Krüppel-like factor 4 (KLF4) inactivation in chronic lymphocytic leukemia correlates with promoter DNA-methylation and can be reversed by inhibition of NOTCH signaling

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Supplemental Figure Legends

Supplemental Figure 1.

(A) Analysis of gene expression profiling of $CD19^+$ B-cells of 9 healthy donor (H) and 15 CLL patient samples (CLL). Shown are the 1866 most differentially expressed genes between CLL and non-malignant samples with $\log_2FC \geq |1|$ and $p \leq 0.05$ (linear models for microarray data, limma). Red color indicates high expression, green low expression. Examples of genes are indicated that are deregulated in our dataset and have previously been shown to play a role in CLL (blue).[1–11] In addition, novel candidate genes are indicated (orange).

(B) DNA-methylation of $CD19^+$ B-cells of 6 healthy donor (H) samples and 13 CLL patient samples that were also used for expression profiling shown in (A) was analyzed using methyl-CpG-immunoprecipitation onto custom promoter arrays. MCIp-array DNA-methylation values of CLL and H are shown, that represent median $\log_2FC$ of signal intensities of single oligonucleotides, normalized to MCIp samples of healthy T-cell pool. Distribution of methylation values of CLL samples show a greater heterogeneity in methylation compared to healthy donor samples ($1^{st}/99^{th}$ percentiles shown in dashed lines, range from 1^{st} to 99^{th} percentile of BC=-0.46/0.37, of CLL=-1.13/0.62, median in solid lines).

Supplemental Figure 2.

KLF4 shows similar promoter DNA-methylation in samples from 4 clinical CLL trials. DNA-methylation in KLF4 MA1 (see Figure 2) was measured with MassARRAY in samples of clinical trials CLL1 (black points), CLL4 and CLL8 (yellow) and CLL2H (blue) conducted by the German CLL Study Group (GCLLSG). All samples were taken at time of study entry. No significant differences in DNA-methylation levels could be detected in patients with Binet stage A (CLL1), in previously untreated patients, with Binet stage B and C (CLL4 and CLL8), and in Fludarabine refractory patients (CLL2H).

Mann-Whitney U test was used to test for significance, horizontal lines represent mean values.

Supplemental Figure 3.

Expression of KLF4 downstream targets *BAK*, *BAX*, *BCL-2* and *CCND1* was measured with qRT-PCR in $CD19^+$ B-cells of up to 55 CLL patients ("CLL") and 23 healthy donor samples ("H").[12–15] *BAK* and *BAX* are significantly down-regulated in CLL cells while *BCL-2* and *CCND1* are overexpressed compared to healthy donor samples. Values are normalized to *PGK* and *HPRT* as house keeper genes (Mann-Whitney U test). Whiskers indicate maximal and minimal values, boxes show $25^{th}/75^{th}$ percentile and median. p-values are indicated by asterisk: **p≤0.01, ***p≤0.001.

(B) Gene expression of *KLF4* shows significant positive correlation with *BAX* and *BAK* and negative correlation with *CCND1* and *BCL-2* (Spearman rank correlation). r Spearman rank correlation coefficient, p p-value.

Supplemental Figure 4.

(A) qRT-PCR measurement confirms overexpression of NOTCH1 in $CD19^+$ CLL cells (n=47) in comparison to $CD19^+$ B-cells from healthy donors ("H"; n=17). (Mann-Whitney U test).

NOTCH1 expression does not correlate with *KLF4* expression in these samples (Spearman rank correlation). Whiskers show maximal and minimal values, box represents 25^{th}/75^{th} percentile and median.

(B) Cell lines MEC1, MEC2, GRANTA-519, JeKo-1, LCL-FM, LCL-MM and PBMCs of 8 CLL patients ($CD19^{+}$ cells ≥91.6%) were treated with 2.5 µM and 5 µM γ-Secretase inhibitor (GSI-I) or 0.002% DMSO as carrier control for 24h. Three independent experiments were conducted per cell line. Cell viability of cell lines (left panel) and CLL patient samples (right panel) was determined after 24h of GSI-I treatment. For cell lines viability was quantified using CellTiter-Glo Luminescent Cell Viability Assay and luminescence units (RLU) are shown relative to untreated control. Viability of CLL cells was determined by Annexin-V/7AAD flow cytometry. Shown are percent living cells ($AnnV^{neg}$/$7AAD^{neg}$) normalized to untreated control. All cell lines and patient samples show significant dose dependent reduction of cell viability after treatment with 5 µM GSI-I (paired t-test).

p-values are indicated by asterisk: *p≤0.05, **p≤0.01, ***p≤0.001, ns non-significant, r Spearman rank correlation coefficient, n sample size

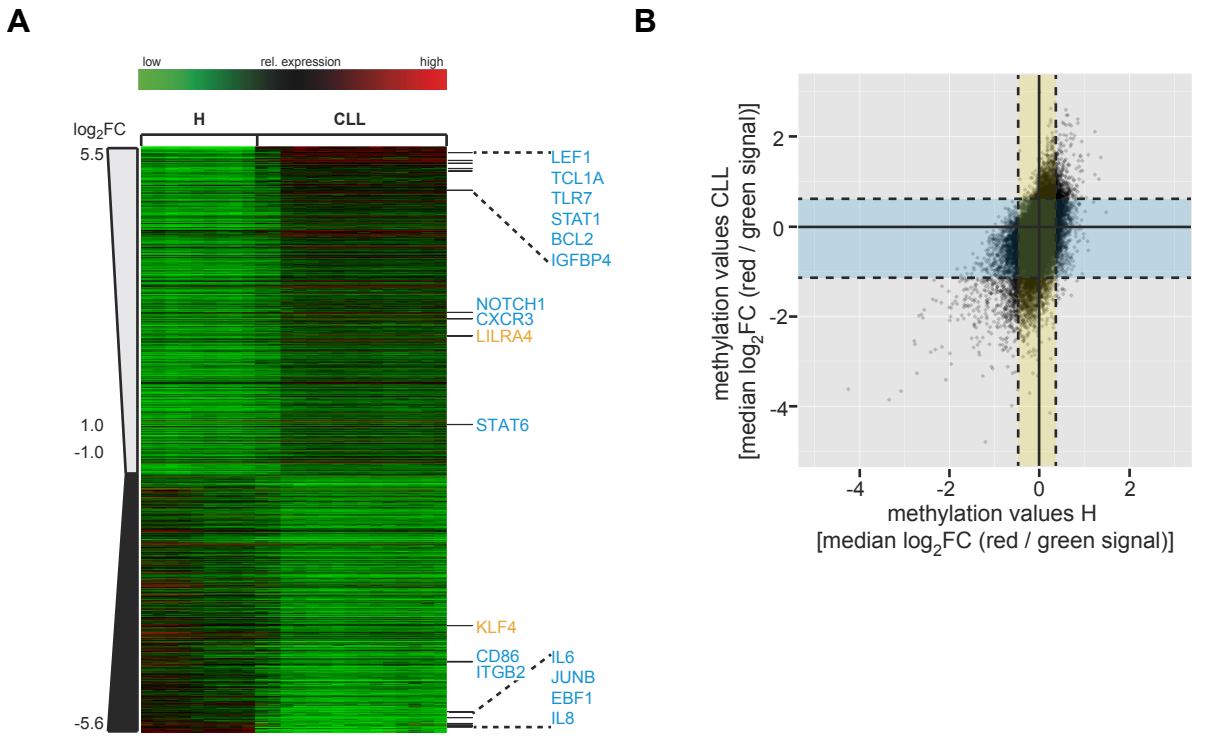
Supplemental Figure 5.

Overexpression of KLF4 in MEC1, MEC2 and JeKo-1 cells compared to empty vector control (EV) 3h, 10h and 24h after nucleofection.

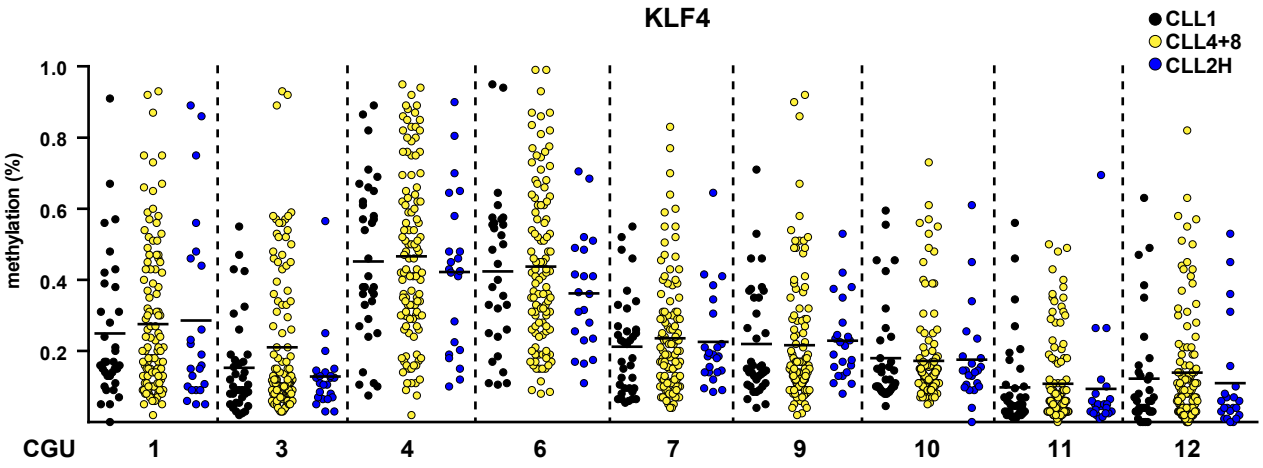
(A) Western Blot for KLF4 shows overexpression in all 3 cell lines 3h, 10h and 24h after transfection. GAPDH was used as loading control.

(B) Scheme representing B-Cell Receptor Signaling. Genes deregulated after KLF4 overexpression in MEC1 (border of symbols) and MEC2 (filling of symbols) are highlighted in green (down-regulation) and orange (up-regulation). Protein interactions are indicated by arrows.

Supplemental Figure 1.



Supplemental Figure 2.



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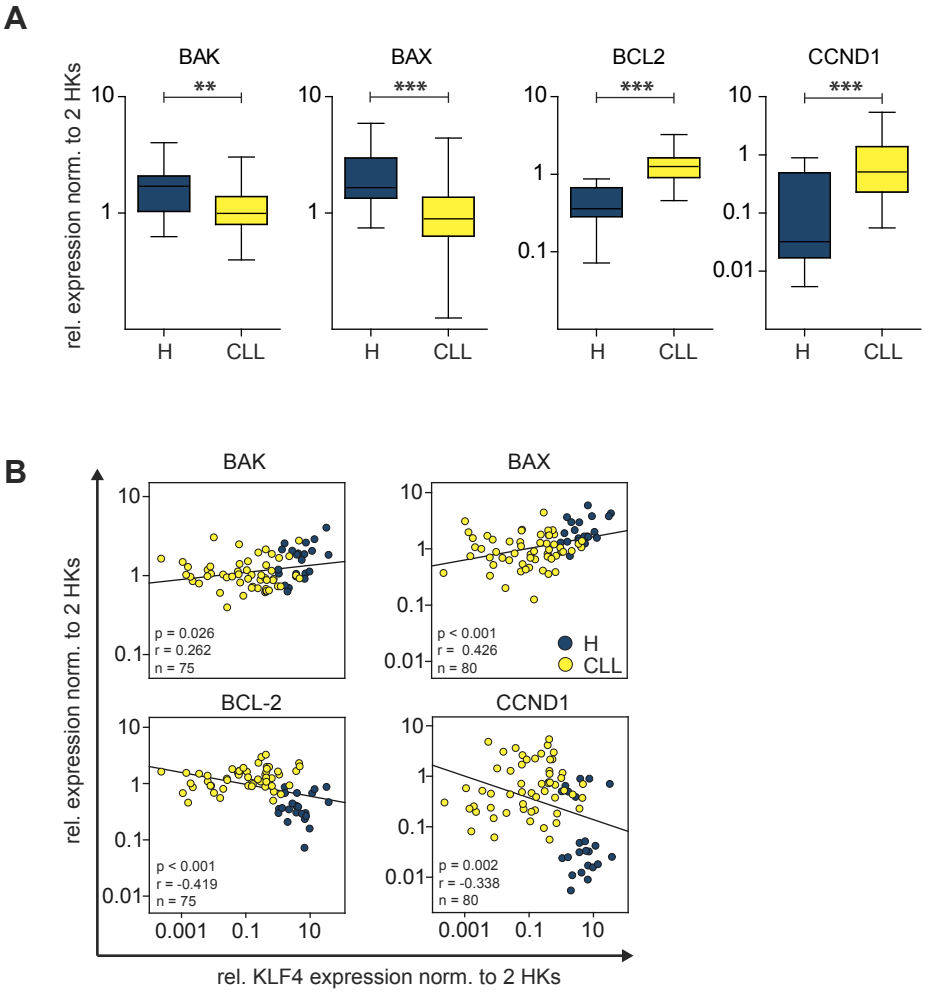
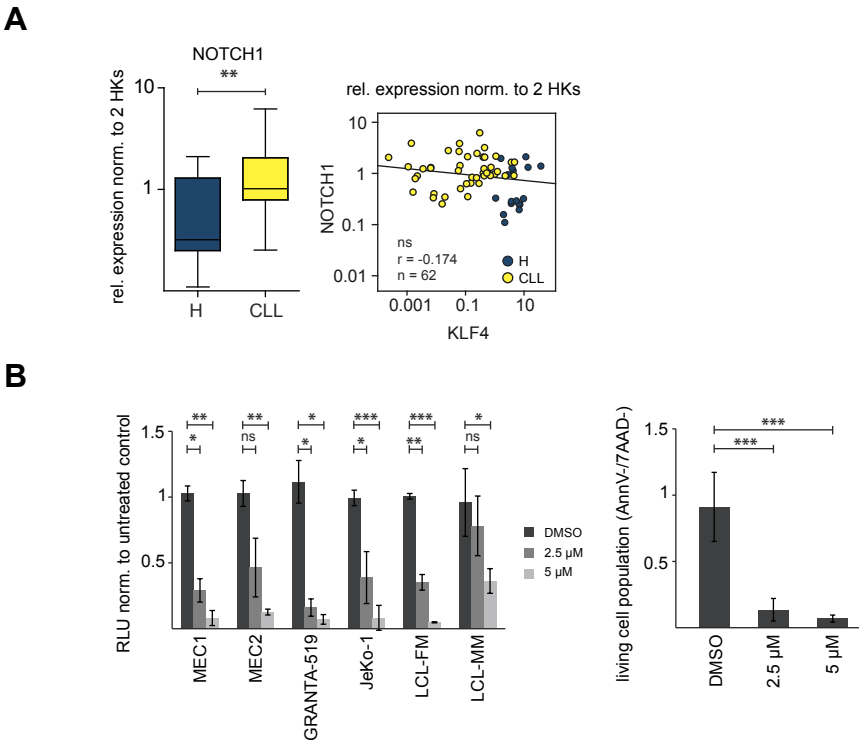
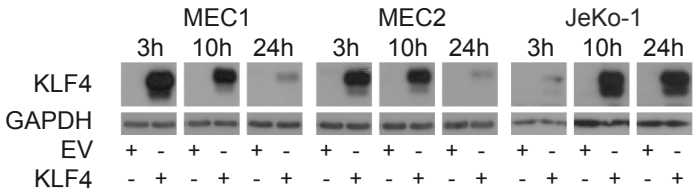


Supplemental Figure 4.

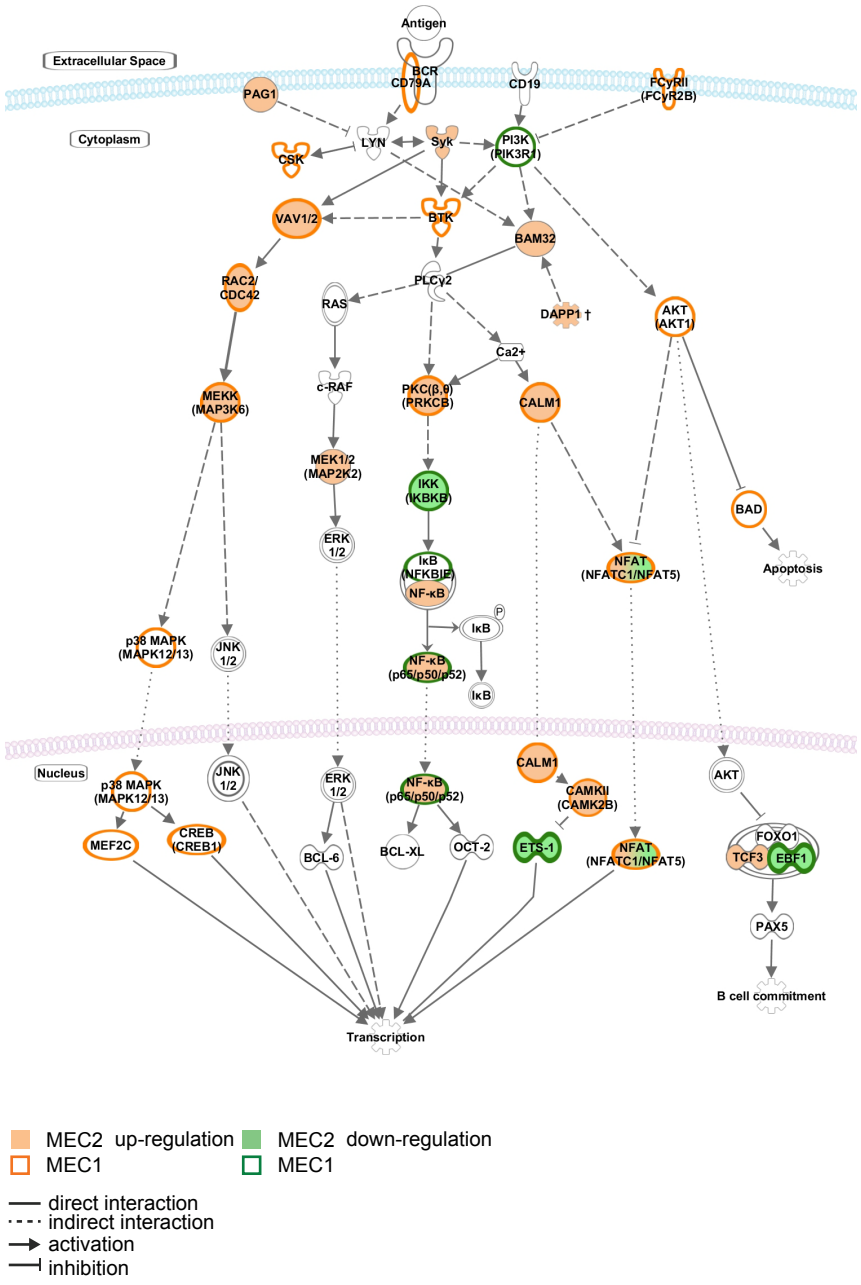


Supplemental Figure 5.

A



B



Supplemental Methods

Sample collection and cell culture conditions

Peripheral blood was collected with informed written consent from CLL patients (Ethics Committee, University of Ulm, approval no.96/08). For healthy donors, buffy coats were obtained from the German Red Cross (DRK, Mannheim, Germany) in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density centrifugation using Biocoll (Biochrom) separating solution. $CD19^+$ PBMCs were purified with CD19 Micro Beads for magnetic positive selection. Purity of PBMCs and $CD19^+$ B-cells was determined by flow cytometry with anti-CD19-APC (555415) or anti-CD20-PE (555623) and anti-CD5-FITC (345781) antibodies (BD Biosciences) on a FACSCanto II device. RNA and DNA was isolated using the AllPrep DNA/RNA Mini Kit as well as the RNeasy Mini and Micro Kits (Qiagen). PBMCs of CLL patients were cultured in DMEM (Sigma-Aldrich), 10% FCS (Biochrom AG) and 20% human type ab serum (MP Biomedicals). MEC1, MEC2, GRANTA-519 were cultured in DMEM with 10% FCS and 5% CO_2. JeKo-1 as well as LCL-MM and LCL-FM were kind gifts of Silke Brüderlein and cultured in RPMI (Sigma-Aldrich) and 20% FCS in 5% CO_2.

GSI-I treatment and transfection

Cells were treated for 24h with 2.5 µM and 5 µM GSI-I (Merck Millipore) or 0.002% DMSO as carrier control. Cells were treated at a density of $1x10^6$ cells/ml (lines) and $6.7x10^6$ cells/ml (PBMCs of CLL patients). $5x10^6$ to $1x10^7$ cells were transfected using the Amaxa Nucleofector 2b device (Lonza), cell line nucleofector kit V, program X-001 and 2 µg pcDNA3.1/V5-His-empty-vector or pcDNA3.1-KLF4 and cultured at a density of $2x10^6$ transfected cells/ml for 3h, 10h or 24h.

Cell viability: CTG and FACS staining

After GSI-I treatment, viability was determined using CellTiter-Glo®Luminescent Cell Viability Assay (Promega) as described in the manufacturer's instructions. Apoptosis was measured using Annexin-V-FITC /7AAD flow cytometry. Up to $1x10^6$ cells were stained in 100 µl Annexin-V-buffer with 3 µl Annexin-V-FITC and 3 µl 7AAD (BD Bioscience) and measured on a BD FACSCanto II device.

Cloning of pcDNA3.1-KLF4

For pcDNA3.1-KLF4, *KLF4* cDNA was amplified with primers containing restriction sites for HindIII and NotI and cloned into pcDNA3.1/V5-His using the respective enzymes. Primers are given in supplemental table 4.

Gene expression profiling and DNA-methylation analysis using methyl-CpG-immuno-precipitation (MCIp)

Expression profiling was performed on Illumina BeadChip arrays HumanHT-12v4 with 500 ng RNA by the DKFZ core facility (https://www.dkfz.de/gpcf). MCIp-arrays were performed as described previously.[16] Briefly, after cell homogenization, 2 µg DNA was immuno-precipitated using recombinant MBD2-Fc fusion protein coupled to SIMAG protein-A magnetic beads.

Fractionated elution of the DNA was performed using buffers with increasing salt concentrations. Fractions were purified using the MinElute Kit (Qiagen) and high salt fraction containing highly methylated DNA used for analysis on Agilent promoter arrays. High-salt MCIp elution fractions were labeled with Cy5, the common reference (T-cell pool) was labeled with Cy3. Hybridization was performed according to protocol number G4170-90012 for Agilent MicroarrayAnalysis version1.0. MCIp samples were hybridized onto custom designed (Agilent) promoter tiling-arrays covering -3.8 to +1.8 kb from the TSS of human RefSeq genes. GEO accession numbers are GSE67642 and GSE66514. 2 CLL and 3 normal control samples were excluded as not enough material could be recovered after MCIp. Differentially methylated regions (DMRs) were defined as windows of 500 bp represented by at least two oligonucleotides and displayed a median $|\log_2 FC|$ between CLL and healthy of ≥0.5 ($p \leq 0.05$).

MassARRAY analysis

MassARRAY EpiTYPER was performed using a Sequenom MassARRAY® mass spectrometry System as described before.[17] Bisulfite converted fragments of different sizes (CpG units, "CGU") containing one or more CpGs are compared to 0% and 100% methylated genomic DNA as quality control (primers in supplemental table 4). 0.5 to 1 µg of DNA were bisulfite converted using the EpiTect Bislufite Conversion Kit (Qiagen). 10 ng of converted DNA were used for PCR amplification following desphosphorylation, *in vitro* transcription and T cleavage. Samples were spotted on SpectroCHIPs using the MassARRAY Nanodispenser and subjected to MALDI-TOF mass spectrometry.

Quantitative real time PCR (qRT-PCR) and western blot

For qRT-PCR 0.5 to 1 µg RNA was analyzed using QuantiTect RT (Qiagen) and ABsolute SYBR ROX Mix (Thermo Scientific) on a 7900HT qPCR. Amplification of cDNA in qRT-PCR analysis was done in 40 cycles with the following program: 2 min at 50°C, initial denaturation and enzyme activation for 15 min at 95°C, denaturation for 15 sec at 95°C, annealing and elongation for 1 min at 60°C. A dissociation curve was measured in the range of 60°C to 95°C. Standard curves were template dilutions of cDNA generated from Universal Human Reference RNA (Agilent Technologies) and allowed to determine PCR efficiency and exact quantification of template. Results were normalized to house keeper genes *HPRT* and *PGK* (supplemental table 4).

For western blot, 5 to 20 µg of protein were immunoblotted and probed with anti-KLF4 (H-180) (Santa Cruz Biotechnologies), anti-GAPDH (6C5) (Merck Millipore) and anti-Notch1 (mN1A) (Abcam) antibodies in a 1:1000 dilution.

Statistical Analysis

Students paired t-test and Mann-Whitney rank sum test were used and p-values ≤0.05 were considered significant. Correlations were calculated using Pearson or Spearman rank coefficients. Expression arrays were quantile-normalized and analyzed with the limma package[18] and QIAGEN's Ingenuity Pathway Analysis (IPA, Qiagen).

Supplemental tables

Supplemental table 1: Patient characteristics

CD19+ sorted CLL and B-cells used for MCIp-array and expression array

CD19+ sorted CLL used for validation of expression and methylation with qRT-PCR and MassARRAY

Patients were previously untreated and showed cytogenetically normal karyotype or solely 13q14 deletion and thus represent a cohort of clinically comparable patients in which the potential effects of genetic aberrations on gene expression are minimized.[16,19]

GSI-I treatment of PBMCs from CLL patient samples

Supplemental table 2: Expressionprofiling: top genes deregulated 10h after KLF4 transfection

Supplemental table 3: Ingenuity pathway analysis of top deregulated genes 10h after KLF4 overexpression

Supplemental table 4: Genes of top canonical pathways deregulated in MEC1 and MEC2 10h after KLF4 transfection

Supplemental table 5: List of primers

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