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## ***SOX4 enables oncogenic survival signals in acute lymphoblastic leukemia***

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**Abbreviations:** ALL, acute lymphoblastic leukemia; ChIP, chromatin immunoprecipitation; EV, empty vector; IL, interleukin; IM, Imatinib

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**Running title:** Sox4 is required for AKT/PI3K signaling in ALL

**The SOX4 transcription factor mediates early B cell differentiation. Compared to normal pre-B cells, *SOX4* promoter regions in Ph<sup>+</sup> ALL cells are significantly hypo-methylated. Loss- and gain-of-function experiments identified SOX4 as a critical activator of PI3K/AKT and MAPK signaling in ALL cells. ChIP experiments confirmed that SOX4 binds to and transcriptionally activates promoters of multiple components within the PI3K-AKT and MAPK signaling pathways. Cre-mediated deletion of *SOX4* had little effect on normal pre-B cells but compromised proliferation and viability of leukemia cells, which was rescued by BCL2L1 and constitutively active AKT and p110 PI3K. Consistent with these findings, high levels of SOX4 expression in ALL cells at the time of diagnosis predicted poor outcome in a pediatric clinical trial (COG P9906). Collectively, these studies identify SOX4 as a central mediator of oncogenic AKT/PI3K and MAPK signaling in ALL.**

## Introduction

The SOX4 (SRY-related HMG-box) transcription factor is expressed in early B- and T- cell development similar to LEF1<sup>Ref1</sup>. In the absence of SOX4, B cell development is arrested at the pro-B to pre-B cell transition<sup>2</sup>. In Sox4<sup>-/-</sup> mice, pro-B cells fail to proliferate in response to IL7 and to expand and differentiate past the pre-B cell receptor checkpoint<sup>2</sup>. Interestingly, SOX4 functions as transcription factor yet closely interacts with membrane-proximal cytokine receptor signaling<sup>3</sup>. The PDZ domain-containing adaptor protein syntenin (*SDCBP*) recruits the Sox4 protein directly to the cytoplasmic tail of the IL5R $\alpha$  chain<sup>3</sup>. With regard to its receptor-proximal activation, Sox4 resembles the SMAD and STAT transcription factors. In addition to its IL5R $\alpha$  interaction, Sox4/syntenin associates with the cytoplasmic tails of other transmembrane receptors, including syndecans and ephrins<sup>4-5</sup>. Since IL5R $\alpha$  signaling is dispensable for early B cell development, the receptor system that recruits syntenin-mediated activation of Sox4 in early B cell development remains to be identified. A role for Sox4 in acute myeloid leukemia was proposed based on the identification of viral insertions, activating Sox4 expression in leukemias developing in mice carrying endogenous retroviruses<sup>6</sup>. Overexpression of Sox4 was also found to block

differentiation of myeloid progenitor cells<sup>7</sup>. Importantly, Sox4-overexpression causes myeloid leukemia<sup>6</sup> and thereby cooperates with haploinsufficiency of the myeloid differentiation factor PU.1<sup>Ref.8</sup>. In acute lymphoblastic leukemia (ALL), we found SOX4 was strongly upregulated upon tyrosine kinase inhibitor (TKI) treatment and high expression of Sox4 correlates with poor clinical outcome of patients with ALL. However, the role of Sox4 in ALL cells remains unclear. In this study, using a conditional Sox4 knockout mouse model, we showed that the deletion of *Sox4* compromised proliferation and viability of leukemia cells and that SOX4 plays as a central mediator of oncogenic AKT/PI3K signaling in ALL.

## Methods

### *Pre-B and leukemia cell culture*

The work described here involves animal experiments (approved by Children's Hospital Los Angeles IACUC) and analysis of clinical data (No Human Subjects, Exempt #4; IRB approval under COG and ECOG Study protocols). Primary human leukemia cells were cultured on OP9 stroma cells in alpha minimum essential medium without ribonucleotides and deoxyribonucleotides (MEMα, Invitrogen), supplemented with 20% FBS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 100 IU/ml penicillin and 100 mg/ml streptomycin. Human ALL cell lines were maintained in RPMI with GlutaMAX (Invitrogen) containing 20% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin. Mouse BCR-ABL1 transformed pre-B cells were cultured in IMDM (Invitrogen, Carlsbad, CA) with GlutaMAX containing 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µmol/l β-mercaptoethanol. Normal mouse pre-B cells were cultured in the presence of 10 ng/ml IL7.

### *Colony forming assay*

The methylcellulose colony-forming assays were performed with 10,000 BCR-ABL1-transformed mouse pre-B ALL cells. Cells were resuspended in mouse MethoCult medium (StemCell Technologies) and cultured on 3 cm diameter dishes, with an extra water supply dish to prevent evaporation. After 7–22 days, colony numbers were counted.

### *Quantitative RT-PCR*

Total RNA from cells was extracted using RNeasy isolation kit from Qiagen (Valencia, CA). cDNA was generated using a poly(dT) oligonucleotide and the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed with the SYBRGreenER mix (Invitrogen) and the ABI7900HT real-time PCR system (Applied Biosystems, Foster City, CA) according to standard PCR conditions. Primers for quantitative RT-PCR are listed in Table S1.

### *Single-locus quantitative ChIP and ChIP-on-chip analysis*

ChIP assays were performed with modifications as described (Reynaud et al., 2008). Briefly,  $1 \times 10^7$  human pre-B cells were cross-linked with 1% formaldehyde. After sonication by a Q700 (Qsonica), immunoprecipitations were performed using 5 µg Sox4 or control IgG antibody. Complexes were washed with low and high salt buffers, eluted, and reverse-crosslinked, and the DNA was precipitated. Immunoprecipitated DNA sequences were analyzed by qPCR (Antibodies and primer sequences used for qChIP analyses are listed in Table S2C and S1 respectively). *TEAD2* and *SPII* were used as a negative control while *LCK* and an upstream regulatory of *SPII* were used as a positive control<sup>8,20</sup>. SOX4 ChIP-on-Chip was performed in the prostate cancer cell line (LNCaP) in triplicates for SOX4-YFP and in duplicates for YFP empty vector control (GSE11874<sup>Ref.15</sup>). Briefly, the Sox4 ChIP and input from each sample were Cy3/Cy5 fluorescent labeled and co-hybridized to NimbleGen 25K human promoter array set (two microarrays that tile 4000bp upstream and 750bp downstream of the transcription start site of a gene promoter on average). Raw hybridization data were Z-score normalized, and ratios of ChIP to input DNA were determined for each sample. In the enrichment plots, the ratios of ChIP vs Input in the gene promoter regions were shown for the three replicates of Sox4 and for the two replicates of control, respectively, and the horizontal lines represent the 95<sup>th</sup> percentile of the ratios of ChIP to input for Sox4 and control samples respectively.

### *Western blotting*

Cells were lysed in CelLytic buffer (Sigma, St. Louis, MO) supplemented with 1% protease inhibitor cocktail (Pierce, Rockford, IL), 1% Phosphatase inhibitor cocktail (Calbiochem) and 1mM PMSF. Protein samples were loaded on NuPAGE (Invitrogen, Carlsbad, CA) 4-12% Bis-Tris gradient gels and transferred on PVDF membranes (Invitrogen, Carlsbad, CA). For the detection of mouse and human proteins by Western blot, primary antibodies were used together with the WesternBreeze immunodetection system (Invitrogen). The antibodies used for Western blotting are listed in Table S2B.

#### *Flow cytometry*

About one million cells/sample were resuspended in PBS blocked using Fc blocker for 10 minutes on ice, followed by staining with the appropriate dilution of the antibodies for 15 min on ice. Cells were washed and resuspended in PBS with Propidium Iodide (0.2 µg/ml) as a dead cell marker. The antibodies used for flow cytometry are listed in Table S2. For Annexin V stainings, Annexin V binding buffer (BD Biosciences) was used instead of PBS and 7AAD (BD Biosciences) instead of PI. PE labeled Annexin V was purchased from BD Biosciences. For BrdU stainings the BrdU Flow Kit was purchased from BD Biosciences and performed according to the manufacturer protocol.

#### *Rescue assays, PI3K, AKT and BCL2L1*

*BCR-ABL1* transformed murine *Sox4*<sup>f/f</sup> pre-B ALL cells were transduced with inducible Cre-puromycin or an empty vector control. Deletion of *Sox4* in *Sox4*<sup>f/f</sup> pre-B ALL cells was induced with 4-hydroxy-tamoxifen (4-OHT) for two days and cells were transduced with the retroviral vector to express a molecule of interest, myr-AKT, myr-p110 $\alpha$  subunit of PI3K or BCL2L1 (Bcl-xL) tagged to GFP. Percentage of viable cells was measured on day 2. The percentage of viable transduced cells normalized to EV control viability measurement (set as 100%).

#### *In vivo leukemia cell transplantation*

*BCR-ABL1* transformed murine *Sox4*<sup>f/f</sup> pre-B ALL cells were transduced with inducible Cre-puromycin or an empty vector control. Puromycin-resistant cells were selected two days after transduction and 1 x 10<sup>6</sup> leukemia cells were injected into sublethally irradiated (250 cGy) NOD/SCID mice. Seven mice per

group were injected via tail vein injection. When a mouse became terminally sick, it was sacrificed and bone marrow and spleen were collected for flow cytometry analysis.

#### *Patient outcome and gene expression microarray data*

Patient outcome and RMA normalized gene expression microarray data were obtained from the National Cancer Institute TARGET Data Matrix ([http://target.nci.nih.gov/dataMatrix/TARGET\\_DataMatrix.html](http://target.nci.nih.gov/dataMatrix/TARGET_DataMatrix.html)) of the Children's Oncology Group (COG) Clinical Trial P9906 (GSE11877<sup>Ref.14</sup>) and from the German Berlin-Frankfurt-Münster (BFM) Clinical Trial ALL-REZ BFM 2002 (GSE4698<sup>Ref.13</sup>). The end point of the clinical data is overall survival (OS) and risk stratification which were determined by a combination of three prognostic factors: intermediate risk, S2; and high risk, S3/S4.

#### *Survival analysis*

Kaplan-Meier survival analysis was used to estimate overall survival (OS). Basically, patients with B-ALL (COG clinical trial, P9906, n=207) were segregated into two groups according whether they had above or below the median expression level of the *SOX4* probeset. Log rank test was used to compare survival differences between patient groups. R package "survival" version 2.35-8 was used for the survival analysis and Cox proportional hazards regression model in R package for the multivariate analysis (R Development Core Team, 2009).

## **Results**

### **Sox4 function in BCR-ABL1 ALL**

Tyrosine kinase inhibitor (TKI) treatment of Ph<sup>+</sup> ALL cells results in strong upregulation of BCL6, which then enables Ph<sup>+</sup> ALL cells to survive TKI treatment in a state of dormancy and quiescence<sup>9-10</sup>. For this reason, we examined other TKI-induced genes for a potential role in leukemia cell survival and TKI-resistance. SOX4 gene expression was strongly upregulated in human Ph<sup>+</sup> and murine BCR-ABL1 driven pre-B ALL by TKI-treatment as verified by qRT-PCR and Western blot (Figure 1). In order to test *SOX4* function in normal pre-B cells and a mouse-model for Ph<sup>+</sup> ALL, we obtained a conditional knockout

mouse model for *Sox4* and isolated bone marrow B cell precursors from *Sox4*<sup>f/f</sup> mice<sup>11</sup> for culture in the presence of IL7 (pre-B cells) or transformation with BCR-ABL1 (model for Ph<sup>+</sup> ALL). To test the function of *Sox4* in a genetic model, we induced *Sox4*-deletion by 4-OHT inducible activation of Cre for two days (Figure S1). Since previous work demonstrated that loss of *Sox4* results in a profound block at the pre-B cell checkpoint<sup>2</sup>, we also studied *Sox4* function in normal *Sox4*<sup>f/f</sup> pre-B cells. Loss of *Sox4* in normal pre-B cells had only mild effects on viability and the ability of pre-B cells to differentiate (Figure 2A, top). Withdrawal of IL7 from normal pre-B cells strongly induces differentiation and Ig light chain gene rearrangement<sup>18</sup>. To examine whether *Sox4* has a measurable effect on early B cell differentiation, we overexpressed *Sox4* in the presence and absence of IL7. However, gain of *Sox4* function did neither affect IL7-responsiveness of normal pro- and pre-B cells, nor the ability of large cycling pre-B cells (Fraction C' according to Hardy nomenclature<sup>17</sup>) to proceed to small resting pre-B cell stages (Fraction D) and to subsequently rearrange Vκ-Jκ light chain gene rearrangements (Figure S2). Compared to normal pre-B cells, *Sox4* ablation in BCR-ABL1 driven pre-B ALL had a severe effect on viability and apoptosis (Figure 2A, bottom). Furthermore, Cre-mediated deletion of *Sox4* in B cell precursor ALL (Figure S1) resulted in G<sub>0</sub>/G<sub>1</sub> arrest of *BCR-ABL1* ALL cells (Figure 2B). To elucidate the function of *Sox4* on colony forming ability, we studied the effect of *Sox4* deletion in methylcellulose serial replating assays. Absence of *Sox4* leads to loss of self-renewal capacity and failure to form colonies in three serial replatings (Figure 2C).

### Mechanism of *Sox4*-mediated survival signaling in ALL

To study the mechanism of *Sox4* regulation in pre-B ALL, we performed a loss-of-function gene expression array experiment (Figure 3A). Deletion of *Sox4* reduced mRNA levels of multiple genes that play a known role in survival signaling in Ph<sup>+</sup> ALL cells including *Bcl2l1* (Bcl-xL), molecules involved in the MAPK pathway *Mapk8*, *Map2k6*, *Rps6ka2* and PI3K/AKT-related molecules *Pik3r2*, *Pik3r3* and *Mtor* (Figure 3A-C). Interestingly, gain-of-function based on *Sox4* overexpression had the opposite effect: Overexpression of *Sox4* resulted in increased mRNA levels of Bcl-xL (*Bcl2l1*; Figure 3C), *Mapk8*

and PI3K/AKT (*Pik3r2*, *Pik3r3*; Figure S3). Since *Sox4* functions as a transcription factor and carries a Sry-related HMG box DNA-binding domain<sup>1</sup>, we identified *Sox4* target genes via promoter binding. ChIP-on-chip analysis in human prostate cancer cells (GS11874) revealed that *Sox4* binds directly to promoters of *BCL2L1* (BCL-XL), genes of the MAPK pathway and multiple genes that are involved in PI3K/AKT signaling (Figure 4A; Figure S4). Single-locus quantitative ChIP confirmed binding of SOX4 to *PIK3R3*, *MAPK8*, *BCL2L1*, *RPS6KA2* and *PDK1* promoters in human Ph<sup>+</sup> ALL cells via its putative DNA binding motif WWCAAWG<sup>1,19-20</sup> (Figure 4B, S5). Transcriptional regulation of components within the PI3K/AKT pathway is indeed functionally relevant since Cre-mediated deletion of *Sox4* caused multiple de-phosphorylation events (Figure S6), including AKT<sup>S473</sup>, SRC<sup>Y416</sup> and RPS6<sup>S235/6</sup> (Figure 5A). While a global analysis of phospho-tyrosine protein changes (using the 4G10 antibody; Figure S6) revealed some increases of tyrosine-phosphorylation, in the vast majority of cases, deletion of *Sox4* caused loss of tyrosine-phosphorylation (Figure S6). Besides its function as transcriptional regulator, some of the de-phosphorylation events may also reflect the ability of *Sox4* to function as cytoplasmic activator of signaling as described previously<sup>3-5</sup>. In addition, inducible deletion of *Sox4* resulted in strong upregulation of both Arf and p53 protein (Figure 5B). Consistent with previous findings<sup>10</sup>, TKI-treatment (Imatinib) resulted in downregulation of Arf expression (Figure 5C). Negative regulation of Arf was dependent on *Sox4*: loss of *Sox4* prevented the downregulation of Arf expression following imatinib treatment (Figure 5C). Loss of viability upon *Sox4* deletion in the leukemia cells was rescued by transduction with BCL2L1 (Bcl-xL; Figure 5D-E) and transiently rescued by constitutively active (myristoylated; CA) mutants of AKT<sup>CA</sup> and the p110 catalytic subunit of PI3K (p110 $\alpha$ <sup>CA</sup>; Figure S7). These findings demonstrate that *Sox4* mediates critical survival signals in Ph<sup>+</sup> ALL via activation of BCL2L1 and PI3K/AKT.

### **Sox4 mediates leukemic transformation *in vivo* and is a predictor of poor clinical outcome**

To test whether Cre-mediated deletion of *Sox4* affects the course of leukemia development *in vivo*, 1 million *Sox4*<sup>f/f</sup> leukemia cells carrying 4-OHT-inducible Cre or an empty vector control were injected

into sublethally irradiated NOD/SCID mice. Leukemia cells were labeled with firefly luciferase and leukemic expansion was monitored by luciferase bioimaging. *Sox4*<sup>f/f</sup> leukemia cells rapidly expanded (Figure 6A) and caused lethal disease in all recipient mice within 15 days (Figure 6B). Inducible deletion of *Sox4* did not cause significant phenotype changes of leukemia (Figure 7) but delayed the onset of disease and substantially prolonged overall survival of recipient mice (Figure 6A-B). These findings are also relevant to human disease since promoter CpG methylation analysis (HELP assay<sup>12</sup>) revealed lower levels *SOX4* promoter methylation in human Ph<sup>+</sup> ALL cells compared to their normal pre-B cell counterparts (Figure 6C). In addition, samples from patients with high risk ALL (n=31) show a trend towards higher *SOX4* mRNA levels compared to patients with intermediate risk ALL (n=23, p=0.07, Figure 6D; ALL REZ BFM 2002<sup>Ref13</sup>, GSE4698). Analyzing data from a larger study based on 207 patients with ALL (COG P9906<sup>Ref14</sup>) showed a statistically significant association between high mRNA levels of *SOX4* and poor clinical outcome (shorter overall survival; Figure 6E and S8).

To determine if *Sox4* expression represents an independent predictor of poor clinical outcome, we have performed a multivariate analysis of *Sox4* expression comparing overall survival (OS) time in relation to Age, white blood cell count (WBC), IKZF1 and CDKN2A expression in a multivariate Cox proportional hazards regression model in R package (R Development Core Team, 2009). These analyses indeed identified *Sox4* expression levels as an independent predictor of outcome [OS time] with respect to Age, WBC, IKZF1 expression and CDKN2A expression (p= 0.0434, HR=1.6481, 95% confidence interval 1.0150: 2.676; Figure S8).

Collectively, these findings identify *SOX4* as a critical upstream regulator of survival signaling in Ph<sup>+</sup> ALL. Pathways affected by *SOX4* include PI3K/AKT signaling downstream of BCR-ABL1, activation of BCL-xL (*BCL2LI*) and negative regulation of Arf and p53.

## Discussion

In this study, we identified *Sox4* as a critical factor in survival signaling of Ph<sup>+</sup> ALL. *Sox4* regulates survival via Bcl-xL, Mapk8 (JNK1) and genes of the PI3K-AKT-pathway. A recent study based on

pharmacological targeting of the PI3K/AKT downstream signaling highlighted the relevance of this pathway in human Ph<sup>+</sup> ALL<sup>16</sup>. The BCR-ABL1 oncogene has been shown to activate the JNK pathway<sup>21</sup> and disruption of Mapk8 (JNK1) results in defective transformation of BCR-ABL1 driven pre-B ALL<sup>22</sup>. In Ph<sup>+</sup> leukemia, Bcl-xL represents an important survival molecule and Stat5-dependent transcriptional activation of Bcl-xL promotes inhibition of apoptosis<sup>26</sup>. Therefore, the discovery of SOX4 as a critical upstream regulator to enable Bcl-xL, JNK1 and PI3K/AKT signaling in Ph<sup>+</sup> ALL represents an important finding. The specific rescue by Bcl-xL and active mutants of AKT/PI3K of loss of viability upon Sox4 deletion provides mechanistic evidence that Sox4 regulates leukemia cell survival through these components (Figure 5D-E, S7). In addition, Sox4 negatively regulates p53/Arf (Figure 5B-C). Since our ChIP analyses did not reveal direct binding of Sox4 at CDKN2A (arf) and TP53 (p53) promoters, these effects may be indirect, e.g. mediated via PI3K/AKT. Given that Arf and p53 represent critical negative regulators of self-renewal<sup>25</sup>, Sox4-mediated negative regulation of Arf/p53 likely contributes to the important function of Sox4 in mediating leukemia colony formation in serial replating experiments (Figure 2C). In agreement with reduced expression of Bcl-xL (*Bcl2l1*) and increased levels of Arf/p53, the overall outcome of Sox4 deletion is rapid loss of viability and apoptosis in BCR-ABL1-transformed ALL but not normal pre-B cells (Figure 2A).

Previous studies demonstrated that Bcl-xL regulates survival during early B cell development<sup>23-24</sup>, with high Bcl-xL expression levels in pro-B cells<sup>23</sup>. Likewise, Bcl-xL transgenic mice develop large B cell expansions as a result of increased survival during immunoglobulin V<sub>H</sub>-DJ<sub>H</sub> gene rearrangement at the pro- to pre-B cell transition<sup>24</sup>. The stage-specific function and gradient of BCL-xL expression and Sox4-dependent regulation of BCL-xL is consistent with the pro-B cell differentiation block in Sox4-deficient mice. In the absence of Sox4-induced BCL-xL, pro-B cells are destined to die when they undergo V<sub>H</sub>-DJ<sub>H</sub> gene rearrangement at the pre-B cell checkpoint.

Sox4 amplifications have been associated with human cancers including lung, breast, prostate cancer<sup>15,27-29</sup>. The correlation of high SOX4 expression with poor clinical outcome (Figure 6D-E) is novel and

suggests that SOX4 and SOX4-mediated signaling represent a potential therapeutic target for patients with ALL. While transcription factors are typically considered intractable for pharmacological inhibition, work by us and others<sup>10,30</sup> suggests that BCL6 may represent a notable exception, because targeting is based on protein-protein interactions with critical BCL6-cofactors. In the case of Sox4, we hope that identification of critical co-factors that mediate Sox4-mediated transcriptional activation of BCL2L1 (BCL-xL) and PI3K/AKT will make this oncogenic pathway accessible to small molecule targeting as previously exemplified for BCL6.

**Authorship:** PRR, HG, CH, LNC, ZC performed experiments and analyzed data, HJ and VL provided critical reagents, AM, EP, WLC, CLW analyzed data and provided clinical information, MM designed the research and wrote the manuscript. The authors have no conflicts of interest.

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## Figure legends

### Figure 1: Upregulation of SOX4 in response to TKI-treatment in Ph<sup>+</sup> ALL

Ph<sup>+</sup> ALL (BV173, SUP-B15, TOM1 and NALM1; Table S6) and murine pre-B ALL were treated with the tyrosine kinase inhibitor (TKI) Imatinib for 16 hours (IM; A). Based on gene expression analyses we identified *SOX4* as a gene that is highly upregulated. BCR-ABL1-transformed mouse pre-B ALL cells were treated with or without Imatinib (IM; 2 µmol/l) for 16 hours and mRNA levels of SOX4 were measured by quantitative RT-PCR (B). In (C), three human Ph<sup>+</sup> ALL cell lines were treated with or without Imatinib (IM; 2 µmol/l) for 16 hours and SOX4 protein levels were assayed by Western blot using β-actin as loading control.

### Figure 2: *Sox4* is required for pre-B ALL cell survival and proliferation

Effects of inducible activation of Cre or empty vector (EV) control in *Sox4*<sup>fl/fl</sup> pre-B ALL cells was studied on viability/apoptosis (A), cell cycle regulation (B) and colony formation in methylcellulose (C). In (A), *Sox4*<sup>fl/fl</sup> was deleted in IL7-dependent normal pre-B cells (top) and BCR-ABL1-transformed ALL cells and viability changes were measured in both after two days of induction of Cre. In (B), cells were stained with BrdU and 7AAD after 1 day of *Sox4* deletion and percentages of cells in G0/1, S and G2/M phases of the cell cycle are indicated (n=3; P=0.008 for S phase) and shown in a bar graph (bottom). In (C) images for colonies in a serial replating assay are shown and quantitative analysis (right; n=3).

### Figure 3: Identification of transcriptional targets of SOX4 in Ph<sup>+</sup> ALL

We deleted *Sox4* upon an inducible activation of Cre in *Sox4*<sup>fl/fl</sup> leukemia cells (Figure S1) and studied gene expression changes after 1 day of activation of Cre or an empty vector control (EV; A). *Sox4*-dependent gene expression changes (GSE36543) involve the PI3K/AKT, MAPK and critical survival pathways. Target genes were verified in a qRT-PCR (B-C; Figure S3). In (C), *BCL2L1* is verified as SOX4 target gene in BCR-ABL1 ALL cells through Cre-mediated deletion and retroviral overexpression of *Sox4*.

**Figure 4: SOX4 binds to promoters of PI3K-AKT and MAPK8 pathway genes in Ph<sup>+</sup> ALL**

ChIP-on-chip data of human prostate cancer cells for SOX4 binding<sup>15</sup> was analyzed (GSE11874) and specific binding at BCL-XL (*BCL2L1*), MAPK and PI3K/AKT-related loci is shown (A). Specific binding of SOX4 to PIK3R3, MAPK8, BCL2L1, RPS6KA2 and PDK1 promoters in human Ph<sup>+</sup> ALL cells was verified by single-locus quantitative ChIP (B). The putative DNA bindinig motif (grey box) and primer (arrows) are indicated in the diagram for each of the specific gene locus.

**Figure 5: Sox4 regulates survival signaling via Bcl-xL and the PI3K-AKT pathway in Ph<sup>+</sup> ALL**

Effects of Imatinib-treatment (IM) on phosphorylation status of survival signaling molecules (Stat5, AKT, SRC, RPS6) were compared to effects of Sox4 deletion (A). In the absence of Sox4, activation of AKT (p-AKT<sup>S473</sup>), S6 (p-S6<sup>S235/6</sup>) and SRC (p-SRC<sup>Y416</sup>) were decreased. Effects of Cre-mediated deletion on protein levels of Arf (in the presence and absence of Imatinib-treatment) and p53 were studied by Western blot in (B) and (C). In (D), Sox4<sup>f/f</sup> ALL cells were transduced with an expression vector for Bcl-xL upon induction of Cre-mediated deletion of Sox4. The effects of Bcl-xL leukemia cell survival (D) and quantitative analysis (E;  $P=0.004$ ) are shown.

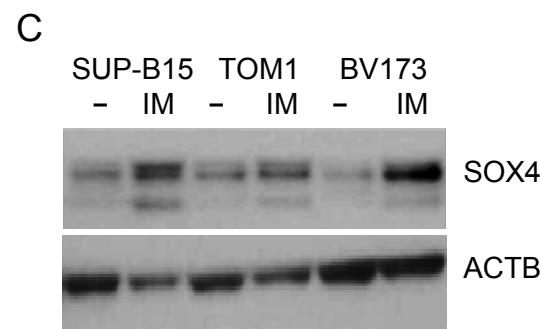
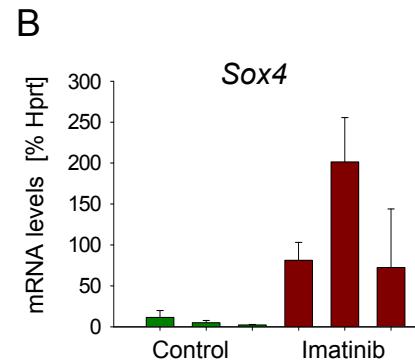
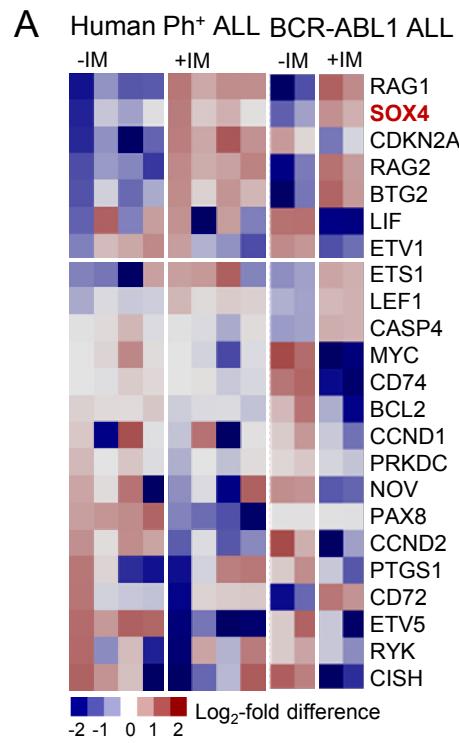
**Figure 6:** *Sox4 promotes leukemic transformation in vivo and is correlated with poor clinical outcome*

Sox4<sup>f/f</sup> ALL cells were transduced with 4-OHT-inducible Cre or an empty vector control (EV) and labeled with firefly luciferase. 1 x 10<sup>6</sup> cells were injected into sublethally irradiated (2.5 Gy) NOD/SCID mice and leukemic expansion was tracked by luciferase bioimaging (A). In (B) overall survival of transplant recipient mice is shown in a Kaplan-Meier analysis (7 mice per group; P=0.0007). The methylation status of the SOX4 promoter was studied (HELP assay<sup>12</sup>; C) in normal human bone marrow pre-B cells (n=12) and bone marrow biopsies of human Ph<sup>+</sup> ALL (n=83; p=1.5e-6; data from ECOG E2993 and ECOG leukemia tissue bank). In (D), samples from patients with high risk ALL (n=31) are compared to patients with intermediate risk ALL in relation to their SOX4 mRNA levels (n=23, p=0.07; ALL REZ BFM 2002<sup>Ref.13</sup>, GSE4698). In (E), we analyzed clinical and gene expression data from the pediatric ALL trial COG P9906<sup>Ref.14</sup> (GSE36543). Samples from 207 patients were divided into two groups according to higher (red curve; n=103) or lower (green; n=104) than mean expression of *Sox4* among all patients. The Kaplan-Meier analysis of overall survival shows a significant difference between the two groups (P=0.046; H).

**Figure 7:** *Phenotype of pre-B ALL upon Sox4 deletion in vivo*

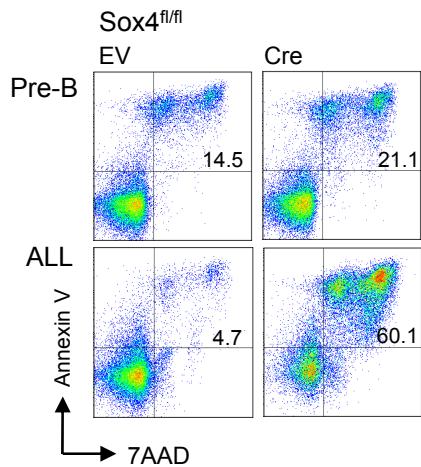
BCR-ABL1-transformed mouse pre-B ALL from bone marrow of Sox4<sup>f/f</sup> mice were transduced with 4-hydroxy tamoxifen (4-OHT)-inducible Cre (Cre) or an empty vector control (EV). 2 x 10<sup>6</sup> leukemia cells were injected into NOD/SCID recipient mice in each group. Phenotypic changes upon deletion of Sox4 were studied by flow cytometry following isolation of leukemia cells from sacrificed animals and staining for c-kit, Sca-1, CD19, B220, IgM, Igκ and Igλ light chains, CD21, CD23, CD43, IL2RA (CD25), IL7R (CD127) and AA4.1 (CD93). BCR-ABL1 transduced ALL cells from donor bone marrow of Sox4<sup>f/f</sup> mice express CD45.2 but not CD45.1. Donor-derived leukemia cells can be identified as CD45.2<sup>+</sup> cells after injection into CD45.1<sup>+</sup> NOD/SCID recipient mice.

**Figure 1:** Upregulation of *SOX4* in response to TKI-treatment Ph<sup>+</sup> ALL

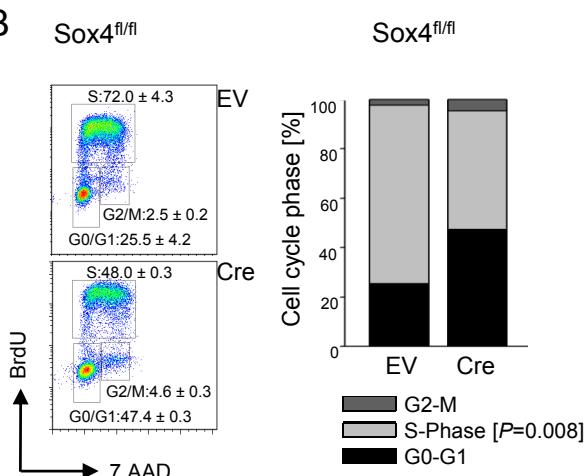


**Figure 2: Sox4 is required for pre-B ALL cell survival and proliferation**

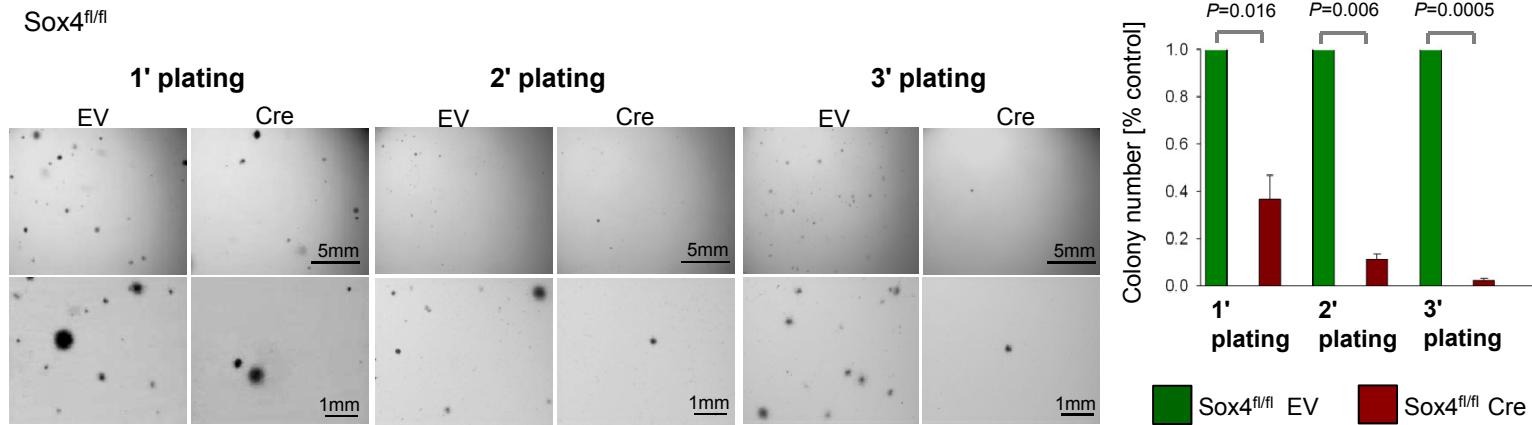
**A**



**B**

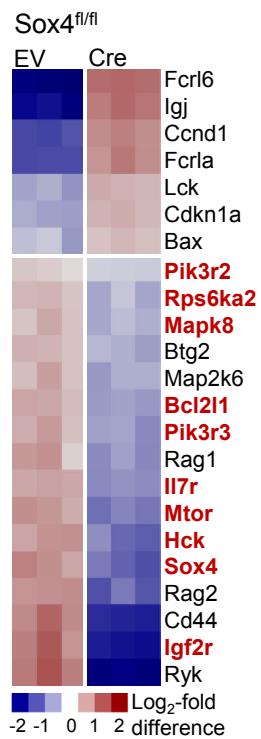


**C**

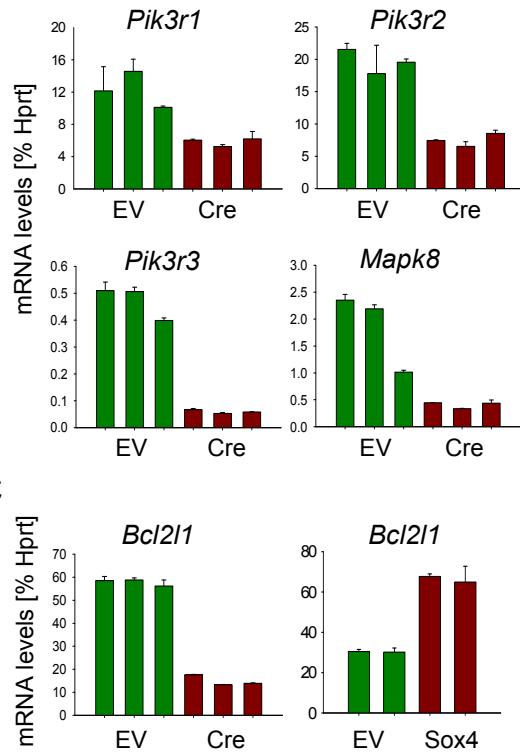


**Figure 3: Identification of transcriptional targets of SOX4 in  $\text{Ph}^+$  ALL**

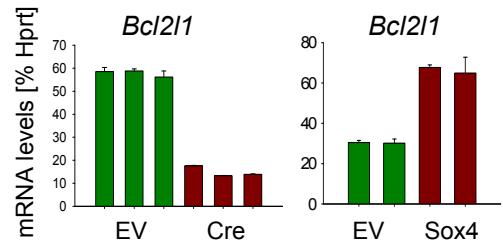
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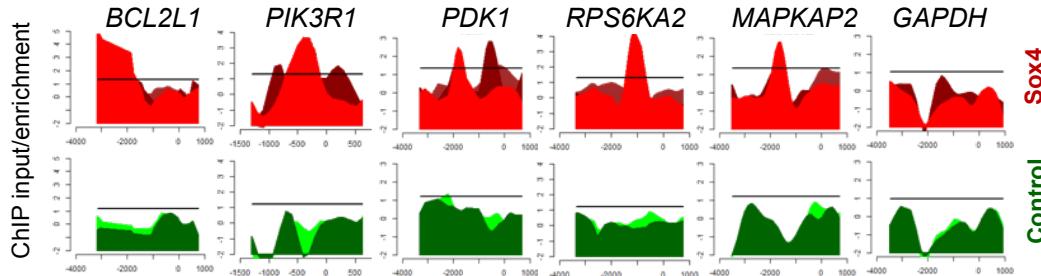


**C**



**Figure 4: SOX4 binds to promoters of PI3K-AKT and MAPK pathway genes in  $Ph^+$  ALL**

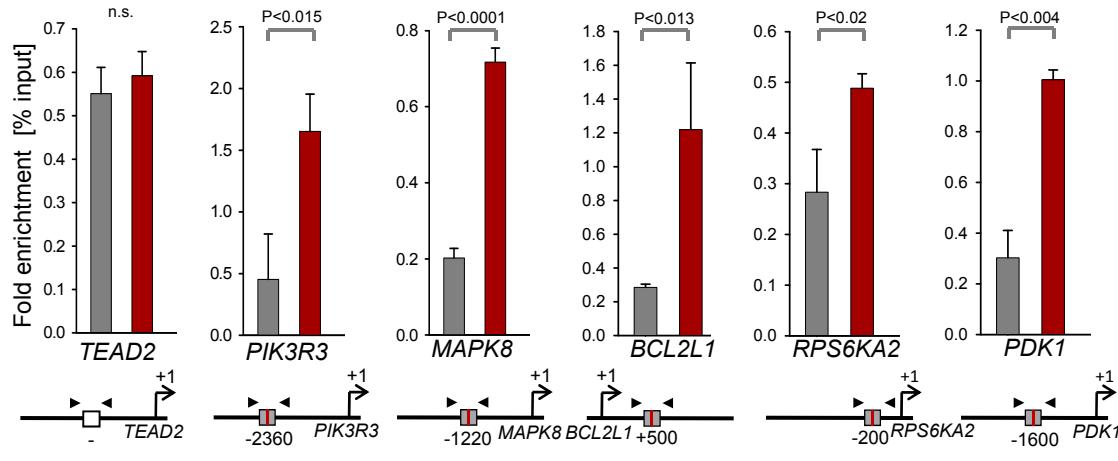
**A**



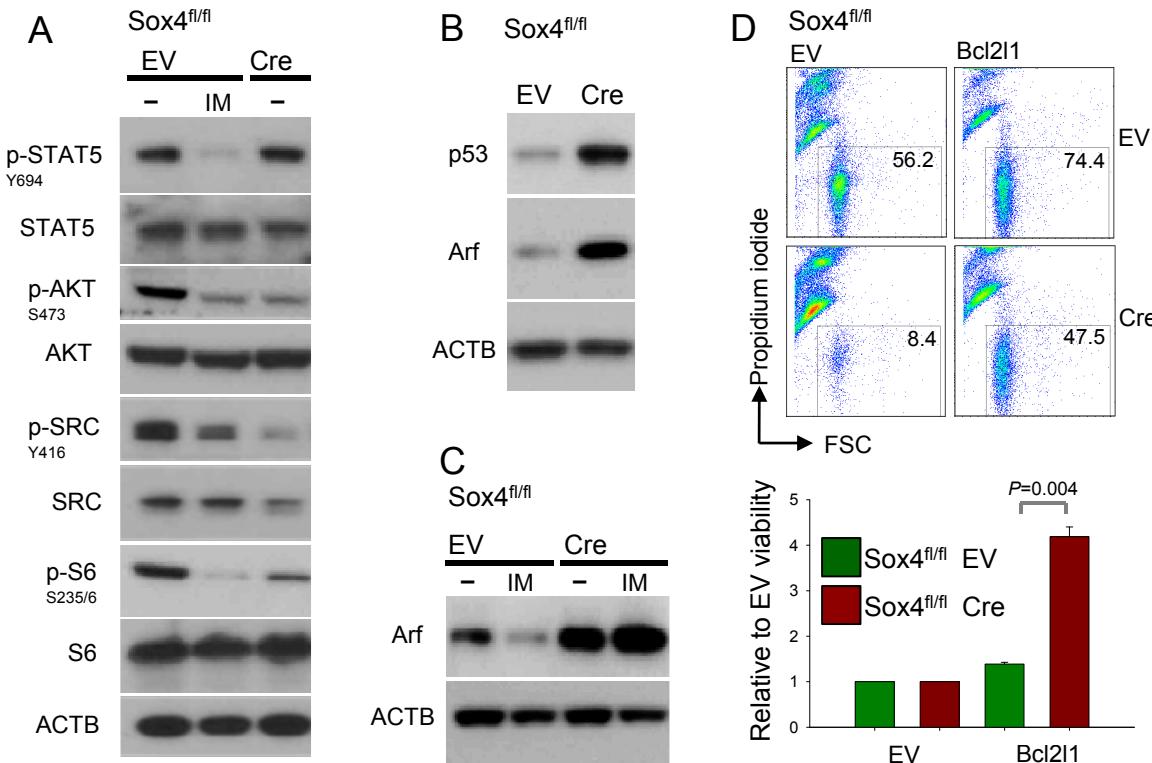
**Sox4**

**Control**

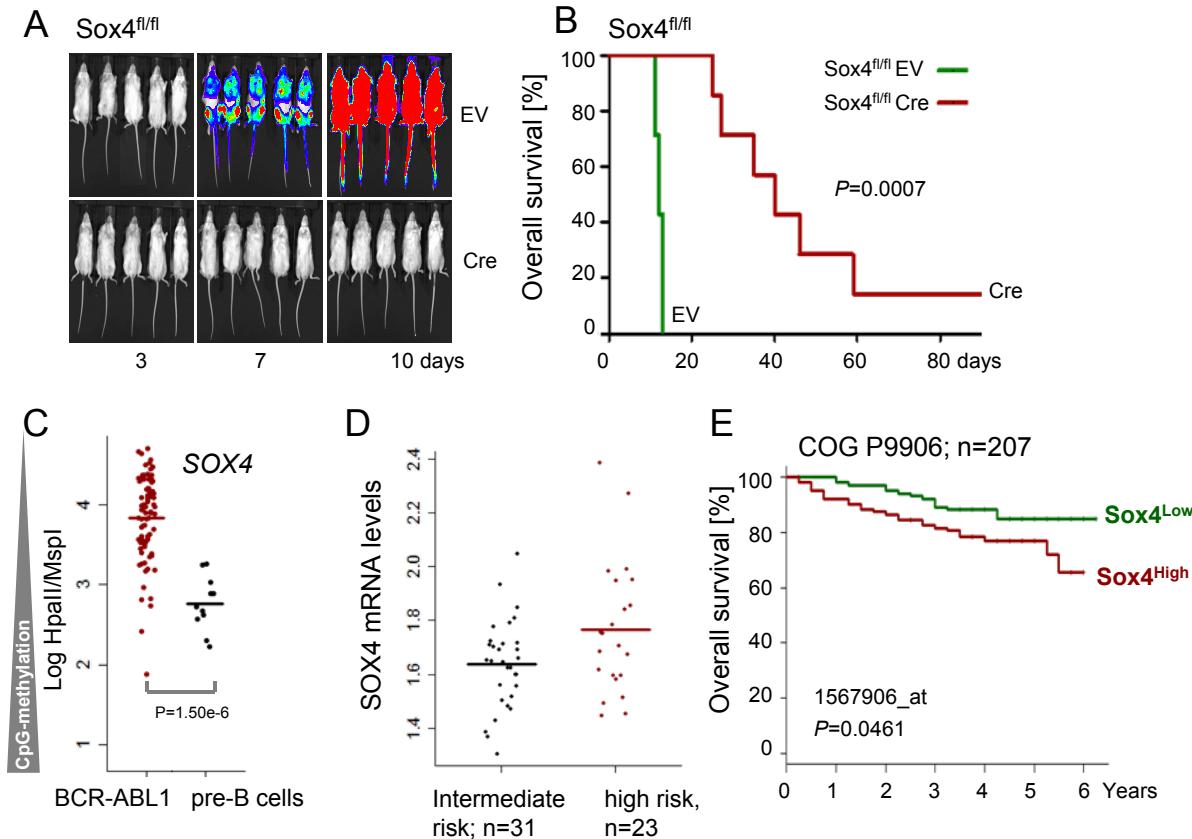
**B**



**Figure 5: SOX4 regulates survival signaling via *Bcl-xL* and the PI3K-AKT pathway in *Ph<sup>+</sup>* ALL**

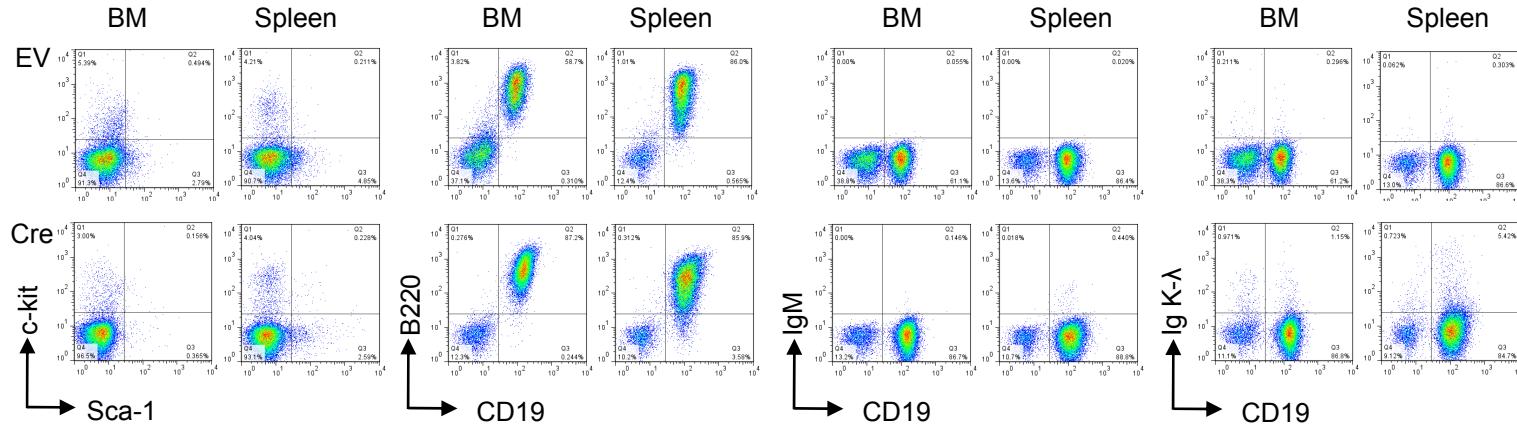


**Figure 6: *Sox4* is promotes leukemic transformation in vivo and is correlated with poor clinical outcome**



**Figure 7: Phenotype of pre-B ALL upon Sox4 deletion in vivo**

Sox4<sup>fl/fl</sup>



Sox4<sup>fl/fl</sup>

