Supplementary information for

SOX4 enables oncogenic survival signals in acute lymphoblastic leukemia

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Table S1: *Sequences of oligonucleotide primers used*

Quantitative RT-PCR

Btg2_F	5'-GATGGCTCCATCTGTGTCCT-3'
Btg2_R	5'-TATACGGTGGCCTGTTGTCA-3'
Rag1_F	5'-TAACAACCAAGCTGCAGACA-3'
Rag1_R	5'-CCTCTGAGGAATCCTTCTCC-3'
Rag2_F	5'-GCAGATGGTAACAGTGGGTC-3'
Rag2_R	5'-ATTGCAGGCTTCAGTTTGAG-3'
Hprt_F	5'-GGGGGCTATAAGTTCTTTGC-3'
Hprt_R	5'-TCCAACACTTCGAGAGGTCC-3'
Mapk8_F	5'-AGCACCAGAGGTCATTCTCG-3'
Mapk8_R	5'-CTGAGTCAGCTGGGAAAAGC-3'
Pik3r1_F	5'-GGATGTGAAGTTGCTCTACCC-3'
Pik3r1_R	5'-GGATTTCCTGGGAAGTACGG-3'
Pik3r2_F	5'-ACAAGACCAGGTGGTGAAGG-3'
Pik3r2_R	5'-TGCATTTCTCCTGTGTCTGG-3'
Pik3r3_F	5'-GATCCAGCTGCGTAAGATCC-3'
Pik3r3_R	5'-TCTGCAACCACAGAACAAGC-3'
Sox4_F	5'-CTGGGGTTGTACGAAGATGG-3'
Sox4_R	5'-AGTTTGAGCTGGGGTTCAGG-3'
Bcl2l1_F	5'-ACAGCAGCAGTTTGGATGC-3'
Bcl2l1_R	5'-AACTTGCAATCCGACTCACC-3'

Quantitative single-locus ChIP

PU.1_region1_F	5'-CAGGGACAGCAAGGAAAAGA-3'
PU.1_region1_R	5'-CCAGGCAAGGGAAGTTTGT-3'
PU.1_region2_F	5'-TTTTCTCAGGCGCCGGCCTT-3'
PU.1_region2_R	5'-CCCCTTCCTCTTGGCGGAAGC-3'
TEAD2_F	5'-CCAAATCCTGAGTTCCTACC-3'
TEAD2_R	5'-ACACACCCCAAAGCAGGATC-3'
MAPK8_F	5'-ACTGTATTGCCTGGCACTGAAT-3'
MAPK8_R	5'-TCTGGTGATTCTGAAAGGGAAC-3'
PIK3R3_F	5'-ATGGCAGGGCACTGGTTTA-3'
PIK3R3_R	5'-TGGTGGGGTTTCAGTTTCT-3'
LCK_F	5'-GGTCTGATAAGTCAGGTCTC-3'
LCK_R	5'-CCTGAGATTGTGAGATCCTG-3'

Table S2: *Antibodies used for flow cytometry and Western blotting*

Antigen	Clone ID	Purchased from
Flow cytometry		
CD19	1D3	BD Biosciences
B220	RA3-6B2	BD Biosciences
Ig κ	187.1	BD Biosciences
Ig λ	JC5-1	Southern Biotech
IgM	R6-60.2	BD Biosciences
CD93		BD Biosciences
CD21	7G6	BD Biosciences
CD23	B3B4	BD Biosciences
c-kit	2B8	BD Biosciences
Sca-1	D7	BD Biosciences
CD43	S7	BD Biosciences
CD25	7D4	BD Biosciences
CD127	A7R34	eBioscience
CD45.1	A20	eBioscience
CD45.2	104	eBioscience
Western blot		
STAT5	#9363	Cell Signaling
STAT5pY^{694}	#9351	Cell Signaling
Sox4	H90	Santa Cruz Biotech.
AKT	#9272	Cell Signaling
AKTpS^{473}	#9271	Cell Signaling
GlobalpY	4G10	Millipore
SRC	36D10	Cell Signaling
SRCpY^{416}	#2101	Cell Signaling
S6	5G10	Cell Signaling
S6pS$^{235/236}$	2F9	Cell Signaling
p19 Arf	ab80	Abcam
p53	1C12	Cell Signaling
ACTB	Polyclonal (ab8227)	Abcam
Chromatin Immunoprecipitation		
SOX4	C20	Santa Cruz Biotech.
IgG control	sc-2028	Santa Cruz Biotech.

Table S3: *Retroviral vectors and transduction methods*

Constitutive expression

BCR-ABL1-IRES-Neo	BCR-ABL1
myr-p110α-IRES-GFP	p110α
myr-AKT-IRES-GFP	Akt
Sox4-IRES-GFP	Sox4
Bcl-xL-IRES-GFP	Bcl-xL
Cre-ER^{T2}-IRES-Puro	Cre
IRES-GFP	empty vector (EV)
IRES-Neo	empty vector (EV)
IRES-Puro	empty vector (EV)

The murine Sox4 was amplified from MSCV-Sox4-puro vector which was a kind gift from Dr. Yang Du (Hematology Branch, NHLBI, Bethesda, MD) and subcloned into an MSCV-IRES-GFP.

Table S4: *Genetic mouse models used in this study*

Mouse strain	Source	Purpose
[a]*Sox4$^{fl/fl}$*	Veronique Lefebvre	Cre mediated deletion of Sox4
NOD/SCID	Jackson Laboratories	Transplant recipient mice

Notes: [a]Penzo-Méndez A, Dy P, Lefebvre V. Generation of mice harboring a Sox4 conditional null allele. *Genesis* 2007*;* 45*:*776-780.

Table S5: *Summary of accession numbers for publicly available microarray and ChIP-data*

GEO Accesion	GEO description	Figure
GSE36543	Effects of inducible *Sox4*-deletion	Figure 1B
GSE11874	ChIP-on-chip data for SOX4	Figure 1D, S5
GSE36543	Clinical and gene expression data; COG P9906	Figure 2H
GSE11877	COG P9906 gene expression studies	Figure 2H
GSE5314	ECOG E2993 data	Figure 2G
GSE4698	ALL-REZ BFM 2002; Berlin-Frankfurt-Muenster	Figure S8

Table S6: *Overview over patient-derived samples of Ph^+ ALL*

Case	Cytogenetics	Gender/Age	*BCR-ABL1*		Clinical course
Primary Cases					
BLQ5	FISH der(9), der(22)	f	p190	T315I	Relapse (Imatinib)
SFO2	t(9;22)(q34;q11)	m/7	p210	unmutated	At diagnosis
TXL3	t(9;22)(q34;q11)		p210	unmutated	At diagnosis
Cell Lines					
BV173	add(1)(q42), der(22)t(9;22)(q34;q11)	m/45	p210	unmutated	LBC
Nalm1	der(7)t(7;9;15)(q10;?;q15), t(9;9)(p24;q33-q34) t(9;22), (q34;q11), der(15)t(7;9;15)	f/3	p210	unmutated	LBC
SUP-B15	t(1;1)(p11;q31) der(4)t(1;4)(p11;q35), t(9;22)(q34;q11)	m/9	p190	unmutated	Relapse
TOM1	der(9)del(9)(q13q34), p190 der(22)t(9;22)(q34;q11)	f/54	p190	unmutated	Refractory

Notes: All primary samples are bone marrow biopsies, blast content >80%; LAX, Los Angeles; BLQ, Bologna; TXL, Berlin; SFO, San Francisco; ICN, Seoul; n.d., not done; f, female; m, male; LBC, lymphoid blast crisis.

Supplemental Methods

Retroviral supernatant production and transduction

Transfections of the various retroviral constructs were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with plain DMEM media (Invitrogen). Retroviral supernatant was produced by co-transfecting 293FT cells with the plasmids pHIT60 (gag-pol) and pHIT123 (ecotropic env; kindly provided by Donald B Kohn, UCLA). Cultivation was performed in high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with GlutaMAX containing 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25mM HEPES, 1 mmol/l sodium pyruvate and 0.1 mmol/l non-essential amino acids. Regular media was replaced after 16 hours by growth media containing 10 mmol/l sodium butyrate. After 8 hours incubation, the media was changed back to regular growth media. 24 hours later, the virus supernatant were harvested, filtered through a 0.45 μm filter and loaded by centrifugation (2000 x g, 90 min at 32 °C) two times on 50 μg/ml RetroNectin (Takara, Madison, WI) coated non-tissue culture 6-well plates. 1-2 x 10^6 pre-B cells were transduced per well by centrifugation at 600 x g for 30 minutes and maintained for about 48 hours at 37°C with 5% CO_2 before transferring into culture flasks. The various retroviral vectors used in this study are mentioned in Table S3.

Murine pre-B cell culture and BCR-ABL1 transformation

Bone marrow from inducible knockout mice was harvested and bone marrow cells were cultured either in the presence of 10 ng/ml IL7 or retrovirally transformed by BCR-ABL1 as described below. All pre-B cells derived from bone marrow of mice were maintained in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, CA) with GlutaMAX containing 20% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 10 ng/ml recombinant mouse IL-7 (Peprotech, Rocky Hill, NJ) at 37°C in a humidified incubator with 5% CO_2. Two days post transduction with BCR-ABL1, IL-7 was slowly diluted in the culture to select for IL7 independent BCR-ABL1 transformed cells. The different mouse strains used in this study are mentioned in Table S4. All mouse experiments were subject to institutional approval by Children's Hospital Los Angeles IACUC.

Gene expression - microarray analysis

Total RNA from cells used for microarray was isolated by RNeasy purification. Biotinylated cRNA was generated and fragmented according to the Affymetrix protocol and hybridized to 1.0 ST mouse microarrays (Affymetrix). After scanning (GeneChip Scanner 3000 7G; Affymetrix) the GeneChip arrays, the generated .cel files were imported to Partek Genomics Tool (licensed to University of Southern California) and processed using the RMA average algorithm for normalization and summarization.

Separately for each perturbation (overexpression, deletion or drug treatment) dataset, each gene row was mean centered and scaled by the standard deviation. For each perturbation and each gene a *perturbation score* was calculated. This *perturbation score* combines the fold-change difference and t-test between the perturbation samples and controls and corresponds to the projection of the ($\log_2$ average) fold-change and the (signed $\log_{10}$) t-test values onto their first principal component axis. A combined perturbation signature was calculated by averaging the *perturbation scores* of individual signatures. Pair-wise Spearman rank correlation coefficients (ρ) were calculated for the perturbation signatures and the corresponding correlation matrix was clustered by a complete-linkage hierarchical clustering procedure with 1- ρ as the distance metric (R package). In addition, the overlap between signatures was assessed with the RRHO algorithm (Plaisier et al., 2010).

Array-based methylation analysis using HELP

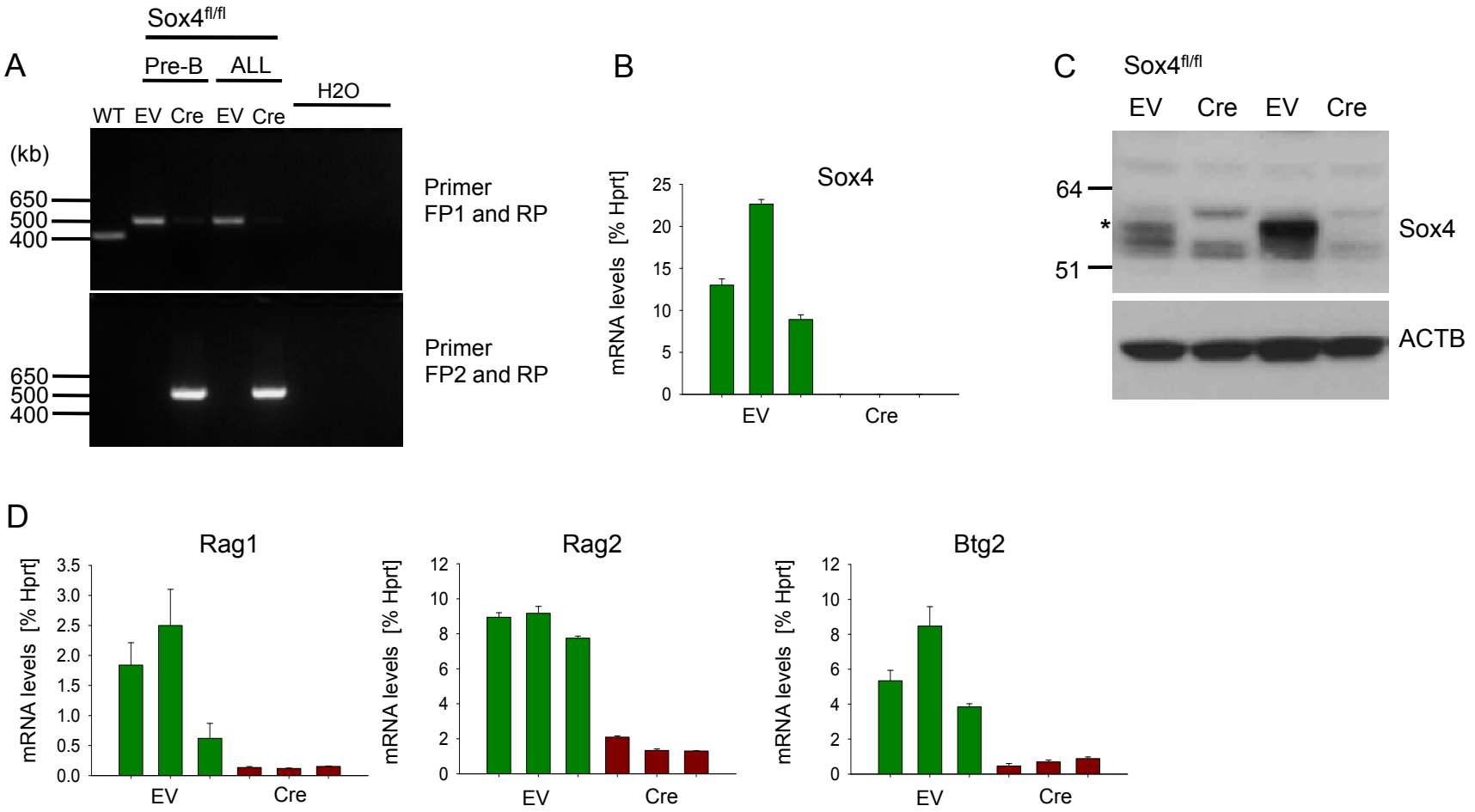
The HELP (HpaII tiny fragment enrichment by ligation mediated PCR) assay was performed as previously published (Khulan et al. 2006). One microgram of high molecular weight DNA was digested overnight with isoschizomer enzymes HpaII and MspI respectively (NEB, Ipswich, MA). DNA fragments were purified using phenol/chloroform, resuspended in 10mM Tris-HCl pH 8.0, and used immediately to set up the ligation reaction with MspI/HpaII-compatible adapters and T4 DNA ligase. Ligation-mediated PCR was performed with enrichment for the 200 to 2000 base pair (bp) products and then submitted for labeling and hybridization onto a human HG_17 promoter custom-designed oligonucleotide array covering 25,626 HpaII amplifiable fragments within the promoters of the genes. HELP data analysis was performed as described previously (Thompson et al. 2008), using R software. Basically, signal intensities at each HpaII amplifiable fragment were calculated as a robust (25% trimmed) mean of their component probe-level signal intensities. Any fragments found within the level of background MspI signal intensity, measured as 2.5 mean-absolute- deviation (MAD) above the median of random probe signals, were considered as "failed" probes and removed. A median normalization was performed on each array by subtracting the median log-ratio (HpaII/MspI) of that array (resulting in median log-ratio of 0 for each array).

References

B Khulan, RF Thompson, K Ye, MJ Fazzari, M Suzuki, E Stasiek, ME Figueroa, JL Glass, Q Chen, C Montagna, E Hatchwell, RR Selzer, TA Richmond, RD Green, A Melnick, JM Greally, Comparative isoschizomer profiling of cytosine methylation: the HELP assay. *Genome Res* 16, 1046-1055 (2006).

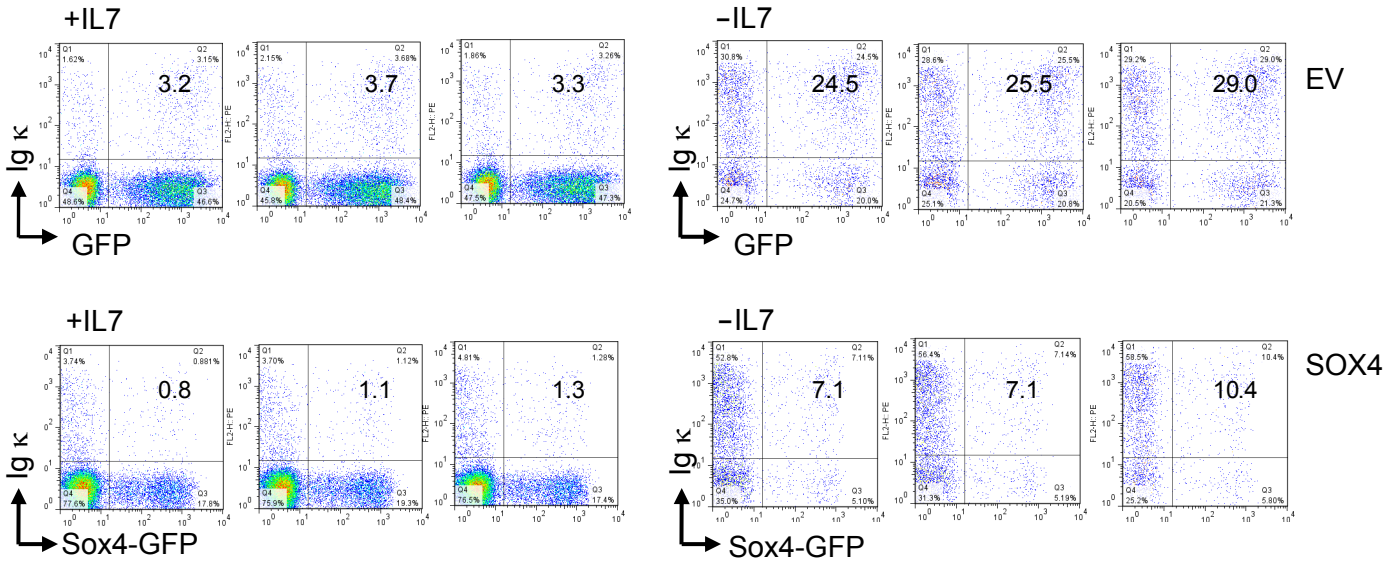
RF Thompson, M Reimers, B Khulan, M Gissot, TA Richmond, Q Chen, X Zheng, K Kim, JM Greally, An analytical pipeline for genomic representations used for cytosine methylation studies. *Bioinformatics* 24, 1161-1167 (2008).

Figure S1: *Inducible activation of Cre and deletion of Sox4*



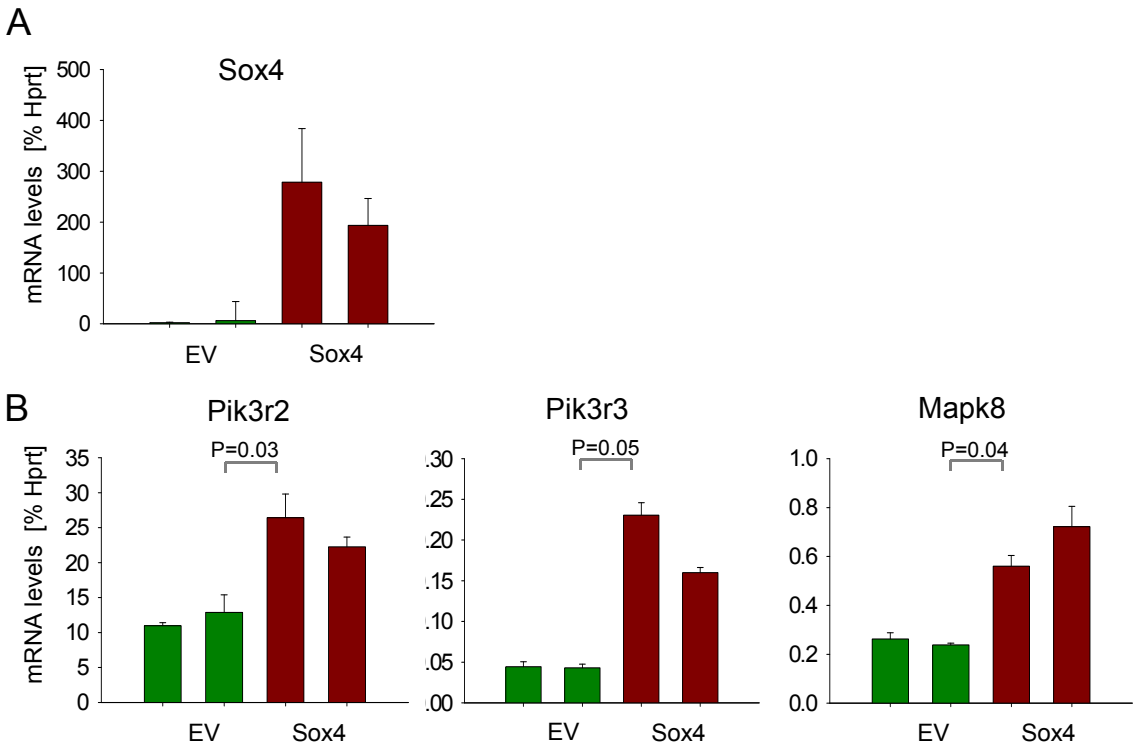
Pre-B cells and BCR-ABL1-transformed mouse pre-B ALL from bone marrow of *Sox4*$^{fl/fl}$ mice were transduced with 4-hydroxy tamoxifen (4-OHT)-inducible Cre (Cre-ERT2) or an ERT2 empty vector control (EV). Cre-mediated deletion of Sox4 was induced by treatment with 4-OHT for 2 days. Deletion of *Sox4* in pre-B cells and pre-B ALL cells was verified at the genomic locus by PCR (A), at the mRNA level by quantitative RT-PCR (B) and protein level by Western blot using β-actin as loading control (C). In (A), two primer sets were used to distinguish between *Sox4* wildtype and *Sox4*^{fl} alleles (FP1-RP) and to amplify the genomic locus after deletion of *Sox4* including the LoxP sites (FP2-RP). Figure 1B shows a microarray analysis of Sox4-dependent gene expression changes. In (D), verification of differential expression of Rag1, Rag2 and Btg2 upon Cre-mediated deletion of Sox4 are shown (quantitative RT-PCR).

Figure S2: *Deletion of Sox4 does not affect IL7-responsiveness and differentiation beyond the pre-B cell checkpoint*



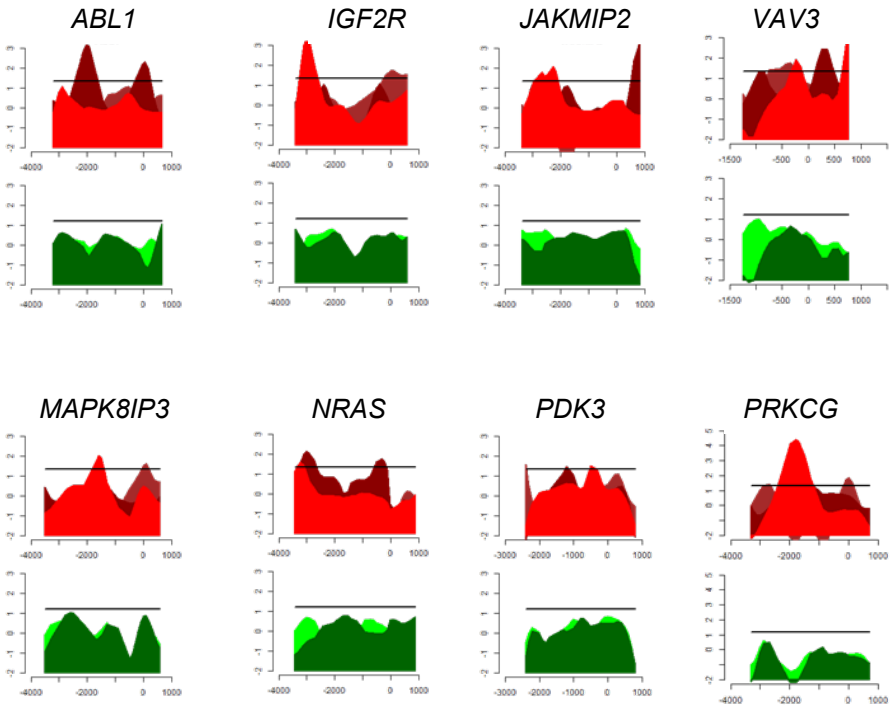
IL7-dependent pre-B cells were transduced with a retroviral (MSCV-based) expression vector for Sox4 tagged to GFP. We then used withdrawal of IL7 to induce differentiation of pro- and pre-B cells. As shown by us and others, withdrawal of IL7 results in Vκ-Jκ immunoglobulin light chain gene rearrangement and subsequent surface expression of κ light chains[9-10]. Here we tested if overexpression of Sox4-GFP changes the ratio of surface κ light chain expressing pre-B cells. No significant differences were observed.

Figure S3: *Overexpression of Sox4 increases expression levels of mediators of PIK3/AKT and MAPK signaling BCR-ABL1 ALL*



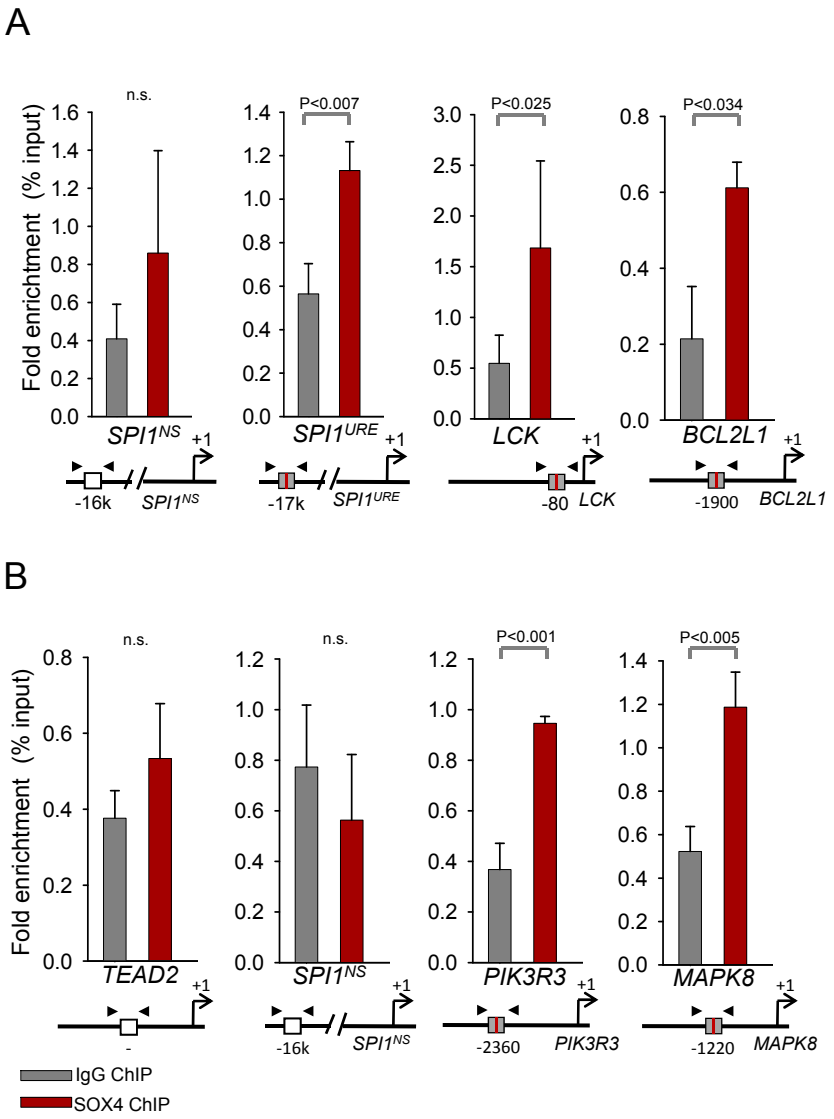
BCR-ABL1-transformed mouse pre-B ALL cells were transduced with an MSCV-based retroviral expression vector for Sox4 tagged with GFP. Overexpression of Sox4 was verified by quantitative RT-PCR. Figure 3-4 demonstrated binding of Sox4 to Pik3r2, Pik3r3 and Mapk8 loci and loss of Pik3r2, Pik3r3 and Mapk8 mRNA expression upon inducible deletion of Sox4. The quantitative RT-PCR analysis here shows that Sox4 overexpression has the opposite effect.

Figure S4: *SOX4 binds to promoter regions of genes involved in PI3K-AKT and MAPK signaling*



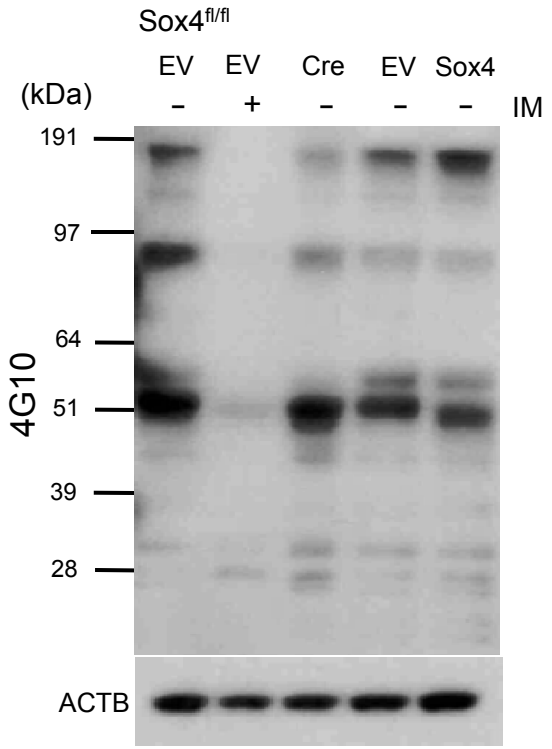
In addition to the loci depicted in Figure 2D, other genes with known roles in AKT/PI3K and MAPK signaling are direct targets of SOX4 binding. The profiles above represent analyses based on data from Scharer et al.[15] (GSE 11874). The red histograms indicate enrichment [% input] using a SOX4 IgG (Table S2), whereas the green histograms indicate negative controls for the same loci using an IgG isotype control.

Figure S5: *Single locus quantitative ChIP confirms SOX4 binding to promoter regions of genes involved in PI3K-AKT and MAPK signaling*



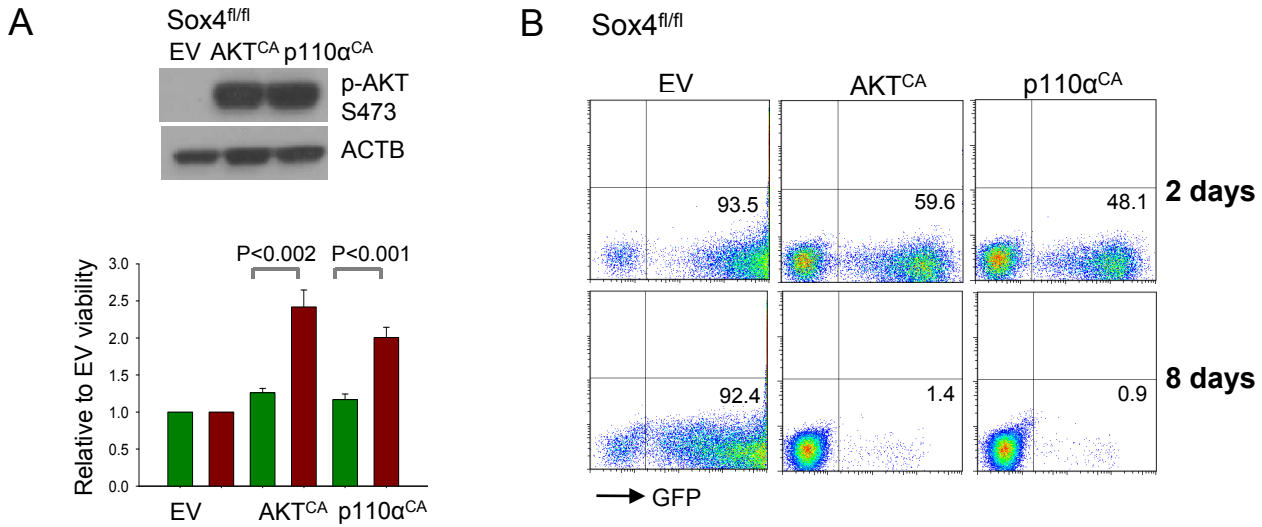
Single locus quantitative ChIP verified promoter binding of SOX4 target genes. In addition to the loci depicted in Figure 2F, an upstream regulatory element (URE) in *SPI1* along with *LCK* served as a positive signal, while a random region in the *SPI1* locus confirmed no enrichment for SOX4 (A). SOX4 binds to the locus of *BCL2L1* in addition to Figure 2F at a different region. In (B), verification of target genes of single locus quantitative ChIP is shown in BLQ5 cells.

Figure S6: *Inducible deletion of Sox4 affects tyrosine phosphorylation events*



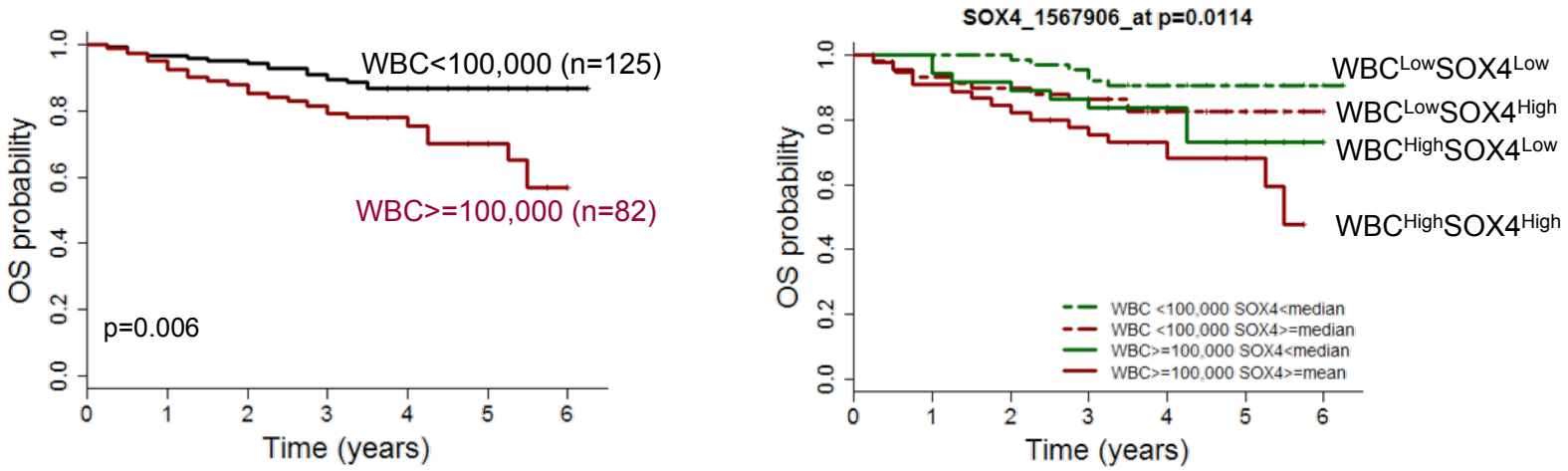
BCR-ABL1-transformed mouse pre-B ALL from bone marrow of *Sox4*$^{fl/fl}$ mice were transduced with 4-hydroxy tamoxifen (4-OHT)-inducible Cre (Cre), Sox4 (Sox4) or an empty vector control (EV). Based on this system, we tested the effect of three expression levels of Sox4 on global tyrosine phosphorylation in BCR-ABL1-transformed mouse pre-B ALL. Global tyrosine phosphorylation events were measured as endpoint and visualized by Western blot using a global p-Y (4G10) antibody and β-actin as negative control. Imatinib-treatment (IM) was used as a reference.

Figure S7: *Loss of viability upon Sox4 deletion is rescued by active mutants of AKT and P110α*



BCR-ABL1-transformed mouse pre-B ALL from bone marrow of *Sox4*$^{fl/fl}$ mice were transduced with 4-hydroxy tamoxifen (4-OHT)-inducible Cre, Sox4 or an empty vector control (EV). Upon deletion of Sox4, the cells were transduced with a retroviral vector of myr-AKT or myr-p110α tagged to GFP. Percentage of viable cells was measured on day 2 (A) and normalized to EV (empty vector control) viability measurement (set as 100%). The percentage of GFP^{+} cells carrying the active mutants of AKT and p110α was abolished after a few days (B).

Figure S8: *Multivariate analysis for SOX4 as outcome predictor in COG P9906*



Patients with B ALL (COG clinical trial, P9906, n=207) were segregated into four groups based on high or low WBC counts (White Blood Cells) and on the greater or lower expression of SOX4 in respect to the median expression value of the *SOX4* probeset. A log rank test was performed to compare survival differences and significance between patient groups (p=0.0114).