SUPPLEMENTARY MATERIALS and METHODS

Cell cultures and retroviral transduction

Three different murine wt pre-BI cell populations were isolated from fetal liver (called LY5.1FL, FLB6-67 and B6BAFL cells) and PAX5<sup>−/−</sup> pro-B cells were purified from bone marrow of a PAX5-deficient mouse. Both cells were cultured on OP9 bone marrow stroma cells in Iscove’s modified Dulbecco’s medium supplemented with 2% FBS, 0.03% w/v primatone and 5% IL-7.(1-2) The bicistronic vector pMSCV-IRES-GFP (MIGR) was used for the retroviral transduction which allows the expression of PAX5/ETV6 and GFP.(1) On day +3, sorting of GFP positive cells was performed (FACSAria, BD Biosciences).

Microarray, statistics and bioinformatics analyses. B6BAFL cells were suspended in Trizol reagent (Invitrogen, Life Technologies) immediately after sorting for GFP. RNA extraction was performed following the manufacturer’s protocol and processed by Affymetrix protocols (Genopolis Consortium, University of Milano-Bicocca, Milano, IT). Microarray analysis was carried out on the Affymetrix 430A 2.0 mouse gene chip, and data (as a.CEL file) have been deposited with Array Express repository (accession E-MEXP-3095).

The Gene Expression Profile (GEP) by microarray analysis has been executed in two independent biological transduction experiments, in B6BAFL pre-BI cells.

| Expression measures for each probe set were obtained using GCRMA, a multi-array analysis method that estimates probe set signals by considering the physical affinities between probes and targets. Normalization was performed by a quantile method. Two successive steps of data filtering were applied. At first from the dataset were filtered out the probe sets with Absent call in all the samples. Then an empirical threshold was determined on the intensity signal of the 95° percentile of the distribution of the Absent call signals. The algorithm selected the probe sets with an intensity signal higher than the threshold. 12,761 probe sets passed |
the filtering step, and subsequently probes whose signal was below 40 in all 4 gene chip were further discarded as non efficient. The filtered data were annotated using Bioconductor.

The algorithm selected the probe sets with a signal higher than the threshold (black dotted line).

Absent calls: red line, Present calls: blue line.

![Graph showing threshold and signal distribution](image)

The log2 (signal) has been calculated by the ratio of the PAX5/ETV6 mean signal over the MIGR-GFP mean signal. Specifically, we considered a differentially expressed gene if log2 (signal) was greater than +0.59 (corresponding to a fold change greater than 1.5) or lesser than -0.50 (corresponding to a fold change lesser than 0.7). For pathway analysis, GEP data were analyzed by on-line software: the Database for Annotation, Visualization and Integrated Discovery (DAVID, [http://david.abcc.ncifcrf.gov/home.jsp](http://david.abcc.ncifcrf.gov/home.jsp)) and Ingenuity Pathway Analysis (IPA, [www.ingenuity.com](http://www.ingenuity.com)).

**Fisher’s exact test.** In order to verify whether the enrichment of PAX5-targets was statistically significant, the Fisher’s exact test has been performed. In details, we proceeded as follows: i) we identified 581 probe sets of known PAX5-target genes in any cell environment, derived from the literature (3-6); ii) 502 over 581 probes have been identified on the Affymetrix 430A 2.0 mouse gene chip; iii) we tested our dataset of 12,761 probes and only 389 PAX5-target probes were present in the filtered and normalized dataset, therefore we considered this probe set for Fisher’s exact test analysis; iv) 34/389 PAX5-target genes were present among the 340 DEGs, while 355/389 belonged to the group of 12,421 non-DEGs.

**Reverse transcription-PCR and real-time quantitative-PCR assays.** RNA extraction was performed using Trizol (Life Technologies). Superscript II enzyme (Life Technologies) was used for cDNA synthesis. Real-time analysis was performed on Light Cycler 480II with Universal Probe Master system (Roche
Diagnostics; F. Hoffmann-La Roche Ltd.). Primers and probes were selected according to the Software Probe Finder (Roche Diagnostics) and are reported in Supplementary Table S8. Data were expressed using the comparative \( \Delta\Delta Ct \) method, using HPRT gene as reference and MIGR-GFP cells as standardization control;\((7-8)\) both t test and SD values refer to triplicates of a single experiment and \( N=3 \) biological independent experiments were performed for each gene.

**MiRNA analysis.** RNA was quantified by Nanodrop2000c (Thermo Fisher Scientific Inc). Reverse transcription with a miRNA-specific primer has been done using the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) and followed by real-time PCR with TaqMan® MicroRNA Assays for each single miRNA (mmu-miR-675-5p assay ID n°1940, -3p assay ID n°1941 and assay ID n°001093 for RNU6B snRNA, which has been used as reference miRNA). The comparative \( \Delta\Delta Ct \) method has been applied.\((8)\)

**Antibodies and flow cytometry.** Phycoerythrin-conjugated antibodies anti-CD19 (MB19-1), anti-CD22 (2D6), anti-SLAMF6/LY108 (13G3-19D) and allophycocyanin-conjugated antibodies against CD44 (IM7) were used (e-Bioscience Inc). Data were analyzed using CellQuest Software (BD Biosciences).

**Protein array and western blot analysis.** Protein array was performed on cell supernatant, obtained from B6BAFL cell culture, using RayBio® Cytokine Antibody Arrays - Mouse Array III-IV (Raybiotech Inc), following the manufacturer’s protocol. Western blot analysis was performed following either Andrews’s extraction method (9-10) or standard RIPA buffer. Anti-beta-actin antibody was used at working dilution 1:1000 (AC-15, Sigma-Aldrich), anti-N-term PAX5 was used at 1:545 (AB4227, Chemicon) and anti-C-term PAX5 at 1:200 (AB15164, Abcam). A StripAblot Stripping Buffer (Euroclone S.p.A.) was used to recover membranes. Densitometry analyses were performed using Alliance instrument and Uviband software (Uvitec Cambridge, UK).

**IL-7 starvation assay and IgM/\( \mu \) heavy chain analysis.** Cells were washed out by IL-7 and plated in IMDM+2% FBS+0.5% IL-7 on OP9 stroma layer. At day 0 and day +5, staining for \( \mu \) heavy chain of IgM was performed using the allophycocyanin-conjugated antibodies against IgM/\( \mu \) heavy chain (II/41) (e-Bioscience Inc).

**VCAM1 adhesion assay.** The adhesion of pre-BI cells to VCAM1-coated coverslips was performed as described elsewhere.\((6)\) Briefly, 15mm Ø round coverslips were coated with recombinant mouse VCAM1-Fc
protein (25 µg/ml; R&D Systems) and placed in 12-well dishes, containing 0.5 ml IMDM+2%FBS medium, with 5x10^5 pre-BI cells, in the presence or lack of 100 ng/ml CXCL12 (Peprotech Inc). The coverslips were mounted on glasses in the presence of DAPI (8µl). Each coverslip was analyzed by a fluorescence microscope, acquiring 30 representative fields, and the single cells were counted.

SUPPLEMENTARY RESULTS

PAX5/ETV6 repressed physiologically activated PAX5-target genes.

In GEP analysis, the value of log2(signal) of CD22 was equal to -1.39 (fold change FC=0.38), and, from RQ-PCR analysis, we obtained fold changes of 0.07 (t test, p=0.000034) in LY5.1FL cells, of 0.29 (t test, p=0.006058) and of 0.42 (t test, p= 0.000034) and in FLB6-67 and B6BAFL pre-BI cells respectively (Supplementary Table 2). More important, phenotypic analysis confirmed previous data, as shown in Supplementary Figures S1B, S2A and S3A, where CD19 overlay is shown as internal control of PAX5/ETV6 activity. In details, considering the CD22 ratio between the Mean Fluorescent Intensity (MFI) of PAX5/ETV6 cells and the MFI of control cells, we obtained a correspondent mean protein repression of -42% in LY5.1FL (range -19% to -77%, in 9 independent experiments), -44% MFI ratio in FLB6-67 (range -42% to -45%, in 2 experiments) and of -42% MFI ratio (range -20% to -74%, in 6 experiments) in B6BAFL (Supplementary Figures S1C, S2B and S3B). SLAMF6/LY108 gene was repressed in GEP analysis with a log2(signal)=-0.55 (FC=0.68) confirmed by RQ-PCR analyses (Supplementary Table 2), in LY5.1FL (FC=0.23, t test, p=0.00081), in FLB6-67 (FC=0.59, t test p=0.002125) and in B6BAFL, with a FC=0.57 (p=0.014589). In addition, phenotypic analysis was in agreement with these data, indeed, in LY5.1FL cells (Supplementary Figure S1D), SLAMF6/LY108 was down-regulated of -44% MFI ratio and of -33% MFI ratio in FLB6-67 cells (range -20% to -46%, in 8 experiments, Supplementary Figure S2C) and this result was consistent in B6BAFL, with -54% MFI ratio (Supplementary Figure S3C), range -38% to -71% in 3 experiments. Similarly, in LY5.1FL we observed a 41% decrease in CD44 protein expression on PAX5/ETV6 cells (MFI ratio range from -19% to -64% in 26 experiments, Supplementary Figure S2E), while the FC was 0.72 by GEP and 0.60 by RQ-PCR (Supplementary Table S2); correspondingly, as shown in Supplementary Figure S2D by FACS analysis, in FLB6-67 cells CD44 was significantly repressed of 28%.
(in 26 experiments, MFI ratio range -19% to -46%), as well as in B6BAFL (Supplementary Figure S3D), where CD44 was repressed of 43% (in 16 experiments, MFI ratio range -28% to -78%).

**PAX5/ETV6 activated physiologically repressed PAX5-target genes.**

By RQ-PCR assay, we demonstrated that H19 and the two micro-RNAs were up-regulated in PAX5/ETV6 LY5.1FL pre-BI cells, which expressed both mir-675-3p and -5p at appreciable levels (Supplementary Figure S6A-B). On the other end, in the FLB6-67, nor mir-675-3p or -5p were amplified with good quality curves, thus we proceeded to a pre-amplification cycle followed by standard RQ-PCR; with this strategy, we technically rescued only mir-675-3p, whereas -5p experiment presented again unacceptable curves of amplification (data not shown). As shown in Supplementary Figure S6C, in PAX5/ETV6 cells, mir-675-3p was significantly up-regulated with a fold increase of 8.75 (t test p=0.014). In addition B6BAFL pre-BI cells, mir-675-3p was present at detectable levels especially in PAX5/ETV6 cells compared to control, leading to a tremendous fold increase equal to 128.27 (t test p=0.005, Supplementary Figure S6D); whereas, mir-675-5p was very low expressed and probably, because of this, we didn’t find any statistical difference (data not shown).

**PAX5/ETV6 blocked pre-BCR expression and repressed molecules associated to its internal signaling.**

We further extended the analysis of genes involved in pre-BCR signaling, but not represented in the microarray. In fact, we showed that PAX5/ETV6 cells repressed PAX5-regulated components of BCR at the surface level, such as CD19 and CD22 (Supplementary Figures S1-S2-S3), CD79a/Igα, CD37, as well as molecules associated to its intracellular signaling, such as IRF8, IRF4, IKZF2/HELIOS and IKZF3/AIOLOS (Supplementary Tables S2-S6) as well as CD79a and BLNK, as demonstrated in our previous study.(1) Furthermore, many molecules involved in lymphocyte signaling and activation, but not regulated by PAX5, are modulated by PAX5/ETV6 (Supplementary Figures S12-S13).

**PAX5/ETV6 represses molecules important for B-cell adhesion.**

Modulation of genes responsible for extra-cellular binding as well as the molecules involved in the intracellular adhesion signaling supported the role of PAX5/ETV6 in adhesion and in particular respect to VCAM1, a physiological B-cell adhesion molecule expressed in bone marrow niche.(6,11-12) We previously showed the down-regulation of several adhesion molecules, known as PAX5-targets, such as SLAMF6/LY108, CD44, BCAR3, SDC4, NEDD9, CAPN2, and PLEKHA2 genes (Supplementary Table S2;
Moreover, we demonstrated by RQ-PCR that PAX5/ETV6 caused the down-regulation of \textit{EDG1} and \textit{EPS8}, two PAX5-activated genes having a role in cell adhesion, whose probes were not present in the microarray platform (Supplementary Table S7). Finally, several genes were identified as being involved in cell adhesion, but not regulated by PAX5; examples are: \textit{RHOB} (Supplementary Table S2), \textit{TNF} Supplementary (Supplementary Figure S1A), \textit{THY1}, \textit{CD47}, \textit{CORO1A}, \textit{ITGB1}, \textit{LYN}, \textit{PAFAH1B1}, \textit{THBD}, and \textit{SLAMF1} (Supplementary Figure S14).

Altogether, this evidence prompted us to investigate the role of PAX5/ETV6 in B-cell adhesion to VCAM1.
**SUPPLEMENTARY FIGURE LEGENDS**

**Supplementary Figure S1.** Validation experiments: protein analysis of down-regulated molecules in LY5.1FL pre-BI cells. (A) TNF alpha protein levels by protein array analysis of B6BAFL cell supernatant, transduced either by PAX5/ETV6 or by MIGR-GFP vector. From (B) to (E), a representative phenotypic analysis in LY5.1FL pre-BI cells is shown. The down-regulation of -49% of CD19 in PAX5/ETV6 cells compared to control MIGR-GFP cells is shown in the histogram overlay analysis in panel (B), as a positive control for fusion protein activity. CD22 expression is -75% (C), SLAMF6 is -44% (D) and CD44 is -50% (E) in PAX5/ETV6 cells compared to control MIGR-GFP cells.

**Supplementary Figure S2.** Representative phenotype analysis in FLB6-67 cells. (A) CD19 expression is down-regulated of -35%, (B) CD22 expression is -45%, (C) SLAMF6 is -46% and (D) CD44 is -46% in PAX5/ETV6 cells compared to control MIGR-GFP cells.

**Supplementary Figure S3.** Representative phenotype analysis in B6BAFL cells. (A) CD19 expression is down-regulated of -50%, (B) CD22 expression is -37%, (C) SLAMF6 is -50% and (D) CD44 is -65% in PAX5/ETV6 cells compared to control MIGR-GFP cells.

**Supplementary Figure S4.** Schematic representation of PAX5-target genes among DEGs.

**Supplementary Figure S5.** Molecular and functional model of PAX5/ETV6 action.

**Supplementary Figure S6.** (A-B) Mir675-3p and mir675-5p expression in LY5.1FL pre-BI cells. (C) Mir675-3p expression in FLB6-67 and (D) in B6BAFL cells. (E-F) Mir675-3p and mir675-5p expression in PAX5+/− pro-B cells. T test, *p<0.05. **p<0.01

**Supplementary Figure S7.** Down-regulation of endogenous PAX5 expression. (A) PAX5/ETV6 and endogenous PAX5 mRNA levels, in two sub-populations of PAX5/ETV6 B6BAFL cells, sorted for low and high levels of GFP-PAX5/ETV6. Probes and primers at 3’-term of wt PAX5 and at fusion mRNA breakpoint were designed, demonstrating an inverse correlation between PAX5/ETV6 and PAX5 mRNA expression, confirming the down regulation of wt PAX5 in presence of increasing levels of the fusion gene (RQ-PCR analysis). (B) Protein analysis by western blot using anti-C-term PAX5 antibody and beta-actin (as internal control) were performed on the same membrane, for each cell population. T test, *p<0.05. **p<0.01 ***p<0.001.
Supplementary Figure S8. A representative phenotypic analysis of CD29 and CD49d expression in (A) LY5.1FL, (B) FLB6-67 and (C) B6BAFL pre-BI cells. The histogram overlay analysis shows PAX5/ETV6 cells and control MIGR-GFP cells.
Figure S1

A

TNF alpha

**

-80%

B

-49%

C

-75%

D

-44%

E

-50%

MIGR-GFP

PAX5/TEL

MIGR-GFP

PAX5/TEL

MIGR-GFP

PAX5/TEL

MIGR-GFP

PAX5/TEL

MIGR-GFP
Figure S2

A

B

C

D
Figure S3

A

PAX5/TEL
MIGR-GFP

B

C

D

CD19

GFP

CD22

GFP

SLAMF6

GFP

CD44

GFP

PAX5/ETV6

MIGR-GFP

PAX5/ETV6

MIGR-GFP

PAX5/ETV6

MIGR-GFP

PAX5/ETV6

MIGR-GFP

PAX5/ETV6

MIGR-GFP

PAX5/ETV6

MIGR-GFP
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- **19/34 (56%)** PAX5-target REJECTED by PAX5/ETV6
- **15/34 (44%)** PAX5-target ACTIVATED by PAX5/ETV6
- **23/34 (68%)** opposite dominance function
- **11/34 (32%)** concordant with PAX5 function

Fisher’s exact test: 1.029 e^-09
Figure S5

PAX5/ETV6

PAX5-DNA BINDING CONSENSUS REGION

mSin3A
HDAC
NCoR

REPRESSION OF
PAX5-ACTIVATED
GENES

IMPAIRMENT OF
BCR ASSEMBLY
and B-CELL
DIFFERENTIATION BLOCK

? ?

PAX5/ETV6

PAX5-DNA BINDING CONSENSUS REGION

? ?

ACTIVATION OF
PAX5-REPRESSED
GENES

B-CELLS
ACQUIRE
NOVEL FEATURES
Figure S6

(A) mir675-3p

(B) mir675-5p

(C) mir675-3p

(D) mir675-3p

(E) mir675-3p

(F) mir675-5p
Figure S7

A

![Graphs showing PAX5 and PAX5/ETV6 expressions](image)

B

![Images showing protein bands and percentages](image)
Figure S8

A
LY5.1FL

B
FLB6-67

C
B6BAFL

PAX5/TEL
MIGR-GFP
SUPPLEMENTARY REFERENCES


