻ lymph node cells were resuspended in RPMI medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 50 μM β-mercaptoethanol followed by incubation for 1 h at 37 °C and 5% CO₂ before stimulation. Non-stimulated cells and cells stimulated with 10 μg/ml goat anti-mouse IgM F(ab')₂ fragment or 2 μM CpG (ODN 1826) were fixed and permeabilized with 1.5% paraformaldehyde in saponin-containing Perm/Wash buffer (BD Biosciences) for 15 min at room temperature followed by 30 min to 1 h on ice. Cells were then washed with Perm/Wash buffer and stained with phospho-specific antibodies. The following modification were used for the phosphorylation analysis of 4E-BP1 and S6 (Fig. S4B). 2% heat-inactivated fetal bovine serum was used, B cells were fixed on ice (unstimulated control) or stimulated with goat anti-mouse IgM F(ab')₂ fragment and incubated for 15 min at 37 °C prior to fixation with eBioscience™ IC Fixation Buffer (Thermo Fisher Scientific).

Calcium fluorimetry

Mature CD43⁻ B cells (2 x 10⁶) from the lymph nodes were loaded with the calcium-sensor dye eFluor 514 (eBioscience) at a final concentration of 1 μM in 1 ml of IMDM medium containing 10% fetal calf serum, 1 mM glutamine and 50 μM β-mercaptoethanol. After incubation for 45 min at 37 °C, the cells were washed twice and the fluorescence emission at 530/30 nm (excitation at 488 nm) was measured in live cells on a Fortessa flow cytometer. The
acquisition of data was initiated 50 s before the addition of the anti-IgM antibody (goat anti-
mouse IgM F(ab')2 fragment; Jackson ImmunoResearch Laboratories) at a concentration of 1
μg/ml. Data were collected for 100 s before the addition of ionomycin (0.6 μg/ml) and
acquisition for another 60 s. The data were analyzed with FlowJo software. The percentage of
fluorescent increase (ΔF/F₀) is plotted against time (t) after stimulation (Fig. 4D). F₀ refers to
the average fluorescence determined between 0 and 50 s prior to antibody addition, and ΔF
corresponds to the fluorescence F(t) (measured at time ‘t’) minus F₀.

**BrdU and EdU labeling of B cells**

*Cd23-Cre Pax5^fl/−* and *Cd23-Cre Pax5^fl/+* mice were intraperitoneally injected with 100 μl of
10 mg/ml BrdU (in PBS) at day 0. At the same time, BrdU was added at a concentration of 1
mg/ml to the drinking water, and the BrdU-containing drinking water, which was protected
from light, was exchanged every day. At day 10, the mice were either sacrificed or received
normal drinking water (without BrdU) for the next 15 days. At day 10 or 25, the BrdU
incorporation into immature and FO B cells of the spleen and FO B cells of the lymph nodes
was analyzed by flow cytometry. Incorporated BrdU was detected by intracellular staining with
an anti-BrdU antibody using the APC BrdU Flow Kit (BD Pharmingen).

For EdU labelling, mice were intraperitoneally injected with 100 μl of 10 mg/ml EdU (in
PBS) and were sacrificed after 2 h to determine the EdU incorporation into pre-B, immature B
and FO B cells by flow-cytometric analysis, using the Click-iT™ Plus EdU Alexa Fluor™ 488
Flow Cytometry Assay Kit (Thermo Fisher Scientific).

**In vitro B cell stimulation**

CD43− FO B cells were enriched from lymph nodes of *Cd23-Cre Pax5^fl/−* and *Cd23-Cre
Pax5^fl/+* mice by immunomagnetic depletion of non-B cells with CD43-MicroBeads (Miltenyi
Biotec). The sorted cells were labeled with 5 μM CellTrace™ Violet dye (Thermo Fisher
Scientific) in PBS for 20 min at 37 °C. After washing with stimulation medium (RPMI-1640
supplemented with 10% heat-inactivated FCS, 1 mM glutamine and 50 μM β-
mercaptoethanol), the cells were seeded at a density of 1 × 10⁶ cells in 2 ml of stimulation
medium containing 1 μg/ml anti-IgM antibody (goat anti-mouse IgM F(ab')2 fragment; Jackson
ImmuoResearch Laboratories) plus 10 ng/ml IL-4 or 2 μg/ml anti-CD40 (HM40-3, eBioscience) plus 10 ng/ml IL-4 or 0.2 μM CpG (ODN 1826, InvivoGen) or 25 μg/ml LPS. At
day 3 or 4, the cell numbers were measured in a CASY cell counter, and the cells were stained
with the Viability Dye eFluor™ 780 (Thermo Fisher Scientific), and the proliferation and/or
IgG1 class switching of the stimulated B cells was assessed by flow-cytometric analysis. The
proliferation index of the stimulated B cells was calculated with the FlowJo’s Proliferation tool.

**Immunization, ELISPOT and ELISA analyses**

Sheep red blood cells (SRBC) were washed in PBS and resuspended at 10⁹ cells/ml followed
by intraperitoneal injection of 100 μl into an adult mouse. The immune response to a specific
antigen was studied by intraperitoneal injection of 100 µg of 4-hydroxy-3-nitrophenylacetyl-conjugated keyhole limpet hemocyanin (NP-KLH) in alum.

The frequencies of NP-specific IgG1 antibody-secreting cells (ASCs) were determined in the spleen by enzyme-linked immunospot (ELISPOT) assay as described (72). NP4-BSA- and NP23-BSA-coated plates were used for capturing high-affinity or total NP-specific IgG1 antibodies secreted by individual cells, respectively. Spots were visualized with goat anti-mouse IgG1 antibodies conjugated to alkaline phosphatase (Southern Biotechnology Associates), and color was developed by the addition of BCIP/NBT Plus solution (Southern Biotechnology Associates). After extensive washing, the spots were counted with an AID ELIspot reader system (Autoimmun Diagnostika).

The serum titer of NP-specific IgG1 antibodies was determined by enzyme-linked immunosorbent assay (ELISA) (72) by using plates, which were coated with 25 µg/ml of NP7-BSA or NP30-BSA to capture high-affinity or total NP-specific IgG1 antibodies, respectively. The serum concentration of NP-specific IgG1 was determined relative to that of a standard NP-specific IgG1 antibody (hybridoma SSX2.1).

**Immunohistological analysis**

Cryosections of the spleen from unimmunized mice were stained with a FITC-anti-IgM antibody (II/41; eBioscience) and a biotinylated CD169 (MOMA-1) antibody (Fig. 1C), and cryosections of the spleen from immunized mice (Fig. 2C,F) were stained with a FITC-anti-IgD (11-26c.2a; BD Biosciences) or FITC-anti-B220 (RA3-6B2; eBiosciences) antibody in combination with biotinylated PNA (B-1075, Vector Laboratories). FITC-labeled antibodies were detected with an alkaline phosphatase-coupled anti-FITC antibody (Roche), which was visualized by incubation with Fast Blue (Sigma). Biotinylated PNA and the biotinylated MOMA-1 antibody were detected with horseradish peroxidase-conjugated streptavidin (BD Pharmingen) followed by incubation with DAB (Sigma).

For the staining shown in Fig. 8A, the spleens from unimmunized mice were fixed with 4% paraformaldehyde in PBS for 1 h, cryopreserved with 30% sucrose in PBS overnight and embedded in Tissue-Tek® O.C.T.™ Compound (Sakura). For immunofluorescence staining, 10 µm cryosections were fixed with cold acetone, hydrated in PBS and blocked with 5% BSA in PBS for 30 min. Sections were stained for 2 h with Alexa Fluor® 647-anti-Pax5 (1H9; BD Biosciences), Brilliant Violet 421™-anti-mouse TCRβ (H57-597; Biolegend), FITC-anti-IgM antibody (II/41; eBioscience) and biotinylated anti-CD169 (MOMA-1, Abcam) diluted in 1% BSA in PBS. Finally, the sections were incubated with Cy3-Streptavidin (Jackson ImmunoResearch) diluted with 1% BSA in PBS and mounted with ProLong™ Diamond Antifade Mountant (Invitrogen). Sections were imaged using an inverted point laser scanning confocal microscope LSM880 (ZEISS).

**Quantification of the MZ B cell layer on histological sections**

For quantification of MZ B cells, images (1024 × 1024 pixels) were acquired using a 20x/0.8 plan-apochromat lens at zoom factor 1 (415 nm/pixel) on the confocal microscope LSM880.
(ZEISS). Images were acquired with identical settings for laser power, detector gain and amplifier offset, with pinhole diameters set for 1 airy unit. Based on the MOMA-1 signal, the marginal zone area was defined as the area outside of the MOMA-1⁺ macrophage ring. MZ B cells were identified by their IgM signal in the marginal zone area and were quantified using a processing pipeline developed in ImageJ (https://imagej.nih.gov/ij/). IgM bright plasma cells were excluded from the analysis. The average number of MZ B cells outside of the MOMA-1⁺ macrophage ring was determined per 10 µm of length of the MOMA-1⁺ ring. The analysis was performed with three independent experiments, each of which contained spleen sections of the different genotypes. Each dot in Figs. 8B and S7H represents the measurement of one lymphoid follicle.

**RT-qPCR analysis**

CD43⁻ FO B cells were enriched from lymph nodes by immunomagnetic depletion of non-B cells with CD43-MicroBeads (Miltenyi Biotec). RNA was extracted using the Trizol reagent (Thermo Fisher Scientific), and residual DNA was eliminated with the DNA-free Kit (Ambion). Reverse transcription was performed with random primers and the SuperScript III First-Strand Synthesis System (Invitrogen). RT-PCR was performed on a CFX96 Real-Time System (Bio-Rad) with a Luna Universal qPCR master mix (New England Biolabs). The *Pten* mRNA expression was normalized relative to the *Tbp* mRNA. The PCR primer sequences were: *Tbp* (5’-TTCGTGCAAGAATGCTGAA-3’ and 5’-CAGTTGTCCGTGCGTCTCT-3’) and *Pten* (5’-GAATTGCTGCAACATGATTGTCA-3’ and 5’-CCTTTTGAAGACCATAACCACC-3’).

**ChIP- and DHS-sequencing**

CD43⁻ FO B cells were enriched from lymph nodes of *Cd23-Cre Pax5fl/fl* and *Cd23-Cre Pax5fl/+* mice and stimulated with anti-CD40 and IL-4 for 2 days. These activated B cells were used for ChIP-seq analysis with an anti-H3K4me2 antibody (07-030, EDM Millipore) and for genome-wide mapping of DNase I hypersensitive sites (DHS-seq), as described (12). Bio-ChIP-seq analysis of Pax5-binding was performed with CD43⁻ FO B cells from lymph nodes of *Pax5Bio/Bio* mice (14), which were stimulated with anti-CD40 and IL-4 for 2 days prior to chromatin precipitation by streptavidin pulldown (Bio-ChIP), as described (12).

**Small-RNA-sequencing**

FO B cells were isolated as CD23⁺ B cells from the spleen or lymph nodes of *Cd23-Cre Pax5fl/fl* and *Cd23-Cre Pax5fl/+* FO B cells by staining with a PE-conjugated anti-CD23 antibody and subsequent immunomagnetic sorting with Anti-PE-MicroBeads (Miltenyi). Total RNA was extracted using the TRIzol reagent (Ambion), phenol-chloroform extraction and isopropanol precipitation. Small-RNA libraries were generated with > 20 µg of total RNA, as described (36). Briefly, the total RNA was size-selected on a 15% denaturing urea polyacrylamide gel (SequaGel, National Diagonstics) for RNA molecules of a 18-30 nucleotide length, which were then eluted from the gel in 0.3 M NaCl, 0.1% SDS overnight. After ethanol precipitation and
verification of the correct size range of the small RNA by using a fragment analyzer, the 3’
linker was ligated and the ligated small RNAs were again size-selected on a 15% denaturing
urea polyacrylamide gel followed by ligation of the 5’ linker and size selection of 60-100
nucleotide long RNA. The linker-ligated small RNA was then reversely transcribed into cDNA
followed by PCR amplification with the KAPA Real Time Amplification kit (KAPA HiFi
HSRM; KAPA Biosystems), using the TruSeq Universal adapter and Index reverse primers
(Solexa). The PCR products were gel-purified on a 2% low-range ultra-pure agarose gel and
eluted using the ZymocleanTM Gel DNA Recovery Kit (Zymo Research). The purified DNA
was subjected to deep sequencing, using the HiSeq system with a read length of 50 nucleotides
(Table S6).

cDNA preparation for RNA-sequencing
All RNA-seq experiments were performed with CD43− FO B cells, which were enriched from
the lymph nodes of Cd23-Cre Pax5fl/fl− and Cd23-Cre Pax5fl/fl+/ mice by immunomagnetic
depletion of non-B cells with CD43-MicroBeads. The CD43− FO B cells were either left
untreated or stimulated for 60 min with 10 µg/ml of a goat anti-mouse IgM F(ab’)2 fragment
(Jackson ImmunoResearch Laboratories) or were stimulated with anti-CD40 (2 µg/ml) plus IL-
4 (10 ng/ml) for 2 days. RNA was isolated with a RNeasy Plus Mini kit (Qiagen), and mRNA
was obtained by poly(A) selection with a Dynabeads mRNA purification kit (Invitrogen),
followed by fragmentation by heating at 94 °C for 3 min (in fragmentation buffer). The
fragmented mRNA was used as template for first-strand cDNA synthesis with random
hexamers and the Superscript VILO cDNA Synthesis kit (Invitrogen). The second-strand
cDNA was synthesized with 100 mM dATP, dCTP, dGTP and dUTP in the presence of RNase
H, E. coli DNA polymerase I and DNA ligase (Invitrogen).

Library preparation and Illumina deep sequencing
About 1-5 ng of cDNA, (Bio-)ChIP-precipitated DNA or DHS-enriched DNA were used as
starting material for generating sequencing libraries with the NEBNext Ultra Ligation Module
and NEBNext End Repair/dA-tailing module, as described (73). For strand-specific RNA-
sequencing, the uridines present in one cDNA strand were digested with uracil-N-glycosylase
(New England BioLabs) followed by PCR amplification with the KAPA Real Time
Amplification kit (KAPA Biosystems). Completed libraries were quantified with the Agilent
Bioanalyzer dsDNA 1000 assay kit and Agilent QPCR NGS Library Quantification kit. Cluster
generation and sequencing was carried out by using the Illumina HiSeq 2000 system with a
read length of 50 nucleotides according to the manufacturer's guidelines (Table S6).

Sequence alignment
ChIP-seq and DHS-seq: Sequence reads that passed the Illumina quality filtering were aligned
to the mouse genome assembly version of July 2007 (NCBI37/mm9) using the Bowtie program
version 12.5 (74). The Pax5 Bio-ChIP-seq data generated with FO B cells stimulated with anti-
CD40 plus IL-4 for 2 days were trimmed to 36-mers and aligned as single-end sequences, to
make them comparable with the already published mature B cell ChIP-seq data (GSM932925 to GSM932931) (12).

RefSeq: Sequence alignment as well as the database generation of RefSeq-annotated genes was performed as previously described (81). To refine the annotation of immunoglobulin genes, the immunoglobulin λ light-chain segments were replaced with their corresponding converted GRCm38.p3 annotations (Ensembl version 79) (82). The resulting number of genes was 24,732.

**Peak calling**

ChIP-seq and DHS-seq: Peaks were called using the MACS program version 1.3.6.1 (75) with default parameters, a read length of 36 or 76, and a genome size of 2,654,911,517 bp (mm9). For the analysis of DHS data, the read-shifting model building was turned off. Peaks were further filtered for \( P \) values of \(< 10^{-10} \).

**Peak-to-gene assignment**

Pax5 target genes were identified by peak-to-gene assignment as described (12). Peaks were assigned to genes in a stepwise manner by prioritizing genes containing peaks in their promoter and/or gene body. For this, peaks overlapping with the promoter (-2.5 kb to +2.5 kb relative to the TSS) or gene body (including +2.5 kb past the TES) were first assigned to the corresponding gene. Other peaks within a specified region of 50 kb upstream of the TSS or downstream of the TES were assigned to the gene containing peaks in the promoter or gene body. All other peaks within the same specified region were assigned to the nearest gene, and all non-assigned peaks were classified as intergenic.

**Motif discovery analysis**

All peak sequences of the Pax5-Bio-Chip-seq data shown in Fig. S3B were searched for the presence of the Pax5 motif (12) by using the MotifLocator program (83), with the threshold parameter (-t) set to 0.75 such that no more than 20% of random sequences obtained a hit. Random sequences were constructed by using DNA sequences 10 kb downstream of each peak, and subsequent shuffling was performed with emboss_6.5.7 shuffleseq (84).

**Analysis of RNA-seq data**

For analysis of differential gene expression, the number of reads per gene was counted using HTseq version 0.6.1p1 (76) with the overlap resolution mode set to ‘union’. The datasets were grouped according to genotype and type of stimulation and were analyzed using the R package DESeq2 version 1.8.2 (77). Sample normalizations and dispersion estimations were conducted using the default DESeq2 settings. Variance-stabilizing transformations were computed with the blind option set to ‘FALSE’. Variance-stabilized counts were transformed from \( \log_2 \) to \( \log_{10} \) scale for the scatterplots shown in Figs. S3E and S5A,D. The default DESeq2 pairwise setup (model design formula: ‘~ genotype’; Wald test) was used for comparison of the RNA-seq data of knockout (Cd23-Cre Pax5flo/flo) versus control (Cd23-Cre Pax5flo/+)) activated B cells stimulated for 2 days with anti-CD40 and IL4.
Untreated and 1-hour anti-IgM-stimulated knockout and control lymph node B (LN B) cells were analyzed, considering the main effects of the genotype (I, II) and time (III, IV) as well as their ‘interaction’ effect (V; see Fig. S5E), which quantifies if the time effect differs across genotype (model design formula: “~ genotype + time + genotype:time”; Wald tests). If not mentioned otherwise, genes with an adjusted $P$ value $< 0.05$ and an absolute fold change $> 3$ as well as a mean TPM (averaged within conditions) $> 5$ were called as significantly regulated. Immunoglobulin and T cell receptor genes were filtered from the list of significantly regulated genes, but were included in the TPM (transcripts per million) calculations.

**Classification of Pax5 (in-)dependent immediate early genes upon anti-IgM stimulation**

Genes, which were significantly $> 3$-fold upregulated in control LN B cells after one hour of anti-IgM stimulation compared with unstimulated control LN B cells (III; Fig. S5E), were chosen for further classification. For those 636 genes, Pax5 dependency (Fig. S5F) was assumed if the following two criteria were met: (1) a significant $> 2$-fold downregulation in stimulated knockout compared with stimulated control B cells (II; Fig. S5E) and (2) a significant $> 1.5$-fold downregulation of the interaction effect (V; Fig. S5E).

Pax5 independency was determined by providing DESeq2 with log$_2$ fold change thresholds for construction of Wald tests of significance. As the estimated absolute log$_2$ fold changes were tested for undermining the defined thresholds, the function DESeq was called with betaPrior set to ‘FALSE’. For classification as Pax5-independent gene (Fig. S5F), genes needed to meet the following three criteria: (1) $< 2$-fold difference in knockout versus control B cells before stimulation (I), (2) $< 2$-fold difference in knockout versus control B cells after stimulation (II) and (3) $< 1.5$-fold difference of the interaction effect (V; Fig. S5E). For these criteria, an adjusted $P$ value $< 0.05$ was sufficient to be considered (no additional fold change or TPM filter was applied). In addition, many immediate early genes failed to meet the criteria for the definition of a Pax5-dependent or Pax5-independent gene and were therefore referred to as non-classified (Fig. S5F).

**Expression of the constant genes of the Igh locus**

For the analysis of constant genes of the immunoglobulin heavy-chain (Igh) locus, Ighm, Ighd, Ighg, Ighe and Igha were manually curated to provide detailed exon annotations and were split into constant, membrane, secreted, hinge and I regions. This annotation was then used to recount the reads as described above (Analysis of RNA-seq data) and were used for TPM calculations.

**Analysis of the small-RNA-seq data**

Adaptor clipping, sequence alignment against the mouse genome (mm10) and counting of the small-RNA-seq library reads were performed by applying the nextflow pipeline available at https://gitlab.com/tburk/smallRNA-meth (78) with maxMultialign set to 1,000. miRNA annotations were derived from miRBase v22 (79). As the mature form of each miRNA can be of the -3p or -5p type, we defined the mature isoform of each miRNA by its predominance of
> 50 % in all small-RNA-seq samples. Differentially expressed miRNAs were identified by DESeq2 version 1.18.1 (77) with the default DESeq2 pair-wise setup (model design formula: ‘~ genotype’; Wald test).

Total context++ scores were downloaded for miRNA families, which are i) broadly conserved among vertebrates and ii) potentially targeting the *Pten* mRNA according to TargetScanMouse Release 7.2 (37). The total context++ score density for *Pten* targeting miRNAs was calculated to define a conservative threshold (< -0.47; Fig. S6C) for the identification of final *Pten*-targeting miRNAs. Color scales in MA plots (Fig. 6C and S6B) were calculated by multiplying the miRNA family’s total context++ score by the sum of the miRNA family’s expression (DESeq2 baseMean).

**Statistical analysis**

Statistical analysis was performed with the GraphPad Prism 8 software. Two-tailed unpaired Student’s *t*-test analysis was used to assess the statistical significance of one observed parameter between two experimental groups. One-way or two-way analysis of variance (ANOVA) was used, when more than two experimental groups were compared, and the statistical significance was determined with Šídák’s or Tukey’s multiple comparison test. The statistical evaluation of the RNA-seq and small-RNA-seq data is described under ‘Analysis of RNA-seq data’ and ‘Analysis of small-RNA-seq data’.

**Data availability**

RNA-seq, ChIP-seq, DHS-seq and small-RNA-seq data, which were generated for this study (Table S6), are available at the Gene Expression Omnibus (GEO) repository under the accession number GSE103260.
Figure S1. Characterization of mature B cells upon conditional Pax5 loss. (A) Deletion of the floxed Pax5 exon 2 in sorted FO (B220⁺CD21<sup>int</sup>CD23<sup>hi</sup>, fl/+), FO (B220⁺CD21<sup>hi</sup>CD23<sup>hi</sup>, fl/–) and MZ (B220⁺CD21<sup>hi</sup>CD23<sup>lo</sup>/–) B cells from the spleen of Cd23-Cre Pax5<sup>fl/–</sup> (fl/–) and Cd23-Cre Pax5<sup>fl/+</sup> (fl/+), as determined by PCR analysis of genomic DNA. The positions of the PCR fragments corresponding to the deleted (Δ) or intact (fl) floxed Pax5 allele and the wild-type (+) or null (–) Pax5 allele are indicated to the right of the agarose gel. A PCR artifact is denoted by an asterisk. (B) Immunoblot analysis of Pax5 protein expression in whole-cell extracts of FO B cells, which were enriched by MACS depletion of CD43<sup>+</sup> splenocytes from Cd23-Cre Pax5<sup>fl/–</sup> and Cd23-Cre Pax5<sup>fl/+</sup> mice. The expression of Pax5 and Hsp90 was detected with specific antibodies. (C) BrdU labeling of B cells in the spleen (left) and lymph nodes (right) of control Cd23-Cre Pax5<sup>fl/+</sup> mice (upper rows) and experimental Cd23-Cre Pax5<sup>fl/–</sup> mice (lower rows). BrdU was added to the drinking water for 10 days followed by a 15-day chase period without BrdU in the drinking water (see Methods). BrdU<sup>+</sup> immature B and FO B cells of the spleen as well as BrdU<sup>+</sup> FO B cells of the lymph nodes were determined by flow cytometry at day 10 (after continuous BrdU labeling) or day 25 (after the chase period), and the percentage of BrdU<sup>+</sup> cells is shown in the indicated gates. (D) Short-term labeling of B cells by intraperitoneal injection of 5-ethynyl-2'-deoxyuridine (EdU) into Cd23-Cre Pax5<sup>fl/–</sup> (black dot) and control Cd23-Cre Pax5<sup>fl/+</sup> (gray dot) mice. EdU<sup>+</sup> immature and FO B cells from the spleen, EdU<sup>+</sup> FO B cells from lymph nodes and EdU<sup>+</sup> pre-B cells (CD25<sup>+</sup>Kit<sup>–</sup>B220<sup>+</sup>CD19<sup>+</sup>IgM<sup>–</sup>IgD<sup>–</sup>) from the bone marrow were identified by flow-cytometric analysis 2 hours after EdU injection. (E) No rescue of the Pax5 mutant phenotype of FO B cells by transgenic expression of the pro-survival protein Bcl2. The percentage of FO B cells (B220⁺CD21<sup>int/lo</sup>CD23<sup>hi</sup>) was determined by flow-cytometric analysis of the spleen or lymph nodes (LN) from experimental Vav-Bcl2 Cd23-Cre Pax5<sup>fl/–</sup> mice and control Vav-Bcl2 Cd23-Cre Pax5<sup>fl/+</sup> mice. Statistical data (D,E) are shown as average percentages with SEM and were analyzed by the two-tailed unpaired Student’s t-test; **P < 0.01, ****P < 0.0001. Each dot corresponds to one mouse.
Figure S2. Loss of GC and memory B cells upon conditional Pax5 inactivation. (A-C) GC B cell formation in the spleen of Cd19-Cre Pax5^{fl/+} (fl/+) and Cd19-Cre Pax5^{fl/-} (fl/-) mice at 10 days after immunization with sheep red blood cells (SRBCs). GC B cells were detected as Fas^{+}PNA^{+}B220^{+} cells by flow cytometry (A) or as PNA^{+} cells on spleen sections (B) that were stained for expression of IgD (blue) and binding of peanut agglutinin (PNA, brown), as described in the Methods. Note that the GC size and FO B cell area are smaller in Cd19-Cre Pax5^{fl/-} mice compared with control Cd19-Cre Pax5^{fl/+} littermates. (C) Deletion of the floxed Pax5 allele as determined by PCR analysis of genomic DNA isolated from sorted GC B cells of the indicated genotypes. The positions of the PCR fragments corresponding to the deleted (Δ) or intact (fl) floxed Pax5 allele and the wild-type (+) or null (−) Pax5 allele are indicated to the left of the agarose gel. Asterisks denote PCR artifacts. (D) GC B cell differentiation in Aicda-Cre Pax5^{fl/+} and Aicda-Cre Pax5^{fl/-} mice at day 5 and 8 after SRBC immunization. GC B cells were detected by flow-cytometric analysis (left) and visualized as PNA^{+} cells by staining of spleen sections for PNA (brown) and IgD (blue) expression (right). Arrowheads indicate GCs. (E) Deletion of the floxed Pax5 allele in GC B cells sorted from the spleen of Aicda-Cre Pax5^{fl/+} and Aicda-Cre Pax5^{fl/-} mice at day 5 after SRBC immunization. Deletion of the floxed Pax5 allele was detected by PCR, and the positions of the distinct PCR fragments (see C for explanation) are indicated to the left. (F) Loss of GC B cells in Peyer’s patches upon conditional Pax5 inactivation. GC B cells (left) and their absolute numbers (right) were determined by flow-cytometric analysis of Peyer’s patches of unimmunized Aicda-Cre Pax5^{fl/+} (fl/++; gray dots) and Aicda-Cre Pax5^{fl/-} (fl/–; black dots) mice. (G,H) Detection of memory B cells in the spleen of Cd23-Cre Pax5^{fl/+} (fl/++; gray dot) and Cd23-Cre Pax5^{fl/-} (fl/–; black dot) mice at day 14 (G) and 28 (H) after immunization with NP-KLH (in alum). NP-IgG1^{+} memory B cells (NP^{+}IgG1^{+}CD38^{hi}CD19^{+}B220^{+}Lin^{-}) and NP-IgG1^{+} GC B cells (NP^{+}IgG1^{+}CD38^{lo}CD19^{+}B220^{+}Lin^{-}) were identified by flow cytometry (G, left), and their absolute cell numbers were determined at day 14 (G, right) and day 28 (H). Statistical data (F-H) are shown as mean value with SEM and were analyzed by the two-tailed unpaired Student’s t-test; *P < 0.05 and **P < 0.01. Each dot represents one mouse. Scale bars (B,D) represent 400 µm.
**Figure S3. Molecular analysis of Pax5-deficient B cells stimulated with anti-CD40 and IL-4.** (A-E) Identification of Pax5 target genes in activated B cells. CD43− FO B cells from the lymph nodes of Pax5^{Bio/Bio} mice (I4) were stimulated with anti-CD40 and IL-4 for 2 days prior to Bio-ChIP-sequencing (12). (A) Identification of 9,047 Pax5 peaks with a P value of < 10^{-10}, as determined by MACS peak calling. (B) Presence of the consensus Pax5 recognition motif (12) in 89% of all Pax5 peaks. A white line (at 20%) indicates the frequency of motif detection in random DNA sequences. (C) Pax5 binding (black bar) at 25.7% of all DNase I hypersensitive (DHS) sites (black and grey) that were mapped in stimulated control Cd23-Cre Pax5^{fl/+} B cells. (D) Identification of 5,049 Pax5 target genes by peak-to-gene assignment (see Methods). (E) Scatter plot of gene expression differences between Cd23-Cre Pax5^{fl/+} (Pax5^{Δ−}) and Cd23-Cre Pax5^{fl/+} (Pax5^{Δ+}) lymph node B cells that were stimulated with anti-CD40 and IL-4 for 2 days prior to RNA-seq analysis. The expression data of individual genes in the two B cell types were plotted as average variance-stabilizing transformed values (norm vst) on a log_{10} scale. Each dot represents one gene. Activated and repressed genes, which are regulated by Pax5 more than 2- or 3-fold, are colored in blue or red, respectively (Tables S1 and S6). (F) The expression of genes involved in class switch recombination, base-excision repair, mismatch repair and non-homologous end joining is shown as mean expression value (TPM) with SEM based on two independent RNA-seq experiments for each genotype (Cd23-Cre Pax5^{fl/+} [gray dots] or Cd23-Cre Pax5^{fl−} [black dots]). (G) Analysis of the Aicda gene in lymph node B cells stimulated with anti-CD40 and IL-4 for 2 days. Pax5 peaks, DHS sites, the histone mark H3K4me2 and RNA expression are visualized as RPM (reads per million mapped sequence reads) values in Cd23-Cre Pax5^{fl/+} (Pax5^{Δ+}, black) and Cd23-Cre Pax5^{fl−} (Pax5^{Δ−}, red) B cells. Horizontal bars denote Pax5-binding regions identified by ‘peak calling’. The promoter (P) and enhancer (E1-E5) regions (85) of the Aicda locus are indicated together with the corresponding genomic mm9 coordinates. (H,I) Analysis of the Cγ1-Cγ2b gene region (H) and Tbp locus (I) in lymph node B cells stimulated with anti-CD40 and IL-4 for 2 days. Pax5 peaks, DHS sites and gene transcripts are shown as RPM values at the Cγ1/Cγ2b (H) and Tbp (I) gene regions in Pax5^{Δ+} (black) and Pax5^{Δ−} (red) B cells. A schematic diagram indicates the intronic (I) promoter, switch (S) region and constant (C) gene segments together with the corresponding genomic mm9 coordinates.
**Figure S4. Expression of genes encoding signaling components in Pax5-deficient FO B cells.** (A) Schematic diagram depicting the calcium and PI3K signaling pathways downstream of the BCR. Arrows indicate phosphorylation events that lead to the activation of downstream signal transducers, which are shown in different colors. Signal transducers that are inactivated by phosphorylation are shown in grey. The phosphorylation of serine (S), threonine (T) and tyrosine (Y) residues that were analyzed by phosflow analysis in this study are indicated. The phosphorylation of AKT at S473 by mTORC2 depends on a high abundance of phosphatidylinositol-3,4,5-triphosphate (PIP3) in the plasma membrane and is thus exquisitely sensitive to PTEN activity, which converts PIP3 to phosphatidylinositol-4,5-triphosphate (PIP2). In contrast, phosphorylation of AKT at T308 by PDK1 is less dependent on high PIP3 concentration and is thus less sensitive to PTEN activity (86). (B) Phosphorylation of the 4E-BP1 and S6 protein in response to BCR stimulation. Lymph node FO B cells of Cd23-Cre Pax5^fl/fl-^ (black) and Cd23-Cre Pax5^fl/fl+^ (gray) mice were stimulated for 15 min with anti-IgM followed by intracellular staining (left) with antibodies specific for p-S6 (p-Ser240/244) or p-4E-BP1 (p-Thr37/46) and quantification of the median fluorescence intensity (MFI, right). (C) Genes coding for signal transducers acting in the mTORC, PI3K-AKT, ERK-MAPK and calcium signaling pathways downstream of the BCR were similarly expressed in quiescent FO B cells (before anti-IgM stimulation) from the lymph nodes of Cd23-Cre Pax5^fl/fl-^ (black dot) and Cd23-Cre Pax5^fl/fl+^ (gray dot) mice. The mRNA expression of the indicated genes is shown as mean expression value (TPM) with SEM based on two independent RNA-seq experiments for each genotype. (D) CD19 protein expression on FO B cells from lymph nodes and the spleen of Cd19^+/^- (red), Cd23-Cre Pax5^fl/fl-^ (black) and Cd23-Cre Pax5^fl/fl+^ (gray) mice, determined by flow-cytometric analysis and quantification of the median fluorescence intensity (MFI). (E) Comparison of AKT phosphorylation (p-S473) in Cd19^+/^- and Cd23-Cre Pax5^fl/fl-^ FO B cells upon BCR stimulation. Lymph node FO B cells were stimulated for 15 min with anti-IgM followed by intracellular staining with a p-AKT (p-S473)-specific antibody and quantification of the median fluorescence intensity (MFI). Statistical data (B,E) are shown as mean value with SEM and were statistically analyzed by two-way ANOVA with Šídák's multiple comparison test: *P < 0.05, **P < 0.01, ***P < 0.001.
Figure S5. Identification of Pax5-dependent immediate early genes induced by BCR signaling. (A) Scatter plot of gene expression differences between control Cd23-Cre Pax5\textsuperscript{fl/+} (Pax5\textsuperscript{Δ+/+}) and Cd23-Cre Pax5\textsuperscript{fl/-} (Pax5\textsuperscript{Δ-/-}) lymph node CD43\textsuperscript{-} FO B cells before (left) or after 1 h (right) of anti-IgM stimulation. The RNA-seq expression data of individual genes between the two genotypes are plotted as average variance-stabilizing transformed values (norm vst) on a log\textsubscript{10} scale. Each dot represents one gene. Activated and repressed genes, which are significantly (adjusted \(P\) value < 0.05) regulated > 3-fold by Pax5, are colored in blue or red, respectively. (B) Functional classes of regulated Pax5 target genes. The RNA-seq data shown in (A) were analyzed together, and genes were only considered for further analysis, if they fulfilled the following criteria: > 3-fold Pax5-dependent regulation with an adjusted \(P\) value < 0.05 before (0 h) and/or after 1h of anti-IgM stimulation, expression of > 5 TPM in at least one condition and Pax5 binding (Bio-ChIP-seq) at the gene locus in FO B cells. (C) Expression of Pax5 target genes implicated in cell cycle regulation and proliferation in FO B cells after 1 h of anti-IgM stimulation. The expression of the indicated genes is shown as mean expression value (TPM) with SEM based on two independent RNA-seq experiments for each genotype. (D) Scatter plot of gene expression differences in control Cd23-Cre Pax5\textsuperscript{fl/+} (Pax5\textsuperscript{Δ+/+}) lymph node B cells that were left untreated (0 h) or stimulated for 1 h with anti-IgM prior to RNA-seq. The expression data of individual genes between the two treatment conditions are plotted as described in (A). Each dot represents one gene. The indicated color code highlights the genes, which were significantly (adjusted \(P\) value < 0.05) down- or up-regulated by a factor of 3-6-fold, 6-9-fold or > 9-fold. The gene number in each category is shown in brackets. (E) Schematic illustration of the test setup for detecting differentially expressed genes based on RNA-seq data of untreated and one-hour anti-IgM-stimulated knockout (red, Cd23-Cre Pax5\textsuperscript{Δ-/-}) and control (blue, Cd23-Cre Pax5\textsuperscript{Δ+/+}) lymph node B cells. Depicted are the main effects of the genotype at time point 0 (I) and after 1 h of stimulation (II) as well as the main effects of the time in control B cells (III) and knockout B cells (IV), as well as the ‘interaction’ effect (V), which were used for bioinformatic analysis (see Methods). (F) Pax5 dependency of immediate early gene expression. The percentage and number of non-classified (white bar), Pax5-independent (gray bar) and Pax5-dependent (black bar) immediate early genes are shown for the different induction ratios (> 3-fold, > 6-fold and > 9-fold, all with an adjusted \(P\) value < 0.05) determined for control FO B cells before and after anti-IgM treatment (D). See methods for detailed description of the three class of immediate early genes. The annotation, induction ratio and Pax5 binding of the 39 Pax5-dependent immediate early genes (significantly > 9-fold induced in control B cells) are shown in Table S3.
**Figure S6. Identification of Pax5-dependent miRNAs in FO B cells.** (A) Pten mRNA expression in lymph node FO B cells from Cd23-Cre Pax5^{fl/fl} (black), Cd23-Cre Dicer1^{fl/fl} (red), Cd23-Cre Pax5^{fl/fl} (orange) and control (grey) mice, as determined by RT-qPCR analysis. The Pten mRNA level was normalized to that of the Tbp mRNA (see Methods). The genotypes of the control mice were Pax5^{+/+}, Pax5^{fl/+}, Pax5^{fl/+} Pten^{fl/+} or Pax5^{fl/fl} Pten^{fl/fl}. (B) MA plot of miRNA expression differences between Cd23-Cre Pax5^{fl/fl} (Pax5^{Δ−}) and Cd23-Cre Pax5^{fl/+} (Pax5^{Δ+}) FO B cells, which were isolated from the spleen as CD23^{+} cells by immunomagnetic sorting. Two small-RNA-seq experiments were performed per genotype. The abundance of individual miRNAs in the two B cell types is plotted as mean value of the normalized counts versus the log2-fold change in abundance between Pax5^{Δ−} and Pax5^{Δ+} FO B cells (Tables S4 and S6). The statistical significance of the observed differences is indicated by gray and black circles (adjusted \(P\) value < 0.05) or gray dots (adjusted \(P\) value > 0.05). Adjusted \(P\) values were determined by DESeq2. Each symbol represents one miRNA. Pten-targeting miRNAs are highlighted by the color corresponding to their position on the scale bar. The scale bar was generated by multiplying the sum of the normalized read counts of all members of a miRNA family with the total context++ score of the miRNA family (Table S5). (C) Identification of Pten-targeting miRNAs by their total context++ score (37). The small-RNA-seq analysis of lymph node FO B cells (Fig. 6C) identified 417 miRNAs (Table S5) that can potentially target the Pten 3’UTR according to the TargetScan algorithm (v7.2; targetscan.org). Analysis of the total context++ score of these 417 miRNAs revealed a cutoff of -0.47, below which a miRNA is strongly predicted to target the 3’UTR of Pten mRNA. (D) Differential abundance of the four miRNAs of the miR-29 family in Pax5-deficient (black) and control (gray) FO B cells from lymph nodes (left) or the spleen (right). The miRNA abundance is displayed as normalized read counts determined by small-RNA-seq, and its fold decrease in Pax5-deficient FO B cells is indicated for each miRNA. As miR-29b-1 and miR-29b-2 have identical sequences, the respective read counts were added up and are shown as one bar for this miRNA pair. (E) Phosphorylation of AKT at Ser473 in lymph node FO B cells of miR-29a/b-1^{−/−} (blue dot), Cd23-Cre Pax5^{fl/fl} (black dot) and control Cd23-Cre Pax5^{fl/+} (gray dot) mice after BCR stimulation for 15 min with anti-IgM. AKT phosphorylation was determined by intracellular staining (left) and quantification of the median fluorescence intensity (MFI, right). The MFI values are shown relative to those of the control FO B cells. Statistical data are shown as mean value with SEM and were statistically analyzed by two-way ANOVA with Šidák's multiple comparison test: **\(P\) < 0.01, ****\(P\) < 0.0001. (F) Pax5 peaks at the two miR-29 loci. Pax5 binding (blue) was determined by Bio-ChIP-seq analysis of Pax5^{Bio/Bio} FO B cells from lymph nodes (GSM932925 to GSM932929), while the nascent transcripts giving rise to the individual miR-29 species were mapped in control FO B cells by GRO-seq analysis (GSM3037403). The Pax5-binding and GRO-seq data are visualized as RPM (reads per million mapped sequence reads) values. Strand-specific GRO-seq reads are shown above and below the line.
Figure S7. No rescue of FO B cell proliferation and GC B cell formation in Pten,Pax5 double-mutant mice. (A,B) Analysis of PTEN protein expression in CD43− FO B cells from lymph nodes of Cd23-Cre Pax5^{fl/fl} (black), Cd23-Cre Pax5^{fl/fl} Pten^{fl/+} (green), Cd23-Cre Pax5^{fl/fl} Pten^{fl/+} (blue) and control Pax5^{fl/fl} Pten^{fl/fl} (gray) mice. (A) PTEN protein analysis by intracellular staining and quantification of the MFI values, which are shown relative to the control FO B cells. The dashed line indicates the MFI value corresponding to the absence of PTEN protein in Cd23-Cre Pax5^{fl/fl} Pten^{fl/fl} FO B cells. (B) Analysis of PTEN protein expression by immunoblotting of 2-fold serially diluted whole-cell extracts with antibodies detecting PTEN, GAPDH or Pax5. One of two experiments is shown. (C) Stimulation of CellTrace Violet-stained FO B cells from lymph nodes of the indicated genotypes with CpG oligodeoxynucleotides for 3 days, followed by staining with the Viability Dye eFluor™ 780 and flow-cytometric analysis. The gate and percentage of viable cells are indicated. (D) Stimulation of CellTrace Violet-stained FO B cells with CpG, LPS or anti-CD40 plus IL-4 for 3 days or anti-IgM plus IL-4 for 4 days followed by flow-cytometric analysis, as described in (C). The proliferation index in response to these stimulation conditions is shown for FO B cells of the indicated genotypes. (E,F) No rescue of GC B cell development in Cd23-Cre Pax5^{fl/fl} mice upon additional loss of PTEN. GC B cells (GL7^{+}Fas^{+}B220^{+}CD19^{+}) of the indicated genotypes were analyzed by flow cytometry and intracellular Pax5 staining of B cells from the spleen and Peyer’s patches. The analysis of mice of the indicated genotypes (right) is shown together with the quantification of the data of all mice analyzed (left). (G) Flow-cytometric analysis of TACI and CD1d expression distinguishes MZ B cells (B220^{+}CD19^{+}CD21^{hi} CD23^{lo/−}) and FO B cells (B220^{+}CD19^{+}CD21^{int}CD23^{hi}) in the spleen of wild-type mice. (H) Statistical analysis of a second immunohistological experiment performed with the spleens of 9-10-week-old mice of the indicated genotypes. The average number of IgM^{+} B cells outside of the MOMA-1^{+} macrophage ring was determined per 10 μm of the perimeter of the MOMA-1^{+} ring (see Methods). Each dot represents the measurement of one follicle. Statistical data (A,D,E,F,H) are shown as mean values with SEM and were analyzed by one-way ANOVA with Tukey’s multiple comparison test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Each dot (A,D,E,F) corresponds to one mouse. The genotypes of the control mice (A,D,E,F) were Pax5^{fl/fl}, Pax5^{fl/fl} Pten^{fl/+}, Pax5^{fl/fl} Pten^{+/+} or Cd23-Cre Pax5^{fl/+} Pten^{+/+}. 