EVALUATING THE PRE-B-CELL RECEPTOR AS A POTENTIAL MEDIATOR OF LEUKEMOGENESIS DOWNSTREAM OF THE TCF3-PBX1 ONCOGENIC TRANSCRIPTION FACTOR

by

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Abstract

Approximately 5% of B-progenitor acute lymphoblastic leukemia (B-ALL) harbor a somatic 1;19 chromosomal translocation which generates a fusion gene coding for TCF3-PBX1. Recent studies have shown that leukemic progenitors with t(1;19) occur in a distinct subset of B-ALL cases that often express a functional pre-B-cell receptor (pre-BCR). It is hypothesized that TCF3-PBX1 drives the leukemogenesis of these cells by promoting aberrant pre-BCR signaling through transcriptional activation of genes encoding pre-BCR components (*IGHM*, *CD79A*, *CD79B*, *IGLL1*, *VPREB1* and *VPREB3*). To test this hypothesis, we have established short hairpin RNA (shRNA) knockdown models in two different t(1;19) B-ALL cell lines (RCH-ACV and 697). Expression of the knockdown shRNA against the *TCF3-PBX1* transcript considerably reduced cell culture-initiation and proliferation. We then performed RNA-Seq in both of our knockdown models and determined that *TCF3-PBX1* knockdown surprisingly *activates*, rather than represses, the expression of *IGHM*, *CD79A*, *CD79B*, *IGLL1*, *VPREB1* and *VPREB3*. Despite this, we observed a marked reduction in phosphorylation and activation of AKT and ERK in *TCF3-PBX1*-silenced cells. Additionally, *TCF3-PBX1* knockdown had no effect on SYK phosphorylation. Altogether, our results suggest that TCF3-PBX1 promotes t(1;19) B-ALL proliferation by activating PI3K/AKT and MAPK/ERK signaling in a pre-BCR independent manner. In considering alternative mechanisms by which TCF3-PBX1 promotes cell proliferation, TCF3-PBX1 activates the expression of genes encoding key downstream pre-BCR signaling molecules such as the Src-family kinase BLK and the atypical protein kinase C PKCζ either of which could promote pro-survival signaling pathways. Alternatively, we propose that TCF3-PBX1 promotes Notch or Rho signaling and that these pathways might crosstalk with the pre-BCR

Co-Authorship

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List of Abbreviations

AD	Activation domain
ALL	Acute lymphoblastic leukemia
B-ALL	B-cell acute lymphoblastic leukemia
BCR	B-cell receptor
BSA	Bovine serum albumin
bp	Base pair
bHLH	Basic helix-loop-helix
CaM	Calmodulin
CaM-Ca^{2+}	Calcium ion-bound calmodulin
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELDA	Extreme limiting dilution assay
FDR	False-discovery rate
FBS	Fetal bovine serum
H-chain	Heavy chain
HSC	Hematopoietic stem cell
ICGC	International Cancer Genome Consortium
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP_3	Inositol-3,4,5-triphosphate
IQR	Interquartile range
ITAM	Immunoreceptor tyrosine-based activation motifs
kb	Kilobase pair
KD	Knockdown
KIX	Kinase-inducible domain interacting domain
MZ B	Marginal-zone B-cell
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PIP_2	Phosphatidylinositol-4,5-biphophate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
pre-BCR	Precursor B-cell receptor
qChIP	Chromatin immunoprecipitation followed by quantitative polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SFK	Src-family kinase
shControl	Control short-hairpin ribonucleic acid
shPBX1	Short-hairpin ribonucleic acid targeting *TCF3-PBX1*
shRNA	Short-hairpin ribonucleic acid
siRNA	Small-interfering ribonucleic acid
SLC	Surrogate light chain

t(1;19)	1;19 chromosomal translocation
T-ALL	T-cell acute lymphoblastic leukemia
TALE	Three amino acid loop extension
TBS	Tris-buffered saline
TG	Target gene
Tris	Trisaminomethane
VDJ	Variable, diversity and joining gene segments

Chapter 1

Literature Review

1.1 Acute lymphoblastic leukemia - overview

Acute lymphoblastic leukemia (ALL) is a malignancy caused by the overproduction of immature lymphocytes from hematopoietic precursor cells located in the bone marrow. Approximately 60% of ALL cases occur in individuals under the age of 20 years[1]. The five-year disease-free survival rate for pediatric ALL is approximately 80%, which is relatively high compared to other cancers[2]. However, since ALL is the most common cancer in young children, it remains a major burden in terms of potential years of life lost in North America[3]. It should also be noted that the prognosis for adult ALL is unfortunately much poorer with a long-term disease-free survival rate of approximately 40% [4]. Current treatment generally consists of a combination of chemotherapy drugs. Although these agents have been shown to be relatively effective, they are associated with many serious and long-term side effects such as low resistance to infection, heart problems, thyroid problems and infertility. These considerations highlight the need for new and better treatments.

1.2 Normal B-cell development

B-cell development relies on tightly controlled sequential expression of a variety of transcription factor genes[5–9]. First, the expression of genes encoding transcription factors such as PU.1 and Ikaros control the commitment of hematopoietic stem cells (HSC) towards the lymphoid lineage[10,11]. Other transcription factors such as TCF3, EBF and PAX5 play an important role in lineage commitment and in various checkpoints during B-cell development[5–8]. Once the HSC has differentiated into a common lymphoid progenitor, these transcription factors will cooperatively drive the expression of genes such as *RAG1* and *RAG2* which are necessary for the rearrangement of D_H-J_H gene segments within the immunoglobulin heavy chain locus[9,12]. Successful D_H-J_H rearrangement defines the pro-B stage. These transcription factors also drive the subsequent V_H-DJ_H rearrangement leading to surface expression of Igμ (i.e. IgM) heavy chain which associates with surrogate light chains and other proteins to form a pre-B-cell receptor (pre-BCR)[13]. Cells that successfully express a pre-BCR are known as pre-B-cells. Signals emanating from the pre-BCR eventually lead to kappa (κ) and lambda (λ) immunoglobulin light-chain gene rearrangements which are necessary for the expression of a mature BCR containing a surface-bound IgM[13]. Immature B-cells that express a mature BCR will emigrate from the bone marrow and enter the spleen where they will develop into marginal zone B (MZ B) cells or follicular B-cells depending on the specificity of their BCR[14]. Finally, upon interacting with their cognate antigen, MZ B-cells can develop into short-lived plasma cells while follicular B-cells may develop into long-lived plasma cells or memory B-cells[14].

1.3 Genetic alterations in B-ALL

Approximately 85% of ALL cases develop from precursors of the B-cell lineage (B-ALL)[15]. It is noteworthy that genes that encode B-lymphoid transcription factors are often perturbed by mutations and major chromosomal aberrations in B-ALL[16,17]. Indeed, 75% of B-ALL cases exhibit aneuploidy or harbor non-random chromosomal translocations which often disrupt the function of these transcription factors[17]. These cytogenic alterations define ALL subtypes that respond differently to certain chemotherapies[17,18]. For example, ALL patients whose leukemia cells contain the t(12;21)(p13;q22) *ETV6-RUNX1* rearrangement form a ALL subgroup with an excellent outcome while patients with the t(4;11)(q21;q23) rearrangement involving the *KMT2A* gene have a dismal prognosis with a 5-year event-free survival rate below 35% [19,20]. Despite this, it should be noted that ALL remains a heterogeneous disease[21]. Studies have shown that there are multiple genetic subclones of leukemia-initiating cells in any given patient with ALL[21–23]. These subclones could become resistant to treatment and be responsible for patient relapse[21]. Regardless, to further improve patient outcome, it is vital to understand the underlying cellular processes that are driving these leukemias. This will allow for the development of new therapeutic strategies that target specific ALL subtypes.

1.4 Chromosomal translocation 1;19 and *TCF3-PBX1*

As mentioned earlier, a surprisingly large fraction of ALL cases harbor chromosomal translocations that disrupt the function of key lymphopoietic transcription factors. The t(1;19)(q23;p13.3) translocation (herein referred simply as t(1;19)) is the second most common translocation in ALL and occurs in approximately 5% of B-ALL cases[24]. This translocation generates the *TCF3-PBX1* (formerly known as *E2A-PBX1*) fusion oncogene by joining the 5' *TCF3* sequence to the 3' portion of *PBX1* [25,26]. The *TCF3* gene encodes what is considered as the founder of the basic helix-loop-helix (bHLH) family of transcription factors since the DNA-binding bHLH domain was first characterized in these proteins nearly 30 years ago[27,28]. *TCF3* plays a critical role in regulating B and T-lymphoid lineage commitment in hematopoietic progenitors, making it one of the most-studied hematopoietic transcription factors[10,11,29–32]. It is expressed at early stages of lymphoid development and is required for proper B-cell development and VDJ recombination[7]. The C-terminal bHLH domain mediates binding to DNA at CANNTG "E-box" consensus sequences where TCF3 homo- or heterodimerizes with other bHLH-containing proteins, often in close physical proximity with other hematopoietic transcription factors such as EBF, PAX5 and TAL1 to modulate gene expression[8,11,33–36]. The pro-B-cells of *TCF3*$^{+/-}$ *EBF*$^{+/-}$ compound heterozygous mice display reduced expression of B-lymphoid genes such as *RAG1*, *RAG2*, *CD79A*, *IGLL1*, *VPREB1* and *CD19* while *TCF3*$^{-/-}$ mice manifest blockade of B-lymphopoiesis at an early stage and develop highly-malignant monoclonal T-cell lymphoma in less than 9 months[37,38]. Conversely, enforced expression of *TCF3* in T-lymphoma cells leads to apoptosis[39]. Further studies have also shown that co-expression of *TCF3* and *EBF* synergistically activates the promoter of the B-cell-specific target gene *CD79A* confirming a critical functional relationship between the two transcription factors[8]. These studies strongly suggest that TCF3 cooperates with other transcription factors such as EBF to promote B-cell commitment through activation of B-lymphoid genes.

PBX1, the other gene affected directly by t(1;19), likewise encodes an important transcription factor. PBX1 is a member of the three amino acid loop extension (TALE) superclass of Hox proteins which include the PBC, MEIS, TGIF, IRO and MKX subclasses[40]. TALE transcription factors are highly conserved in evolution and fundamental for embryonic development[41–45]. As their name suggests, they are characterized by an atypical DNA-binding homeodomain (HD) containing 3 additional residues in its first loop[41]. PBX1 and other TALE members are thought to function mainly as DNA-binding cofactors and can modulate the DNA-binding specificities of their Hox binding partners[46–48]. For example, HOXB1 requires combinatorial interactions with both PBX1 and PREP1 to stimulate the *COL5A2* promoter[49]. These interactions are mediated by the tryptophan-containing W motif, independently, or in conjunction with specific PBC interaction motifs which are unique to members of the PBC subclass[46,50]. In addition to its extensive role in embryonic development, some evidence suggests that *PBX1* is important during early stages of lymphopoiesis. Several other Hox genes are differentially expressed and important during specific stages of hematopoiesis and *PBX1*$^{-/-}$ embryonic stem cells fail to generate common lymphoid progenitors in a *RAG1*$^{-/-}$ blastocyst-complemented mouse model[51–55]. However, *PBX1* function is highly dependent on the expression of multiple Hox proteins, therefore it is unclear whether these results can be extrapolated to human hematopoietic stem cells *in vivo*.

1.5 Oncogenic mechanism of TCF3-PBX1

The TCF3-PBX1 fusion protein contains the first 481 amino acids of TCF3 but omits the first 88 amino acids of PBX1[27]. It is important to note that the *TCF3-PBX1* fusion gene retains the sequence coding for the PBX1 HD but loses that encoding the TCF3 bHLH DNA-binding domain[25,26]. There exist two TCF3-PBX1 isoforms. The longer isoform, TCF3-PBX1a, seems to be the dominant isoform in t(1;19) B-ALL cells while the less abundant isoform, TCF3-PBX1b, is shorter due to pre-mRNA splicing events near the end of the 3' region of *PBX1*[27]. Nevertheless, no major functional differences between the two isoforms have yet been reported so TCF3-PBX1a and TCF3-PBX1b are commonly referred simply as TCF3-PBX1[27,49].

The first studies aimed at characterizing the TCF3-PBX1 fusion protein occurred over 25 years ago[25,26]. Surprisingly, its leukemogenic mechanism still has not been fully elucidated. TCF3 has two well-characterized transcriptional activation domains (ADs) named AD1 and AD2. Either AD can induce transcription independently but can, alternatively, cooperate to synergistically activate the expression of a reporter gene[56–59]. Our lab demonstrated that the function of the TCF3 ADs involves an interaction with the KIX domain of the transcriptional coactivator p300[60–62]. The primary role of this coactivator is to acetylate lysine 27 residues on Histone H3 associated with transcriptional enhancers and promoters[63]. This post-translational modification loosens the chromatin which allows the transcriptional machinery to access the promoter[63]. The two TCF3 ADs are retained during the translocation, which allows TCF3-PBX1 to interact with p300[59–61]. Taking into consideration the fact that *PBX1* is not expressed in B-cells[25,26] and that TCF3-PBX1 contains the PBX1 HD but lacks the TCF3 bHLH domain, it is conceivable that TCF3-PBX1 promotes leukemia through ectopic activation of PBX1 target genes through a mechanism that entails local recruitment of p300. Substitution of various key residues in either AD1 or AD2 abrogates the transcriptional activity of TCF3-PBX1 in luciferase-based reporter assays and prevents the *in vitro* immortalization of primary murine bone marrow cells that express TCF3-PBX1[60–62]. These studies provide strong evidence that the interactions between the two TCF3-PBX1 activation domains and the KIX domain of p300 are critical for leukemogenesis in t(1;19) B-ALL cells. It should be noted that p300 can also acetylate important non-histone proteins such as p53 and wild-type TCF3[64,65]. Unlike most cancer types, *TP53* is not commonly mutated in ALL[66]. However, TCF3-PBX1 could promote leukemia through aberrant post-translational modifications of various non-histone proteins such as p53.

Although the simple hypothetical model of TCF3-PBX1-mediated oncogenesis described above is plausible, recent results from our laboratory point to greater complexity. These experiments used chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) to identify TCF3-PBX1 binding sites in two different t(1;19) B-ALL cell lines. The results showed that TCF3-PBX1 does not preferentially bind to the PBX1 consensus sequence. Instead, TCF3-PBX1 binds preferentially to B-lymphopoietic enhancer elements that are also bound by B-lymphopoietic transcription factors including EBF1, ETV1, RUNX1 and wild-type TCF3 (Nanan et al, in preparation). These recent findings suggest an alternative model where TCF3-PBX1 interacts, and possibly interferes, with DNA-bound B-lymphopoietic transcription factors while maintaining local p300 activity.

1.6 The pre-BCR signaling pathway

Despite advances in understanding the molecular mechanism of *TCF3-PBX1*, there has been little progress in directly identifying the TCF3-PBX1 target genes that are driving the leukemogenesis of these lymphoid progenitors. However, many studies have proposed that *TCF3-PBX1* could play an important role in pre-BCR signaling[18,67–69]. The structure of the pre-BCR resembles that of the mature BCR. It contains two IgM heavy-chains (also known as Igμ, μHC or IGHM) and two transmembrane signaling molecules CD79A and CD79B (also known as Igα and Igβ)[70]. However, unlike the mature BCR, the pre-BCR does not express the products of rearranged κ or λ light chain genes[70]. Instead, it expresses two surrogate light-chains (SLC) named Vpreb-1 (*VPREB1*) and λ5 (*IGLL1*) (Figure 1) which are thought to be crucial for downstream signaling[71–74]. The pre-BCR has three key tasks. First, it terminates the heavy-chain (H-chain) rearrangement to prevent the expression of two different H-chains within the same cell[75]. Second, the pre-BCR initiates the rearrangement of the κ and λ light chains[70]. Finally, the pre-BCR promotes proliferation and survival through various signaling pathways. The induction of these signaling pathways causes a proliferation burst in pre-B-cells allowing them to undergo multiple rounds of cell division before differentiating into non-dividing mature B-cells[16,76]. In fact, pre-B-cells are destined to die unless they are rescued by signals originating from the pre-BCR[16]. Thus, expression of a functional pre-BCR serves as a critical checkpoint in B-cell development. Progenitors that survive this checkpoint are called large pre-B-cells and should manifest a $CD19^{+}CD25^{+}\mu HC^{+}kappa^{-}lambda^{-}$ phenotype[77].

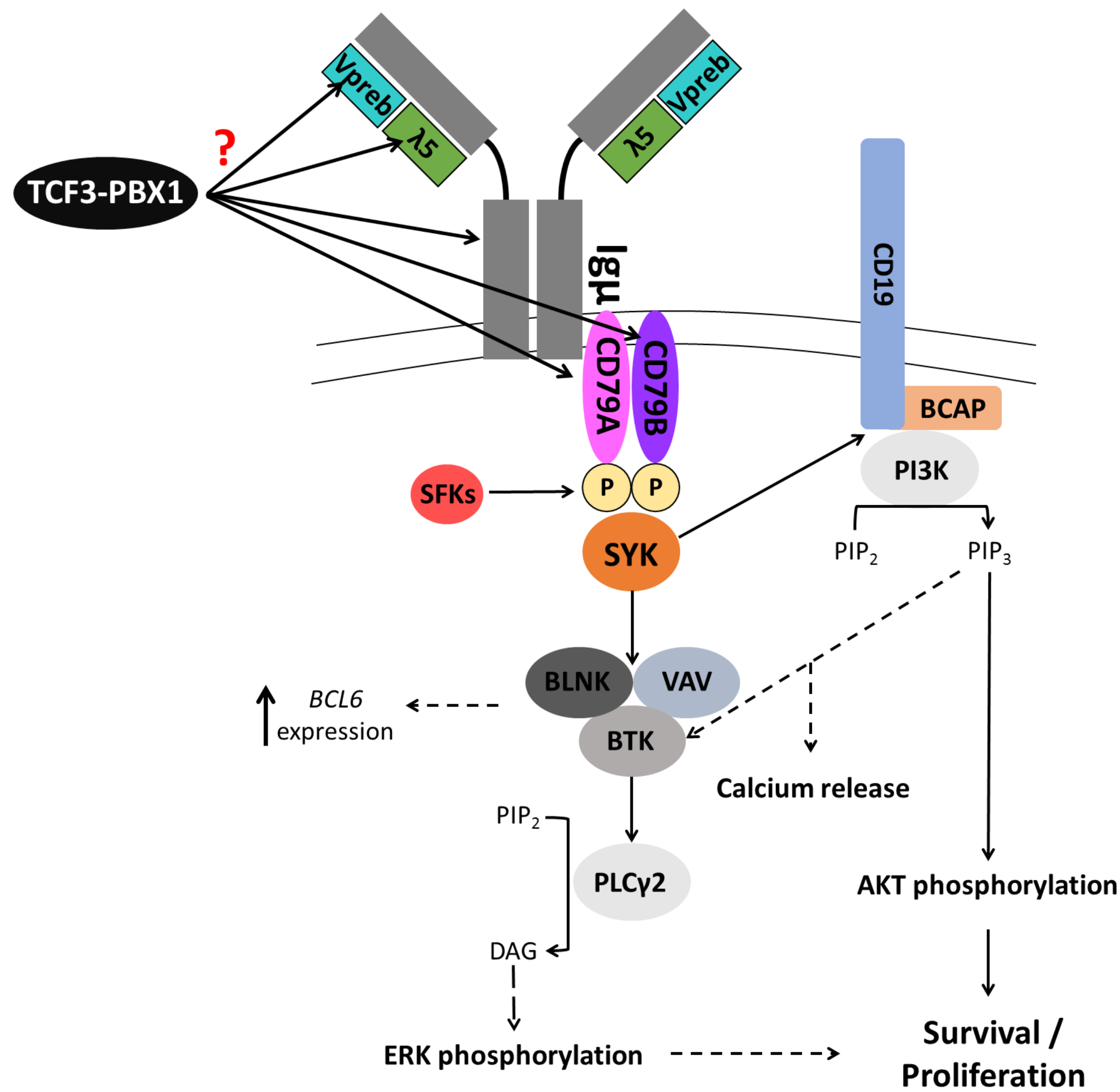

Figure 1. Hypothetical relationship between TCF3-PBX1 and pre-BCR signaling.

Schematic of the pre-BCR and downstream signaling pathways illustrating the proximal position of SYK. Activation of the surface pre-BCR through galectin binding or spurious ligand-independent crosslinking leads to the recruitment of Src-family kinases (SFKs) which phosphorylate the CD79A/B subunits. The phosphorylated residues form a docking site for SYK to which it gets recruited and then activated by tyrosine phosphorylation. SYK is thought to be crucial for downstream activation of the PI3K and MAPK signaling pathways. Dotted lines represent indirect regulatory relationships. It has been proposed that TCF3-PBX1 promotes pre-BCR signaling through direct transcriptional activation of genes that encode the components of the pre-BCR (i.e. *IGHM*, *CD79A*, *CD79B*, *IGLL1*, *VPREB1* and *VPREB3*).

Galectin-1 is a β-galactoside-binding protein that is highly-expressed in bone marrow stromal cells and is thought to be the main ligand involved in ligand-dependent pre-BCR signaling[78]. When galectin-1 binds to the λ5 SLC, the pre-BCR will crosslink other receptors and form oligomerized receptor aggregates, which likely leads to the activation of Src-family kinases (SFK)[79]. Members of the SFK family can also be activated by phosphatases such as CD45 which dephosphorylate an inhibitory phosphorylation site[79,80]. Once activated, the SFKs then phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM) located on the cytoplasmic tails of the CD79A and CD79B subunits of the pre-BCR[81,82]. These transmembrane proteins are thought to be critical for proper pre-BCR function since deletion of either *CD79A* or *CD79B* prevents the differentiation of large pre-B-cells in mice[83]. The phosphorylated CD79A and CD79B ITAMs then form a docking site for SYK[70,84]. Binding of the SYK SH2 domain to the phosphorylated ITAM motifs releases the C-terminal kinase domain which leads to autophosphorylation of several tyrosine residues[85]. SYK is considered as the master kinase of the pre-BCR signaling pathway and its activated form is thought to be a key transducer of survival signals emanating from the pre-BCR[85]. One of the main effectors of the pre-BCR signaling pathway is PI3K[86]. Although SYK has been shown to associate directly with PI3K, SYK likely promotes PI3K signaling through CD19 and BCAP phosphorylation since loss of either protein markedly impairs pre-BCR-dependent activation of PI3K[87–90]. Activation of the PI3K signaling pathway increases the abundance of phosphatidylinositol-3,4,5-triphosphate (PIP_3) thereby leading to AKT and BTK activation[16,91]. The sudden increase in phosphorylated AKT molecules results in the inactivation of several anti-proliferative or -survival proteins such as FOXO, GSKβ and

BAD[16,92–94]. Meanwhile, BTK activates PLCγ2 which induces the hydrolyzation of phosphatidylinositol-4,5-biphophate (PIP_2) to inositol-3,4,5-triphosphate (IP_3) and diacylglycerol (DAG). It is noteworthy that PLCγ2 can also directly associate with SYK suggesting that PLCγ2 may represent a node in this signaling pathway[85]. DAG induces the MAPK signaling pathway while IP_3 molecules bind to specific receptors which allows for the release of calcium ions from the endoplasmic reticulum into the cytosol. Other SYK-binding partners include VAV proteins and BLNK which are also thought to promote proliferation through induction of the MAPK/ERK signaling pathway[85,95]. Indeed, stimulation of the pre-BCR has been shown to dramatically increase the abundance of phosphorylated ERK in a t(1;19)-associated pre-B ALL cell line[96]. Deletion of ERK1 and ERK2 also prevents the pro- to pre-B-cell transition in mice illustrating the importance MAPK signaling, downstream of the pre-BCR[97]. Overall, stimulation of pre-BCR signaling leads to SYK-mediated activation of a variety of effector proteins such as PI3K, PLCγ2, VAV1 and BLNK which forms a signalosome that promotes pro-survival signaling pathways, ultimately leading to calcium release, proliferation and survival (Figure 1).

In addition to ligand-dependent signaling, it should be noted that many studies have suggested that the pre-BCR can participate in low-level tonic signaling (i.e. ligand-independent signaling)[79]. Abundant expression of surface pre-BCR likely leads to autonomous homodimerization and signaling. In fact, structural studies and electron-microscopy have provided strong evidence that the pre-BCR predominantly exists as a dimer[98]. These interactions are thought to be mediated by electrostatic interactions between λ5 chains since mutations in *IGLL1*, the gene that encodes λ5, impair pre-BCR internalization[73]. More recent studies used monovalent quantum dot-labeled probes to measure distances between pre-BCRs in live pre-B-cells[99]. These studies found that the homotypic interactions between pre-BCRs are short-lived but frequent enough to promote downstream *BCL6* expression, which is considered as a measure of pre-BCR activity[18,99]. From these studies, it is expected that an increase in surface pre-BCR expression would consequently lead to an increase in spurious pre-BCR aggregation and signaling.

1.7 *TCF3-PBX1* and the pre-BCR

The association between t(1;19) in B-ALL cells and expression of the pre-BCR was first mentioned by Carrol and colleagues in 1984[100]. The group noted that 4/17 (23.53%) children with pre-B ALL also had a unique 1;19 translocation, which had never been reported before. Interestingly, t(1;19) was not found in any of the other ALL types[100]. Since then, there have been many studies confirming the existence of a strong association between the presence of t(1;19) and the expression of a pre-BCR[18,68,101,102]. For example, a more recent study found that approximately 90% of *PBX1*-expressing ALL also express a pre-BCR (n = 57)[18]. Furthermore, studies also indicate that therapeutic kinase inhibitors targeting the pre-B-cell receptor signaling pathway such as, dasatinib and ibrutinib, are especially effective against t(1;19)-associated ALL cells[18,96]. As mentioned earlier, an abundance of data suggests that the pre-BCR participates in both ligand-dependent and tonic signaling. However, the mechanisms for which ligand-dependent and tonic pre-BCR signaling mechanisms are altered in pre-BCR$^+$ B-ALL and how this prevents the rearrangement and expression of κ and λ light chains remains unclear.

Chapter 2

Introduction

The current consensus in the literature is that leukemic progenitors with t(1;19) express the pre-BCR because TCF3-PBX1 is driving the ectopic expression of pre-BCR components[18,67]. This idea is based on previous correlative studies indicating that t(1;19) B-ALL is associated with pre-BCR expression[18,100,103]. A recent elaborate study by Geng *et al.*[18] demonstrated that TCF3-PBX1 and p300 both bind to the promoter regions of genes that encode key pre-BCR components such as *CD79A*, *CD79B*, *IGLL1*, *VPREB1* and *VPREB3*. Our group has also shown that TCF3-PBX1 binds to regions that are near the *CD79B*, *IGLL1* and *VPREB1* promoters (Nanan et al, in preparation). To our knowledge, the dependence of the pre-BCR on TCF3-PBX1 abundance has never been investigated directly. Since t(1;19)-associated B-ALL cases are overwhelmingly pre-BCR$^+$ and TCF3-PBX1 has been shown to bind near genes encoding pre-BCR components, we hypothesize that TCF3-PBX1 drives the leukemogenesis of B-lymphoid cells by promoting aberrant pre-BCR signaling through transcriptional activation of pre-BCR genes (Figure 1). To test this hypothesis, we generated *TCF3-PBX1* knockdown models using an inducible shRNA system in RCH-ACV and 697, two established t(1;19) B-ALL-derived cell lines. The main objectives of this study are to i) determine the effects of *TCF3-PBX1* knockdown on cell proliferation and culture initiation, ii) determine whether *TCF3-PBX1* knockdown reduces expression of genes that encode pre-BCR components, iii) investigate the consequences of *TCF3-PBX1* knockdown on signaling pathways that function downstream of the pre-BCR and iv) identify other genes that are differentially expressed upon *TCF3-PBX1* knockdown and that may alter the transduction of pro-proliferative or –survival signals.

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Chapter 3

Material and Methods

3.1 Lentiviral infection

HEK-293T cells were plated in poly-L-lysine-coated 10 cm dishes in growth media (DMEM, 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2mM L-Glutamine), and transfected with 2 µg of the pTRIPZ (Dharmacon, Lafrayette, CO, USA) lentiviral backbone plasmid, 2 µg of Pax2 lentiviral packaging plasmid and 1 µg of pMD2.G envelope plasmid using jetPRIME buffer (Polyplus, Strasbourg, France) according to the manufacturer's instructions. The pTRIPZ vector confers doxycycline-regulatable expression of a shRNA targeting the *PBX1* transcript or a control shRNA. The sequence for the control and knockdown shRNA were 5'-AAAATGTCTCCAATGT CCT-3' and 5'-TCACAGATCAGACAAATCT-3', respectively. Four hours after transfection, the media was carefully aspirated and replaced with fresh DMEM supplemented with 10 mM sodium butyrate. The following morning, the media was replaced with growth media. Three days later, the virus supernatants from 2 different batches were harvested, filtered, centrifuged and loaded twice on a 50 ug/ml RetroNectin-coated (Clontech, Mountain View, CA, USA) untreated 6-well plate (Sarstedt, Cat. 83.1839.500). The supernatant was collected once more and added to the rest of the virus to increase the concentration of the virus. After washing the RetroNectin-coated plate with PBS containing 1% BSA, RCH-ACV and 697 cells were transduced by centrifugation. Cells were then resuspended in RPMI supplemented with 10% FBS, Lenti-X (Clontech) concentrated virus and 8 µg/mL polybrene and incubated for 48 hours. Transduced RCH-ACV and 697 cells were selected with puromycin (1 µg/mL) for

7 days. Finally, cells were cloned by limiting dilution in 96-well plates and then clones that displayed maximal doxycycline-inducible silencing of TCF3-PBX1 were subcloned to ensure homogeneity and stability, expanded and frozen in aliquots.

3.2 Cell culture and doxycycline induction

The RCH-ACV (ACC-548) and 697 (AC-42) cell lines were acquired from DSMZ (https://www.dsmz.de/home.html). Unless indicated otherwise, all cell lines were cultured in RPMI supplemented with 10% tetracycline-free fetal bovine serum at 37 Cº under 5% CO_2. To establish our knockdown model, shPBX1 and shControl cell lines were incubated with 1 µg/mL of doxycycline for 3 days before every experiment. Media was replenished with fresh doxycycline every 48 hours to maintain shRNA expression. To avoid clonal drift, cell lines were propagated in culture for no more than 3 months before being replaced by a freshly thawed aliquot.

3.3 Cell proliferation assays

Once the knockdown model was established, $5x10^3$ RCH-ACV- or 697-shPBX1 and shControl cells were plated in a 96-well plate in triplicate. Uninduced shPBX1 cells were included as an additional negative control. Cells were incubated in RPMI supplemented with 10% fetal bovine serum for various indicated times and doxycycline (1 µg/mL) was replenished every 48 hours as necessary. Cell viability was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Exponential regression and doubling times were calculated using GraphPad Prism 7 statistical software (GraphPad Software; http://www.graphpad.com).

3.4 Limiting dilution assays

Once the knockdown model was established, RCH-ACV cells were serially diluted to 1, 2, 4, 8, 16 and 32 cells per 100 μL while 697 cells were diluted to 1, 10, 20, 30, 40 and 50 cells per 100 μL. For each dilution, 100 μL was plated in each of 16 wells of a round-bottom 96-well plate (Falcon, Cat. 353077). After 7 days, wells containing at least 50 viable cells were considered as indicating culture initiation and enumerated by microscopy. Media was replenished with fresh doxycycline (1 µg/mL) every 48 hours to maintain continuous shRNA expression. Data were analyzed using the extreme limiting dilution analysis (ELDA) online tool[104]. Statistical significance was assessed using the chi-squared test.

3.5 RNA-Seq analysis

RCH-ACV-shPBX1, -shControl, 697-shPBX1 and -shControl cell lines were induced with doxycycline (1 µg/mL) for 72 hours. Triplicate RNA samples from each cell line were harvested and isolated using the RNeasy kit (QIAGEN, Hilden, Germany). Library preparation and sequencing was performed by the PM-OICR Translational Genomics Laboratory (https://labs.oicr.on.ca/ translational-genomics-laboratory). Briefly, RNA libraries were synthesized from 200 ng of total RNA using the Illumina TruSeq Stranded Total RNA with Ribozero Gold Sample Prep kit. Total RNA was depleted of ribosomal RNA (including mitochondrial ribosomal RNA). The first and second strand cDNA was synthesized, A-tailed, adapter ligated, and PCR amplified. RNA was sequenced on the Illumina NextSeq550 platform, using a read depth of 80 million clusters, 160 million paired end reads, 75bp X 75bp. The R-Bioconductor package (DESeq2) was used for differential expression analysis. Differentially expressed genes were only considered if they had a fold change equal to or greater than 1.5 in both cell lines. Genes that were differentially expressed in opposite directions in the two cell lines were not considered.

3.6 α-IgM stimulation and pervanadate treatments

Once the knockdown model was established, cells were resuspended at 1×10^6 cells/mL and plated overnight with fresh doxycycline (1 µg/mL). The next morning, the cells were treated with 10 µg/mL F(ab')2 fragment goat anti-human IgM (cat. 109-006-129, Jackson ImmunoResearch, PA, USA) for indicated times. For pervanadate treatments, cells were treated with various concentrations of freshly prepared pervanadate solutions for 30 min. 1X pervanadate solution consisted of 0.2 mM sodium orthovanadate and 1 mM hydrogen peroxide.

3.7 Immunoblotting

Cells were lysed in RIPA buffer. Equal amounts of total protein in Laemmli’s loading buffer were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked in TBS 0.1% Tween-20 supplemented with either 5% non-fat milk or 5% BSA and incubated with primary antibodies overnight at 4 Cº. Antibodies against p-CD79A-Y182 (1:1000, 5173S), p-AKT-S473 (1:1000, 9271S), p-Erk1/2-T202/Y204 (1:1000, 4376S), Erk1/2 (1:1000, 9102S), p-SYK-Y525/526 (1:1000, 2711S), p-ZAP-70-Y319/SYK-Y352 (1:10000, 2717S), SYK (1:1000, 2712S) were from Cell Signaling Technologies (Danvers, MA, USA). Anti-GAPDH (1:40000, 2251-1) and -AKT (1:2000, 1086-1) were from Abcam

(Cambridge, UK). Anti-tubulin (1:15000, T4026-.2ML) was from Sigma-Aldrich (St-Louis, MO, USA). Antibodies against BLK (1:500, sc-65980), CD79A (1:500, sc-20064) and CD79B (1:1000, sc-53210) were from Santa Cruz Biotechnology (Dallas, TX, USA). Wild-type TCF3 and TCF3-PBX1 was detected using the E2A (Yae) antibody (1:1000, sc-416) from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-Mouse (1:3000, Jackson ImmunoResearch) and anti-Rabbit (1:6000, Abcam) secondary antibodies were detected by the Clarity Western ECL Blotting Substrate (#1705061, Bio-Rad Laboratories, Hercules, CA, USA).

3.8 Bioinformatics and data analysis

Normalized array-based gene expression data from the TARGET-ALL project was downloaded from the ICGC portal. Data visualization and pre-processing were performed on MATLAB. Expression data were log2-transformed and boxplots were used to visualize the data. Samples were removed if i) the total read counts were 1.5 interquartile range (IQR) below or above the 25th or 75th percentile, respectively, and ii) Spearman correlations values were 1.5 IQR beneath the 25th percentile. Once the samples were removed, the total read counts and Spearman correlations were re-plotted and samples were repeatedly removed if they satisfied both conditions. Samples where *PBX1* expression was 1.5 IQR above the 75th percentile were considered as t(1;19)-associated ALL samples. Gene-array expression data for putative TCF3-PBX1 target genes were acquired from the TARGET-ALL project. A candidate TCF3-PBX1 target gene was defined as a gene that satisfied the following conditions: i) gene is differentially expressed in RCH-ACV and 697 cells upon *TCF3-PBX1* knockdown, ii) fold change is in the same direction in both cell lines and iii) gene contains a TCF3-PBX1 binding site within 500 kilobases of the transcriptional start site. Differentially expressed genes that were not represented in the TARGET-ALL array were omitted. Principal component analysis was performed on the expression of 126 putative TCF3-PBX1 target genes from 194 primary ALL samples using the MATLAB default settings. The first two principal components were used as the x and y axes. Unsupervised clustering was performed on median-centered expression data and standardized by gene expression. The Euclidean distance was used as a similarity measure and clusters were merged by average links.

3.9 Statistical analysis

Differences in gene expression in our t(1;19) cell-models were assessed by the 2-tailed unpaired Student's *t* test while the non-parametric Mann-Whitney *U* test was used to establish differences in patient gene expression. The chi-squared test was performed to determine differences in cell culture-initiation frequency. Cell doubling-times were calculated by exponential regression. All statistical analyses were performed using Prism 7 (GraphPad Software http://www.graphpad.com).

Chapter 4

Results

4.1 *TCF3-PBX1* knockdown by shRNA

To study the role of *TCF3-PBX1* in pre-BCR signaling, we needed an inducible *TCF3-PBX1*-knockdown model in a human t(1;19)-ALL cell line. We used a lentiviral approach to generate stable cell lines capable of doxycycline-inducible expression of either i) a short-hairpin RNA targeting the *TCF3-PBX1* transcript (shPBX1) or ii) a region of the *PBX1* transcript that is not present in the *TCF3-PBX1* fusion transcript and not expressed in either of these cell lines (shControl, Figure 2A). The two parent cell lines used, RCH-ACV and 697, were each established from pediatric cases of t(1;19)-associated B-ALL.

The abundance of *PBX1* transcript was quantified as a measure of *TCF3-PBX1* expression since wild-type *PBX1* is not normally expressed in B-ALL cells[25,26]. RCH-ACV- and 697-shPBX1 cells had much lower *TCF3-PBX1* expression ($p < 0.0001$) upon doxycycline induction than their shControl counterparts, as determined by RNA-Seq (Figure 2B). As expected, shControl had no effect on TCF3 or TCF3-PBX1 protein in either cell line (Figure 2C). Nevertheless, expression of shPBX1 greatly reduced the abundance of both TCF3-PBX1 protein isoforms in RCH-ACV and 697 cells but had little effect on wild-type TCF3 (Figure 2C). Overall, these results show that shPBX1 specifically targets the *TCF3-PBX1* fusion transcript and can effectively reduce the abundance of both TCF3-PBX1 protein isoforms in two t(1;19) B-ALL cell lines, without disrupting the expression of TCF3.

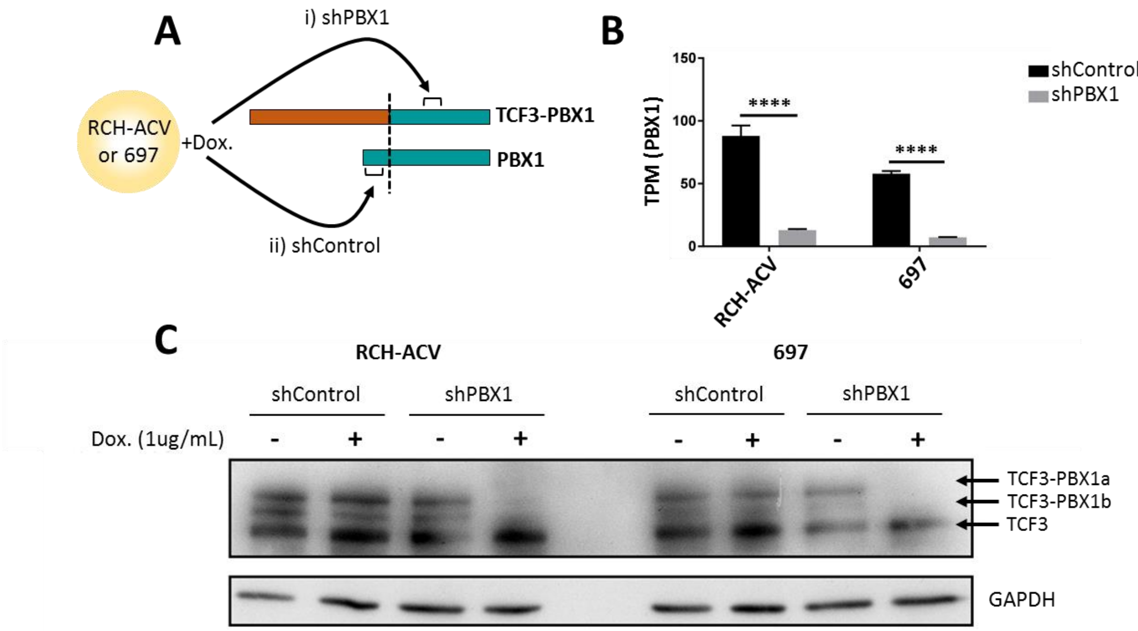


Figure 2. Creation of *TCF3-PBX1* knockdown model in two t(1;19) B-ALL cell lines.

(A) We used the pTRIPZ lentiviral vector (Dharmacon) to generate cell lines that can express a doxycycline-inducible shRNA targeting either i) a region of the *TCF3-PBX1* transcript (shPBX1) or ii) a region of the *PBX1* transcript that is not present in the *TCF3-PBX1* fusion transcript (shControl). This model was established in two t(1;19)-associated B-ALL-derived cell lines RCH-ACV and 697. (B) Expression of shPBX1 dramatically reduces *TCF3-PBX1* transcript in RCH-ACV and 697 cells. Bar graphs depict the mean ± SD transcripts per million (TPM), as determined by RNA-Seq (****$p<0.0001$, two-tailed unpaired Student's t test). (C) Immunoblot confirming that the expression of shPBX1 effectively reduces the two main TCF3-PBX1 isoforms in both t(1;19) B-ALL cell models (RCH-ACV and 697). Cells were induced with doxycycline for 72 hours.

4.2 *TCF3-PBX1* promotes t(1;19)-ALL cell proliferation and cell culture-initiation

The first goal of this study was to investigate the phenotypic consequences of *TCF3-PBX1* KD in our t(1;19) B-ALL cell lines. Rather relying on a single t(1;19) cell line, we generated knockdown models in two distinct t(1;19) lines in order to enable us to filter out transcriptional and phenotypic changes that are associated idiosyncratically with a particular cell line. Our main observation is that *TCF3-PBX1* KD substantially reduces the proliferation and viability of RCH-ACV and 697 cells (Figure 3A-B). Whereas RCH-ACV-shControl and uninduced RCH-ACV-shPBX1 cells had a doubling time of 32.16 and 25.92 h, respectively, RCH-ACV cells expressing shPBX1 conferred a growth disadvantage with a doubling time of 55.30 h. Similarly, the doubling time for 697-shControl and uninduced 697-shPBX1 were 23.17 and 23.69 h, respectively, whereas 697 cells expressing shPBX1 had a doubling time of 31.44 h. The results are representative of at least 5 independent experiments. The observable difference in cell proliferation between RCH-ACV-shControl and uninduced RCH-ACV-shPBX1 cells is likely caused by clonal differences between the two cell lines.

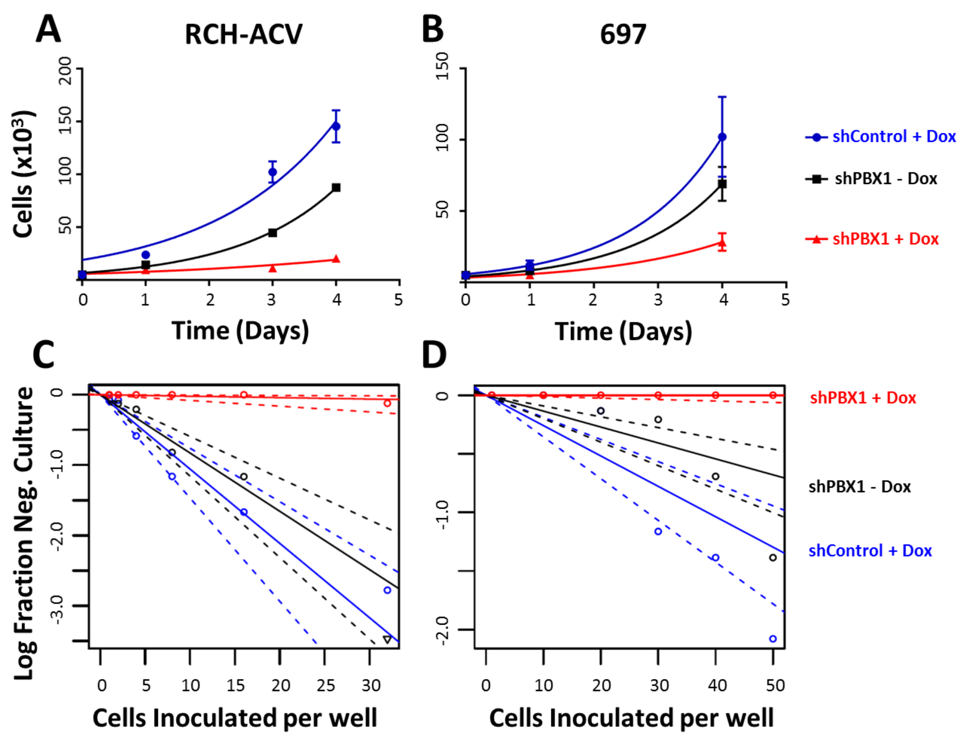


Figure 3. ***TCF3-PBX1* promotes t(1;19) B-ALL proliferation and cell-culture initiation.**

(A-B) Cell proliferation was measured using the Cell Counting Kit-8 assay (Dojindo). (A) RCH-ACV or (B) 697-shPBX1 (red) and -shControl (blue) cells were induced for 72 hours and resuspended in RPMI supplemented with 10% fetal bovine serum for indicated number of days (n=3). Values are mean cell number ± standard deviation. Uninduced shPBX1 cells (black) were used as an additional negative control. (C-D) Enumeration of culture-initiating (C) RCH-ACV and (D) 697 cells by limiting dilution assays in 96-well plates. The slope of the solid line indicates the log-active cell fraction and the dashed lines represent the 95% confidence intervals. Data points with no negative response are designated by down-pointing triangles. The chi-squared test was used to determine difference in cell culture initiation frequencies between any of the groups.

Limiting dilution assays and the online ELDA tool[104] were used to calculate the prevalence of culture-initiating cells relative to the total cell population. The proportions of culture-initiating cells for RCH-ACV-shControl and uninduced RCH-ACV-shPBX1 were 1/7.45 (95% CI, 1/13.2 to 1/6.81) and 1/12.07 (95% CI, 1/16.9 to 1/8.64), respectively (Figure 3C). *TCF3-PBX1* KD considerably decreased the proportion of culture-initiating RCH-ACV cells to 1/487.83 (95% CI, 1/1936.6 to 1/122.8). The pairwise χ^2 test for difference in culture-initiation proportion found that expression of shPBX1 significantly reduced the frequency of culture-initiating cells in comparison to RCH-ACV-shControl ($\chi^2 = 88.1$, $df = 1$, $p < 0.0001$) and uninduced RCH-ACV-shPBX1 ($\chi^2 = 70.5$, $df = 1$, $p < 0.0001$), yet found no significant difference between the two negative controls ($\chi^2 = 1.01$, $df = 1$, $p = 0.32$). *TCF3-PBX1* KD also completely halted the proliferation of 697 cells when plated at extremely low densities (Figure 3D). These results provide strong evidence that *TCF3-PBX1* is important for the proliferation of t(1;19)-associated B-ALL cells and justify the use of our cell line-based experimental model to elucidate the effects of TCF3-PBX1 on signal transduction.

4.3 *TCF3-PBX1* augments pro-survival signaling independent of the pre-B-cell receptor

TCF3-PBX1 is thought to promote pre-BCR signaling in t(1;19) B-ALL through transcriptional activation of genes that encode components of the pre-BCR[18,67,69]. To test this hypothesis, we decided to study the effects of shPBX1 on signal transduction downstream of the pre-BCR. The pre-BCR signaling pathway can be stimulated using an exogenous antibody against IgM[105]. Anti-IgM-induced AKT and ERK phosphorylation was used as a measure of pre-BCR signaling activity. In line with previously published data[96], RCH-ACV and 697 cells both responded to stimulation of the pre-BCR and the maximal response was observed when cells were treated with α-IgM (10 µg/mL) for 2 minutes (Figure 4A). *TCF3-PBX1* KD impaired pre-BCR-induced phosphorylation of AKT and ERK but surprisingly had no effect on SYK phosphorylation (Figure 4B; Supplemental Figure 1). This was true for tyrosine residue 352 (Y352) as well as the dual-tyrosine residues (Y525/526) found in the catalytic loop (Figure 4B; Supplemental Figure 2). This is surprising because SYK is thought to function upstream of AKT and ERK. Similarly, *TCF3-PBX1* KD also failed to reduce the total level of phosphorylated-CD79A (Supplemental Figure 3).

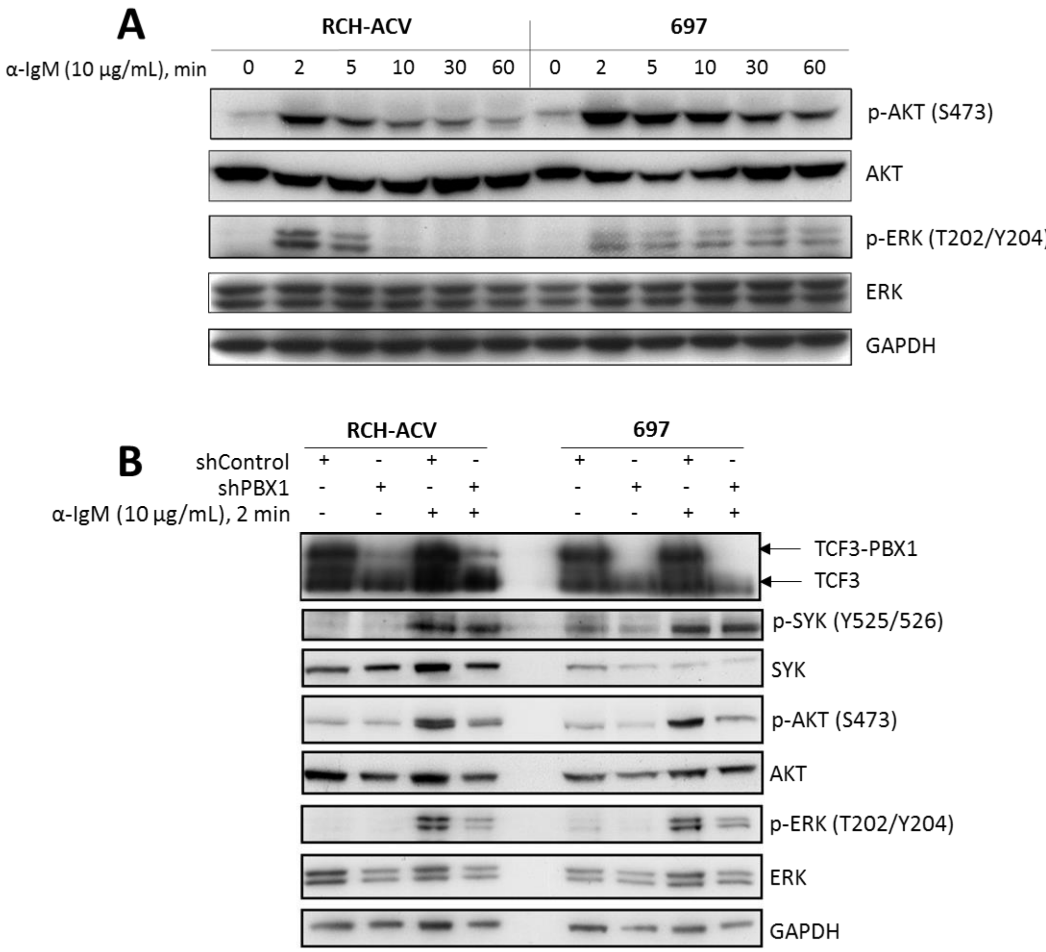


Figure 4. *TCF3-PBX1* promotes downstream pre-BCR signaling through a SYK-independent mechanism

(A) Western blot showing that RCH-ACV and 697 respond to ligand-dependent pre-BCR signaling. (B) *TCF3-PBX1* knockdown results in reduced AKT and ERK phosphorylation in both cell lines but has no effect on SYK phosphorylation. Cells were induced with doxycycline (1 µg/mL) for 72 hours and re-plated overnight with fresh doxycycline prior to stimulation with α-IgM.

Since SYK activity is unaffected by *TCF3-PBX1* KD, we decided to look upstream and study the relationship between TCF3-PBX1 and the pre-BCR. To do so, we examined the expression of the genes that encode the pre-BCR components (*CD79A*, *CD79B*, *VPREB1*, *VPREB3*, *IGHM*, *IGLL1*) in shPBX1 versus shControl-expressing t(1;19) B-ALL cells using RNA-Seq. Surprisingly, the expression of all 6 pre-BCR components significantly *increased* upon *TCF3-PBX1* KD ($p < 0.01$) in RCH-ACV cells while 4/6 pre-BCR components (*CD79A*, *CD79B*, *VPREB3*, *IGHM*) *increased* significantly ($p < 0.01$) in 697-shPBX1 cells (Figure 5A). These findings suggest that, rather than maintaining expression of the pre-BCR, TCF3-PBX1 instead functions as a direct or indirect *repressor* of pre-BCR expression. The genes encoding the critical CD79A and CD79B pre-BCR subunits were also found to be suppressed at the protein level in both cell lines (Figure 5B). Collectively, our data demonstrate that TCF3-PBX1 activates proliferation-promoting signaling pathways downstream of the pre-BCR through a mechanism that does not involve transcriptional induction of genes that encode the pre-BCR components themselves.

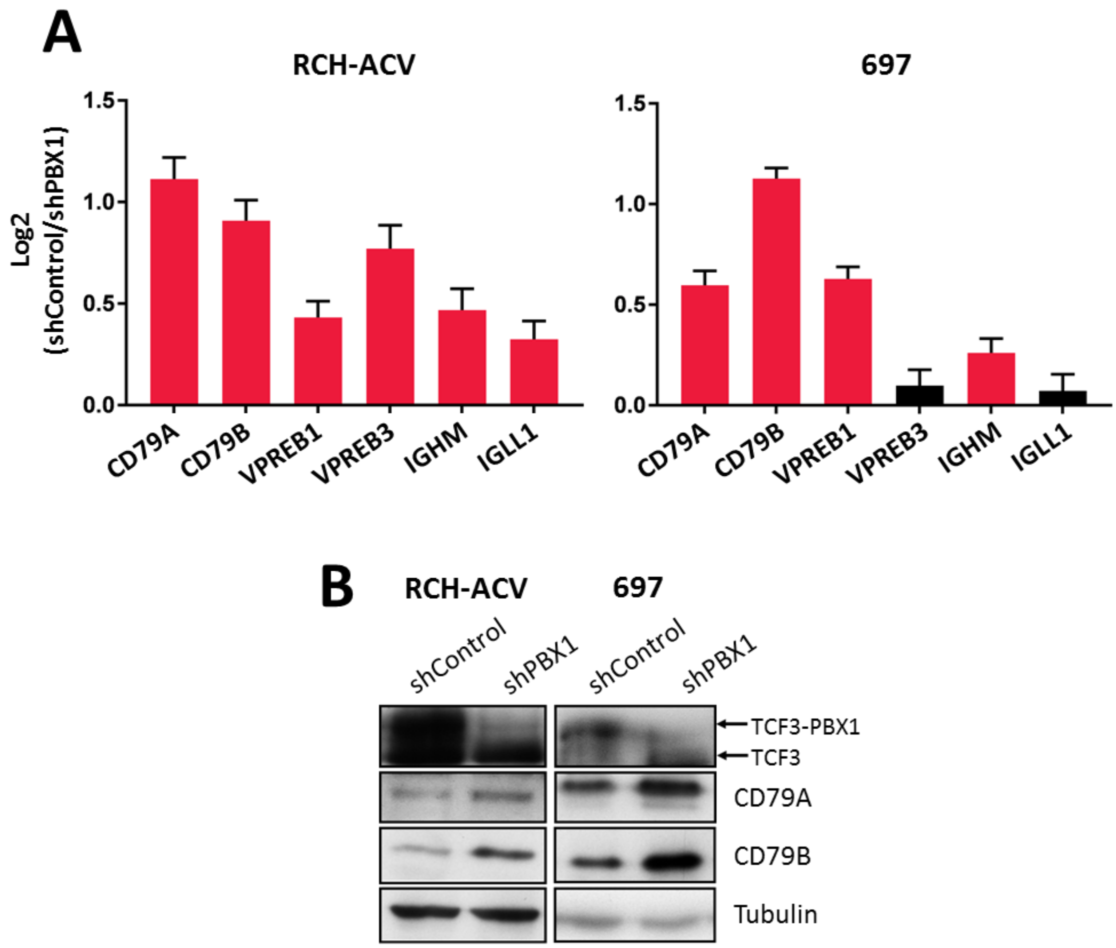


Figure 5. ***TCF3-PBX1*** **does not upregulate expression of the pre-BCR.**

(A) Results of RNA-Seq differential gene expression profiling for pre-BCR transcripts in both RCH-ACV (left) and 697 (right) cells after *TCF3-PBX1* knockdown (n = 3). Red bars indicate genes that are differentially induced ($p < 0.01$, two-tailed Student's t test) in shPBX1-expressing cells. (B) Immunoblots demonstrating that *TCF3-PBX1* knockdown leads to an increase in CD79A and CD79B protein abundance in both RCH-ACV and 697 cells. Cells were induced with doxycycline (1 µg/mL) for 72 hours.

4.4 Identification of TCF3-PBX1 target genes

Since TCF3-PBX1 is clearly not required for maintaining expression of the pre-BCR components in t(1;19) B-ALL, we sought to identify TCF3-PBX1-regulated target genes that might account for the stimulatory effects of TCF3-PBX1 on PI3K/AKT and MAPK/ERK signaling. To this end, we purified RNA from RCH-ACV and 697 cells three days after inducing expression of shPBX1 and carried out RNA-Seq and differential gene expression analysis. We identified transcripts that were up- or down-regulated by greater than 1.5-fold, in both cell lines, upon *TCF3-PBX1* KD. This analysis identified a total of 478 differentially expressed genes. Three hundred and seventy-nine (79.29%) differentially expressed genes were *induced* downstream of TCF3-PBX1, providing further evidence that TCF3-PBX1 promotes ALL largely through aberrant transcriptional activation of gene expression. We then used α-PBX1 ChIP-seq data, provided by Nanan *et al.* (unpublished), to identify candidate TCF3-PBX1 target genes defined by: i) significant perturbation of expression upon *TCF3-PBX1* KD; and, ii) the presence of at least one TCF3-PBX1 binding site within 500 kilobases (kb) of the transcriptional start site. This approach identified 178 genes that are candidates for direct transcriptional regulation by TCF3-PBX1 (Supplemental Table 1). Again, most of these genes (81.5%) were *activated* by TCF3-PBX1 (Figure 6A).

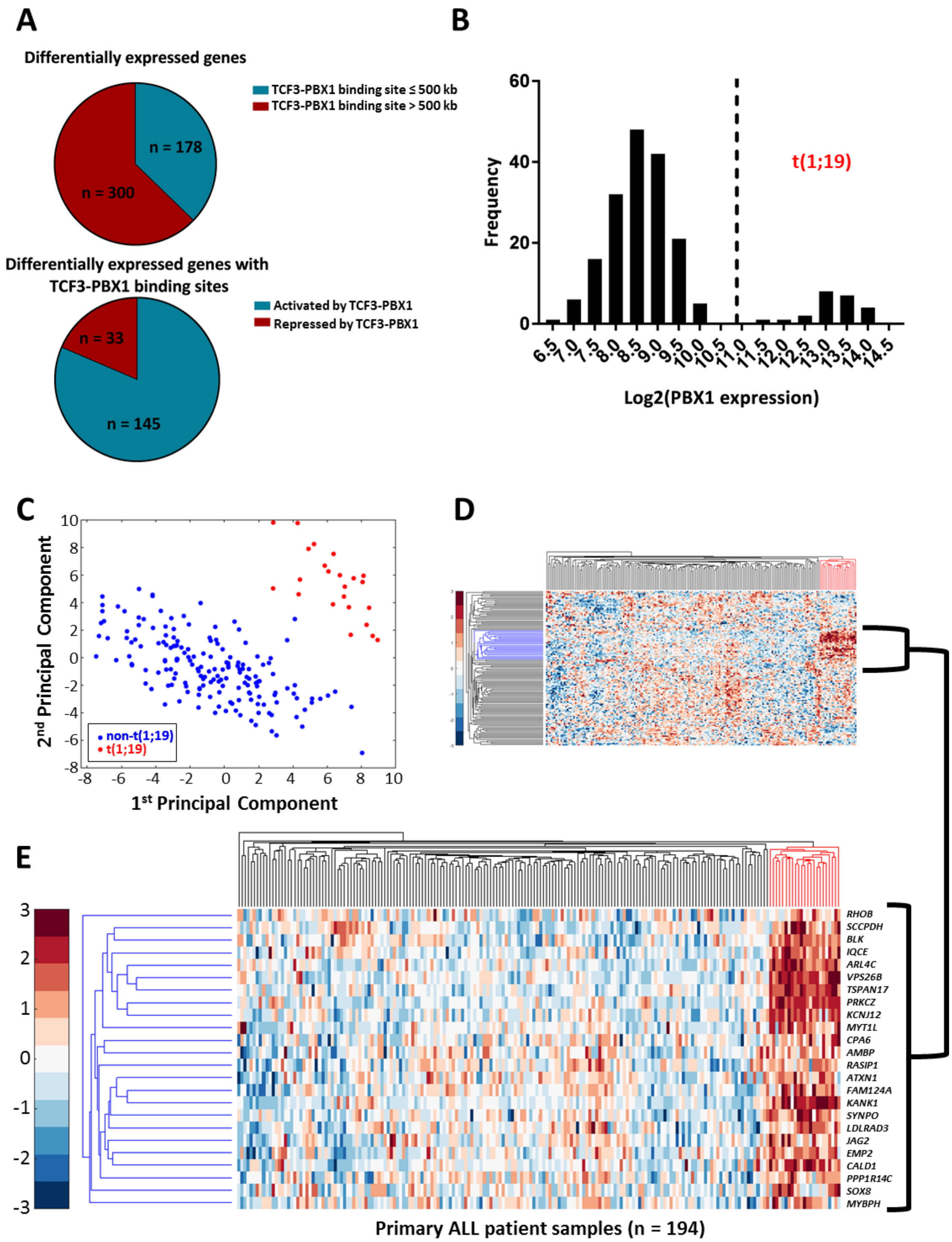


Figure 6. Identification of TCF3-PBX1 target genes.

(A) Upper pie chart depicts the proportion of differentially expressed genes with at least one TCF3-PBX1 binding site within 500 kb of its transcriptional start site. The lower pie chart depicts the proportion of differentially expressed genes with a TCF3-PBX1 binding site (< 500 kb) that are transcriptionally activated or repressed by the presence of TCF3-PBX1. (B) Frequency histogram illustrating the distribution of *PBX1* expression in the TARGET-ALL database that contains expression data from 194 primary ALL samples. The dashed line represents 1.5 interquartile range above the 75th percentile. (C) Two-dimensional principal component analysis (PCA) scatter plot distinguishing t(1;19) samples (red) from non-t(1;19) samples (blue). Expression of genes that respond to *TCF3-PBX1* knockdown in both RCH-ACV- and 697-shPBX1 cells with TCF3-PBX1 binding sites within 500 kb were used as features (n = 126). (D-E) Unsupervised hierarchical clustering of 194 primary ALL samples (columns) using genes (rows) that are significantly perturbed upon *TCF3-PBX1* knockdown with at least one TCF3-PBX1 binding sites within 500 kb as features. The t(1;19) samples are labeled in red in the dendrogram. Log2-transformed expression data is median-centered and standardized by row. Genes that are overexpressed are labeled as dark red. Low expression genes are labeled in dark blue. The clustergram identified a subset of genes that are overexpressed in t(1;19) samples, labeled blue in the dendrogram. (E) Magnified clustergram of putative TCF3-PBX1 target genes (blue dendrogram) that are over-expressed in primary t(1;19) B-ALL samples (red dendrogram).

We next hoped to validate some of the 178 putative TCF3-PBX1 target genes using data from the TARGET-ALL project (https://ocg.cancer.gov/programs/target/acute-lymphoblastic-leukemia), which includes array-based expression data from primary samples of leukemic cells from 194 pediatric pre-B ALL patients that were considered to be at high-risk for relapse. Patients within this dataset do not express the *BCR-ABL* fusion gene and were not known to be hypodiploid. Furthermore, all patients must have achieved remission (fewer than 5% blasts) following a standard two rounds of induction therapy. The rationale for using expression data from primary, patient-derived samples was to strengthen our list of putative *TCF3-PBX1* target genes by eliminating genes that, although respond to *TCF3-PBX1* KD in established cell lines, are not consistently upregulated in primary t(1;19)-associated ALL samples. The microarray-based expression data were accessed from the ICGC portal (https://dcc.icgc. org/projects/ALL-US). Only 126 (70.8%) of the 178 genes that was used for this project were represented on the TARGET expression array. Before validating target genes, the data were pre-processed while outliers and batch effect samples were removed (Supplemental Figure 4).

ALL samples associated with t(1;19) were identified using above-threshold expression of *PBX1* as a surrogate for the translocation. This is justified because *PBX1* is not normally expressed in B-cells or progenitors[25,26]. Using this strategy we removed 13 (6.3%) samples and identified 23 and 171 samples with and without t(1;19), respectively (Figure 6B). Principal component analysis (PCA) was performed on these 194 patient samples using all 126 putative TCF3-PBX1 target genes as features. The 23 t(1;19) B-ALL samples formed a separate cluster in the 2-dimensional PCA scatter plot (Figure 6C). This indicates that t(1;19) ALL samples have similar expression profiles which are distinct from non-t(1;19) ALL samples. The genes that are influencing the principal components likely include genes that are regulated by TCF3-PBX1. Hierarchical clustering also grouped all of the t(1;19) B-ALL samples in a distinct cluster and revealed a subset of 24 transcripts that are dramatically up-regulated in primary t(1;19) B-ALL patients (Figure 6D-E). These 24 genes are important candidates as TCF3-PBX1 target genes since they i) are differentially expressed upon *TCF3-PBX1* KD in two t(1;19)-associated B-ALL cell lines, ii) are consistently up-regulated in primary t(1;19) B-ALL samples and iii) have a TCF3-PBX1 binding site within 500 kb of their respective transcriptional start site. These putative target genes are listed in Table 1, along with other relevant information. With the exception of *ATXN1*, the genes that were expressed at *elevated* baseline levels in primary t(1;19) ALL samples were, as expected, *downregulated* upon *TCF3-PBX1* KD in both t(1;19) B-ALL cell lines. Of interest, genes encoding the SFK member BLK and the Notch ligand JAG2 were identified as candidates for direct transcriptional regulation by TCF3-PBX1. This suggests that TCF3-

PBX1 might promote Notch signaling and expression of genes encoding signaling molecules downstream of the pre-BCR.

Table 1. List of putative TCF3-PBX1 target genes

Gene	Median basal expression from TARGET			shControl / shPBX1 fold change (TPM)		Nearest TCF3-PBX1 binding site (kb)	Function
	Non-t(1;19) n=171	t(1;19) n=23	Fold change	RCH-ACV	697		
RHOB	1893.54	3914.05	2.07	13.39	4.81	75.26	RHO-family GTPase[106]
SCCPDH	1035.38	3334.58	3.22	1.56	1.67	14.34	Function unknown
BLK	840.945	3033.26	3.61	2.99	2.09	0	Src-family kinase[107]
IQCE	351.11	1136.79	3.24	2.90	2.75	30.49	Function unknown
ARL4C	736.19	7425.53	10.09	24.74	4.76	328.11	GTP-binding protein[108]
VPS26B	630.14	5777.92	9.17	6.09	2.96	0	Retromer complex[109]
TSPAN17	554.07	2172.60	3.92	1.87	1.90	110.51	Regulates ADAM10[110]
PRKCZ	293.98	9311.72	31.67	3.01	2.15	115.57	Protein Kinase C[111]
KCNJ12	59.80	802.13	13.41	9.03	1.89	27.08	Potassium Channel[112]
MYT1L	206.52	1041.95	5.05	3.55	6.45	0	Transcription factor[113]
CPA6	65.56	212.05	3.23	5.07	19.61	0	Metallocarboxy-peptidase[114]
AMBP	274.46	439.84	1.60	3.46	15.80	0.15	α_1-microglobulin and bikunin precursor[115]
RASIP1	140.33	297.74	2.12	3.68	3.81	398.19	GTPase signaling[116]
ATXN1	468.54	842.33	1.80	0.06	0.53	30.95	Function unknown.
FAM124A	301.07	633.96	2.11	6.43	10.67	230.38	Function unknown
KANK1	507.53	4318.42	8.51	7.94	16.97	0	Regulation of cytoskeleton[117]
SYNPO	1146.10	1957.35	1.71	3.13	4.97	121.09	Regulation of cytoskeleton[118]
LDLRAD3	165.52	424.37	2.56	2.05	2.49	341.54	Function unknown
JAG2	369.82	679.39	1.84	1.96	2.78	362.68	Notch ligand[119]
EMP2	344.66	1338.84	3.88	4.38	1.82	287.80	Regulates membrane composition[120]
CALD1	379.32	1830.52	4.83	3.75	3.44	11.73	Actin- and CaM-binding protein[121]
PPP1R14C	109.23	296.23	2.71	12.33	4.94	23.35	Inhibits PP1 phosphatase[122]
SOX8	27.99	141.78	2.34	24.76	2.52	110.09	Transcription factor[123]
MYBPH	54.32	135.69	2.49	29.18	2.60	0	Myosin-binding protein[124]

4.5 *BLK* is a TCF3-PBX1 target gene

The approach outlined above identified *BLK* as a putative TCF3-PBX1 target gene. BLK is one of the SFKs implicated in promoting signaling downstream of the pre-BCR through phosphorylation of the CD79A and CD79B subunits[107,125]. Since there are multiple SFKs that could contribute to this function in pre-B-cells[126], we examined the expression profiles of the SFKs in RCH-ACV and 697 cells. *BLK* was the most abundantly expressed SFK in both cell lines (Figure 7A). The Mann-Whitney U test was performed to compare log2-transformed *BLK* expression in primary t(1;19) ALL samples versus non-t(1;19) ALL samples from the TARGET-ALL project. The 23 t(1;19) samples were associated with much greater *BLK* expression (mean ranks = 11.67) than the 171 non-t(1;19) samples (mean ranks = 9.84) and this difference was statistically significant ($U = 129$, $p < 0.0001$, $r = 0.52$) (Figure 7B). Furthermore, *TCF3-PBX1* KD greatly decreased *BLK* expression in both RCH-ACV and 697 cells (Figure 7A) suggesting that *BLK* is induced downstream of TCF3-PBX1 in t(1;19) B-ALL. *TCF3-PBX1* KD also reduced the abundance of BLK protein in RCH-ACV cells (Figure 7C). As mentioned earlier, *TCF3-PBX1* KD curiously had no effect on CD79A phosphorylation (Supplemental Figure 3), suggesting that BLK might be a unique SFK that can promote survival through a mechanism that does not entail phosphorylation of the CD79A and CD79B subunits. Finally, ChIP experiments demonstrated that TCF3-PBX1 binds in association with p300 to three different locations within or near the *BLK* gene locus suggesting that *BLK* expression might be epigenetically activated through a mechanism that entails recruitment of p300, possibly through a direct protein-protein interaction with TCF3-PBX1[59–61] (Figure 7D). Altogether, these data provide strong evidence that *BLK* is a direct TCF3-PBX1 target gene that may mediate induction of signaling pathways that promote cell proliferation and/or culture initiation. However, its precise role in t(1;19) B-ALL and pre-BCR signaling remains to be elucidated.

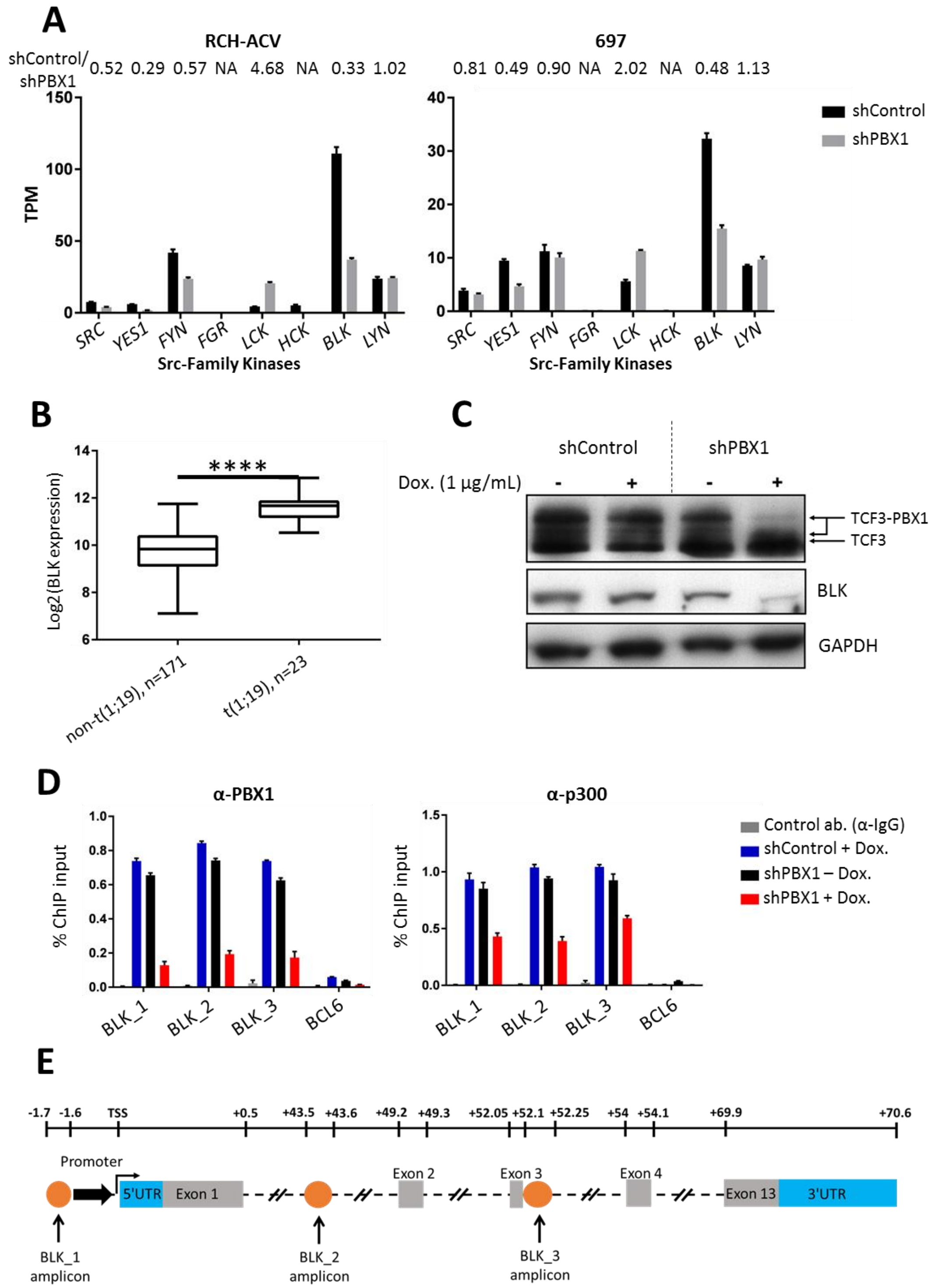


Figure 7. *BLK* is a downstream target of TCF3-PBX1.

(A) Expression profiles of Src-family kinases in RCH-ACV (left) and 697 (right) cells expressing a shRNA targeting the *TCF3-PBX1* transcript (shPBX1) or a control shRNA (shControl). Bars depict the mean ± SD transcripts per million (TPM), as determined by RNA-Seq. Fold changes are indicated at the top. *TCF3-PBX1* knockdown led to a marked decrease in *BLK* expression. Cells were induced with doxycycline (1 μg/mL) for 72 hours. (B) The box and whisker plots depict *BLK* expression in primary ALL cases from the TARGET-ALL project. The whiskers represent minimum and maximum expression values. *BLK* expression levels were significantly higher in t(1;19) samples using the two-tailed Mann-Whitney *U* test (U = 129, ****p < 0.0001, r = 0.52). (C) Western blot showing that *TCF3-PBX1* knockdown reduces the abundance of BLK protein. Cells were induced for 72 hours. (D) qChIP analysis of TCF3-PBX1 (left) and p300 (right) demonstrating binding of both proteins at three sites within the *BLK* locus in RCH-ACV cells expressing shPBX1 (red) or a control shRNA (blue). Bar graphs represent the mean % of ChIP input chromatin ± SD. Mouse IgG was used as a control antibody and the *BCL6* promoter was used as a negative genomic region. *TCF3-PBX1* knockdown reduces p300 recruitment to 3 regions near or within the *BLK* locus. (E) Schematic illustrating the genomic location of the amplicons that were amplified in (D). The upper values indicate the distance (kb) relative to the transcriptional start site (TSS). The first PBX1/p300 binding site, BLK_1, is located immediately before the *BLK* promoter. BLK_2 is in a large intronic sequence in between the first two exons. BLK_3 is situated directly after the third exon.

4.6 Notch signaling components are overexpressed in t(1;19) B-ALL

Since TCF3-PBX1 does not promote pre-BCR signaling through direct regulation of pre-BCR components, we inferred that there must be crosstalk between the pre-BCR and other signaling pathways. Recent findings have suggested that Notch activity can synergize with BCR signaling[127,128]. Interestingly, the Notch ligand *JAG2* was identified as a putative TCF3-PBX1 target gene (Figure 6D-E; Table 1), prompting us to further investigate the Notch signaling pathway in t(1;19) B-ALL. It is noteworthy that Notch receptors (*NOTCH2*, *NOTCH3*) and the Notch ligand *JAG1* were also significantly overexpressed ($p < 0.0001$) in primary t(1;19) B-ALL samples (Figure 8A-D). Likewise, *TCF3-PBX1* KD significantly reduced the abundance ($p < 0.01$) of *NOTCH2*, *NOTCH3*, *JAG1* and *JAG2* expression in RCH-ACV cells (Figure 8E). These findings suggest that the Notch signaling pathway is dependent on the presence of TCF3-PBX1. Activated Notch signaling could be promoting a hyper-responsive pre-BCR in t(1;19)-associated ALL but this remains to be determined.

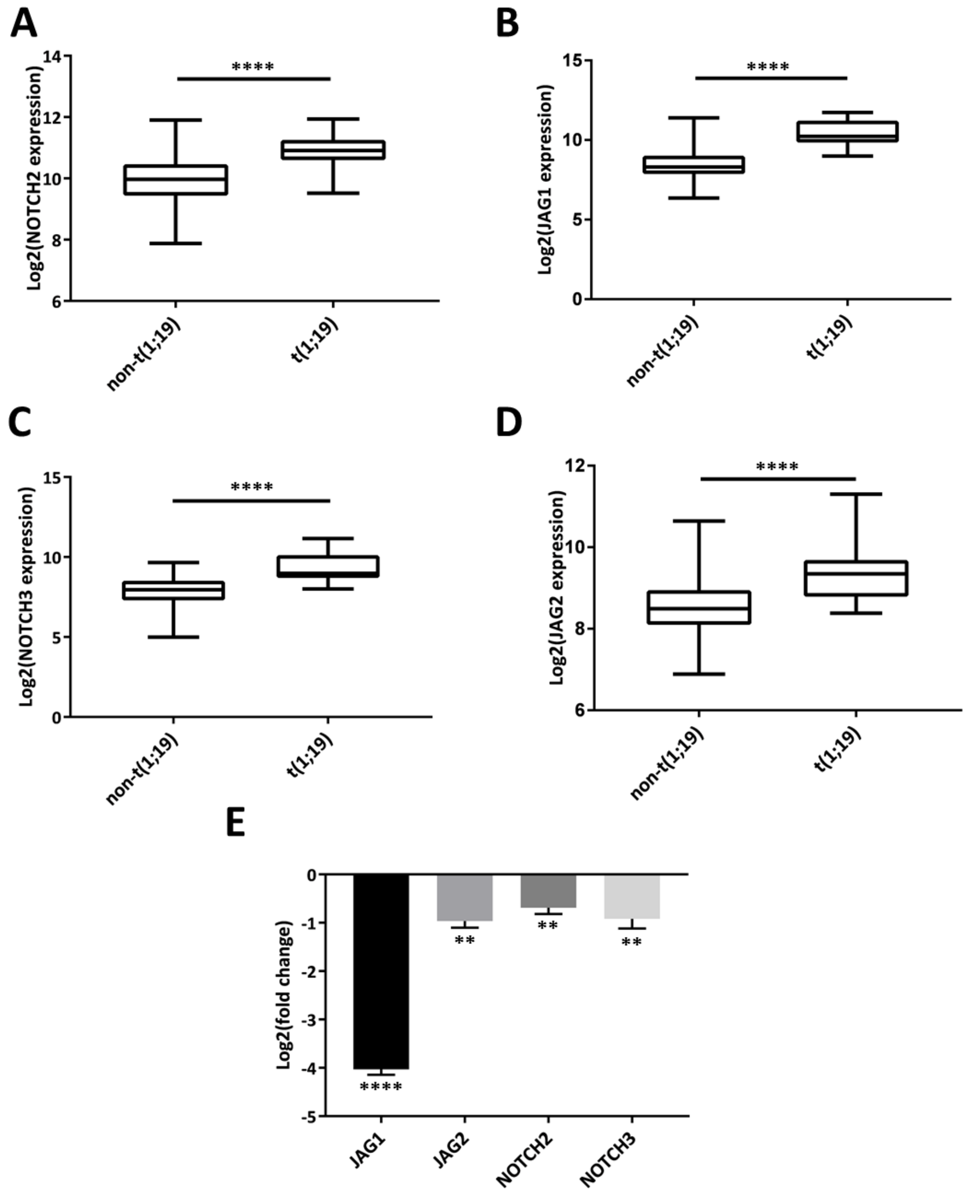


Figure 8. Key players of Notch signaling are overexpressed in t(1;19) B-ALL.

(A-D) The box and whisker plots depict expression of NOTCH receptors and ligands that are overrepresented in primary t(1;19) ALL cases from the TARGET-ALL project. The whiskers depict minimum and maximum expression values (***p < 0.0001, two-tailed Mann-Whitney *U* test). (E) Results of RNA-Seq differential gene expression profiling for NOTCH players that are overexpressed in primary t(1;19) ALL cases subsequent to *TCF3-PBX1* knockdown in RCH-ACV cells. Bar graphs represent the mean ± standard deviation (***p* < 0.01, *****p* < 0.0001, unpaired, two-tailed Student's *t* test). RCH-ACV-shControl and -shPBX1 cells were induced for 72 hours.

Chapter 5

Discussion

In undertaking this research, we considered that the availability of an inducible *TCF3-PBX1*-knockdown model in a human t(1;19)-ALL cell line would greatly advance efforts to elucidate the role of TCF3-PBX1 in pre-BCR signaling. Previous studies have tried to decipher the mechanistic aspects of leukemia induction by TCF3-PBX1 through enforced expression of *TCF3-PBX1* or knockdown strategies using small interfering RNA (siRNA)[129–131]. These transient knockdown strategies are inefficient since the TCF3-PBX1 protein has an exceptionally long half-life of approximately 10 hours[132]. Furthermore, siRNAs are known to have many off-targets which is problematic for transcriptomic studies[133]. Finally, certain TCF3-PBX1 target genes might not be captured using transient knockdown strategies since epigenetic changes are known to be relatively stable and TCF3-PBX1 is thought to promote gene expression through p300-mediated acetylation of H3K27[59–61,134]. These points summarize our rationale for developing stable shRNA-directed *TCF3-PBX1*-knockdown cell models.

Most *TCF3-PBX1* studies have been performed on the 697 cell line, an established human t(1;19)-associated-B-ALL-derived cell line. We decided to generate a knockdown model in two different t(1;19)-associated cell lines to filter out transcriptional and phenotypic changes that are specific to the 697 cell line. We also questioned whether the 697 cell line is appropriate as a single model for studying pre-BCR signaling since it i) does not express BTK, an important mediator of pre-BCR signaling [18,96], ii) is idiosyncratically resistant to the PI3K inhibitor idelalisib[135] and iii) harbors an activating *NRAS* mutation[135,136]. Regardless, here we show that sustained *TCF3-PBX1* knockdown reduces cell proliferation and the prevalence of culture-initiating cells in both RCH-ACV and 697 cells (Figure 2). Interestingly, based on this phenotype, 697 cells are more resistant to *TCF3-PBX1* knockdown than RCH-ACV cells. One possible explanation for this is that aberrant signaling emanating from mutant NRAS is compensating, at least partially, for the reduced effect of TCF3-PBX1 in stimulating signaling pathways at a point further upstream. Conversely, 697 cells are unable to proliferate at extreme dilutions (Figure 2D) which suggests that these progenitors might rely on critical paracrine signaling from cytokines provided by neighboring cells. Finally, there were considerable differences between the expression profiles of our two cell lines. Only 36.3% of genes that were differentially expressed following *TCF3-PBX1* knockdown in 697 cells were also affected in RCH-ACV cells (data not shown). These studies highlight the importance of validating findings in various experimental models.

Our differential expression analysis identified a total of 478 genes that are significantly perturbed upon *TCF3-PBX1* knockdown in both t(1;19)-associated cell lines according to our criteria requiring greater than 1.5-fold change with an FDR-corrected p-value of 0.1 or lower. As expected, these included many genes that were previously reported to be associated with the 1;19 translocation such as *BLK*[18,96,137], *CALD1*[137,138], *KANK1*[138], *PRKCZ*[18,138,139], *NOTCH2*[140] , *ANKS1B*[130,138,141], *FAT1*[138,142,143], *PHACTR3*[138], *ARL4C*[137,138], *VPS26B*[138], *ASNS*[131], *SLAMF1*[137,138], *MYBPH*[137], *RORB*[138] and *EPHA3*[138,143–145]. Several of these genes also have at least one TCF3-PBX1 binding site within 500 kb and were overexpressed in t(1;19) primary ALL samples (Figure 6D-E; Table 1). Charmsaz *et al.*[145] recently investigated the role of the ephrin receptor A3 (EphA3) by performing microarray gene expression profiling on *EPHA3* knockdown pre-

B-cells versus pre-B-cells expressing high-levels of *EPHA3*. Intriguing comparisons can be made with our own results. Notably, *EPHA3* knockdown reduced the expression of TCF3-PBX1 target genes such as *BLK*, *ASNS*, *PRKCZ*, *CALD1*, *FAT1*, *SLAMF1* and *RORB*[145]. Conversely, *EPHA3* knockdown also led to a significant increase in expression of the *CD79A*, *CD79B*, *VPREB1* and *IGLL1* pre-BCR components as well as other genes that are apparently repressed by TCF3-PBX1 such as *MZB1*, *MME* and *FAM129C* (data not shown)[145]. The similarity with our transcriptional studies strongly suggest that TCF3-PBX1 maintains the expression of several target genes through ectopic EphA3 signaling. A human derivative of the EphA3-specific monoclonal antibody, IIIA4, developed by KaloBios Pharmaceuticals Inc., induces EphA3 receptor internalization and is currently under clinical trial as a potential therapeutic agent for various hematological malignancies[145]. Given the strong, apparently functional relationship between *TCF3-PBX1* and *EPHA3*, IIIA4 could be an effective treatment option for t(1;19) B-ALL patients.

Our differential expression analysis validated several TCF3-PBX1 target genes identified by other investigators. On the other hand, our data do not support a study that claimed that *SYK*, *ZAP70* and *LCK* are transcriptionally activated by TCF3-PBX1 in RCH-ACV and 697 cells[69]. Another recent study proposed that *TCF3-PBX1* transcriptionally activates *SETDB2* in RCH-ACV and 697 cells[140]. Our results confirm that *TCF3-PBX1* knockdown does indeed reduce *SETDB2* expression. However, *SETDB2* is expressed at a very low level in these cells and the fold change is minimal compared to other putative TCF3-PBX1 target genes listed in Table 1. Furthermore, non-t(1;19) pre-B ALL samples from the TARGET dataset express higher levels of *SETDB2* than *TCF3-PBX1*-expressing ALL samples. Taken together, it seems unlikely that *SETDB2* is driving the leukemogenesis of t(1;19) pre-B-cells. A curious omission from our differential expression analysis was *WNT16*, a well-established TCF3-PBX1 target gene[130,143,146], whose expression was interestingly unaffected by *TCF3-PBX1* knockdown in RCH-ACV cells (data not shown). We speculate that the regulatory relationship between *TCF3-PBX1* and *WNT16* was lost in this cell line over many years of *ex vivo* culture. Finally, our current strategy assumes that changes in gene expression directly correlate with changes in protein abundance. This does not account for potential effects of *TCF3-PBX1* knockdown on various post-translational modifications that could alter protein stability.

Previous studies demonstrated that the presence of *TCF3-PBX1* is associated with high expression of genes encoding key pre-BCR subunits[18,68,101,102]. However, a direct transcriptional relationship has not been demonstrated. Our *TCF3-PBX1*-knockdown models provide an excellent opportunity to investigate this relationship directly. Intriguingly, our results show that there is an inverse relationship between expression of *TCF3-PBX1* and expression of genes that encode the proteins that constitute the pre-BCR. *TCF3-PBX1* knockdown led to an unexpected increase in expression of all 6 pre-BCR genes in both cell lines (Figure 5). In line with these data, the expression of *BCL6*, a surrogate marker of pre-BCR activity[18], significantly *increased* upon *TCF3-PBX1* knockdown (data not shown). These observations indicate that TCF3-PBX1 is not actively promoting expression of the pre-BCR. Instead, we hypothesize that TCF3-PBX1 is impeding the differentiation of t(1;19) B-ALL cells and that this, in turn, serves to maintain cells in a “pre-BCR-low” state. Indeed, our differential expression analysis indicated that *TCF3-PBX1* knockdown led to an increase in expression of several genes known to be upregulated during the maturation of pre-B-cells to immature B-cells, including *MS4A1*, *IRF8*, *IGJ* and *HLA-DMB* (Supplemental Figure 5). *TCF3-PBX1* knockdown also increased surface CD20 (Supplemental Figure 5), which is encoded by *MS4A1*, but did not sensitize the cells to anti-CD20 (Rituximab) immunotherapy (data not shown).

An alternative hypothesis is that the apparent increase in pre-BCR expression reflects a compensatory mechanism. In fact, several studies have shown that pre-BCR components are often subject to broad feedback regulation[147–149]. Coincidentally, this regulation is thought to be mainly controlled by Ca^{2+}-bound calmodulin-mediated inhibition of wild-type TCF3. Signals emanating from the pre-BCR lead to rapid Ca^{2+} release which is detected by the calcium-sensor protein calmodulin (CaM). The activated Ca^{2+}-loaded CaM complex binds to the TCF3 bHLH domain and silences the expression of pre-BCR components by disrupting cooperative transcriptional activation of the genes that encode the components of the pre-BCR[147,150]. One should note that the *CALD1* gene which encodes the CaM-Ca^{2+} binding protein caldesmon was also revealed earlier as a high-priority TCF3-PBX1 target gene (Table 1). Here we propose four plausible mechanisms for the apparent TCF3-PBX1-mediated repression of pre-BCR components: i) TCF3-PBX1 induces the ectopic expression of *CALD1* which facilitates the formation of the CaM complex. ii) TCF3-PBX1 promotes Ca^{2+}-mediated inhibition of TCF3 via indirect activation of the pre-BCR signaling pathways through various putative target genes (e.g. *BLK*, *PRKCZ*, *NOTCH2*, *JAG2* or *RHOB*). iii) TCF3-PBX1 dimerizes with TCF3 or EBF and disrupts the transcriptional activation of pre-BCR components. iv)

TCF3-PBX1 restrains the expression of the pre-BCR through ectopic EphA3 signaling (Figure 9). However, it seems more reasonable to presume that there are multiple mechanisms simultaneously influencing the expression of pre-BCR components, downstream of TCF3-PBX1, ultimately resulting in a net increase in expression. Keeping that in mind, it might be worth investigating the therapeutic effects of pre-BCR signaling inhibitors such as dasatinib in conjunction with *TCF3-PBX1* knockdown.

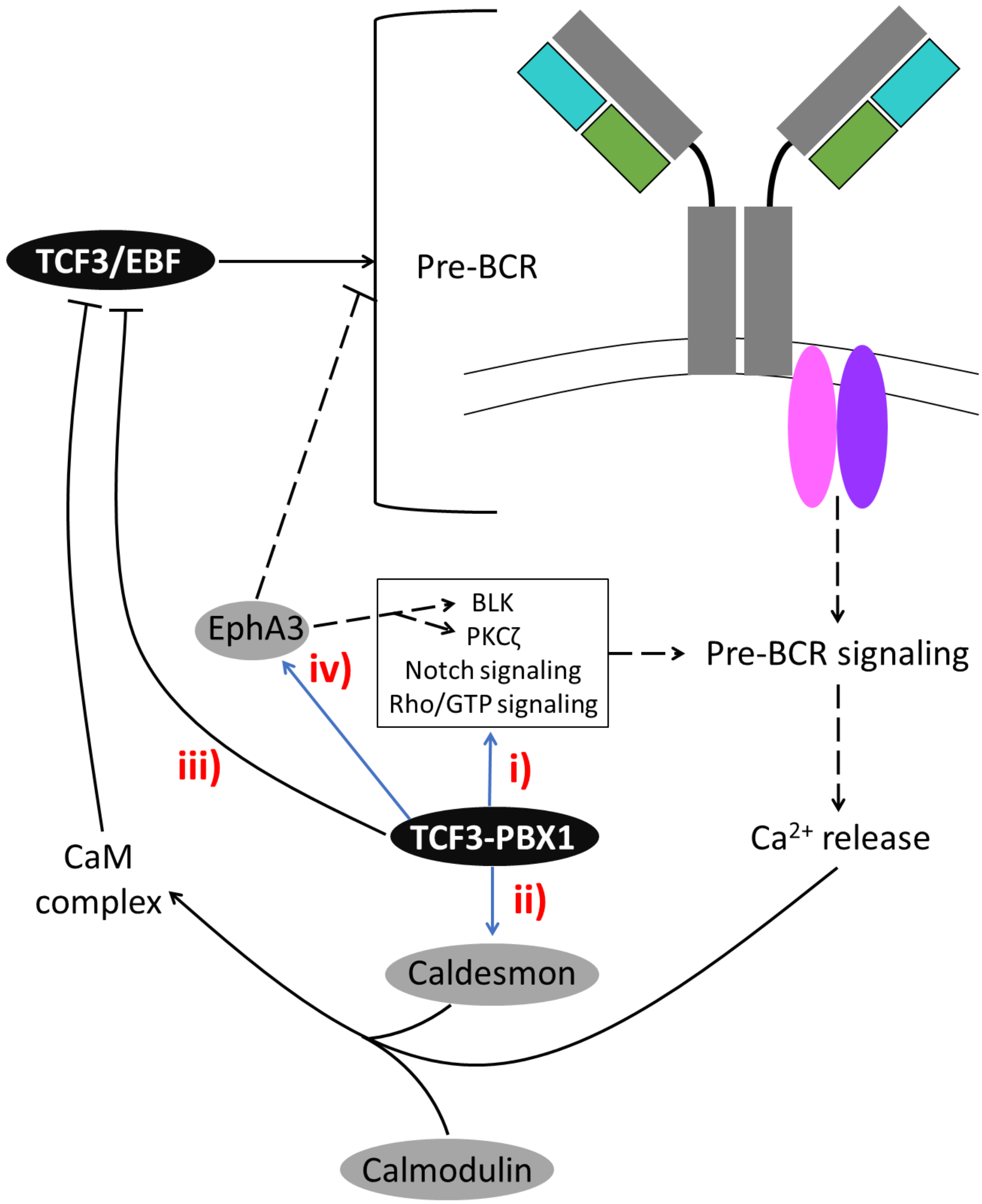


Figure 9. Hypothetical mechanisms for TCF3-PBX1-mediated repression of the pre-BCR.

Schematic of possible mechanisms for pre-BCR repression by TCF3-PBX1. i) TCF3-PBX1 promotes Ca^{2+}-mediated inhibition of TCF3 via indirect activation of the PI3K and MAPK signaling pathways through various putative target genes such as *BLK*, *PRKCZ* and members of the Notch and Rho-GTP signaling pathways. ii) TCF3-PBX1 induces the ectopic expression of *CALD1* (caldesmon) which facilitates the formation of the CaM complex. iii) TCF3-PBX1 dimerizes with TCF3 or EBF and disrupts their synergistic transcriptional activation of pre-BCR components. iv) TCF3-PBX1 represses the pre-BCR via ectopic EphA3 signaling. Dashed arrows represent indirect mechanisms. Blue arrows indicate transcriptional activation.

Our signaling studies suggest that TCF3-PBX1 promotes PI3K and MAPK signaling through a mechanism that is downstream of the pre-BCR. The results from our differential gene expression analysis allow us to speculate as to which genes might be stimulating these pro-survival pathways. *BLK* and *PRKCZ* are two candidate target genes that are likely promoting proliferation in t(1;19) ALL (Figure 10). *BLK* is arguably the most interesting of our putative TCF3-PBX1 target genes since it encodes a SFK and can be easily targeted by kinase inhibitors such as ibrutinib[96]. Pre-BCR crosslinking is thought to activate SFK members including *BLK*. With that said, our studies could not demonstrate an increase in BLK phosphorylation and activation following pre-BCR stimulation (data not shown). *TCF3-PBX1* knockdown did not reduce the total abundance of phosphorylated-CD79A despite the substantial decrease in BLK protein abundance (Supplemental Figure 3). These observations suggest that BLK could stimulate growth- and survival-promoting signaling through downstream effectors other than CD79A/B or SYK. Other studies also support this notion. An activating mutation of *BLK* was shown to promote B-cell proliferation, suppress V_H to DJ_H rearrangement and stimulate κ chain rearrangement in mice lacking a functional pre-BCR[151]. More recently, Kim and colleagues[96] found that *BLK* knockout reduced the proliferation of t(1;19) B-ALL cells but had no effect on pre-BCR-dependent phosphorylation of AKT and ERK. From these results, we infer that BLK might play a special role in t(1;19) pre-B ALL cells.

The second gene, *PRKCZ*, encodes an atypical protein kinase C isoform named PKCζ. Studies have already shown that this isoform is overexpressed in t(1;19) pre-B ALL and is critical for SDF-1-mediated phosphorylation of ERK1/2[139,152]. PKCζ activity seems to be strongly dependent on PI3K suggesting that it might play an important role in conveying pre-BCR-induced PI3K signals to other survival pathways[152]. Expression of PKCζ is clearly dependent on *TCF3-PBX1* and is likely responsible in transmitting at least some of the signals emanating from the pre-BCR. Whether this is important in driving t(1;19) B-ALL and whether it can be targeted therapeutically remains to be determined.

Our multidimensional approach identified several other players that might be promoting the proliferation of t(1;19) B-ALL. Of interest, members of the Notch signaling pathway are overexpressed in patient ALL samples and downregulated upon *TCF3-PBX1* knockdown (Figure 8). This includes the Notch ligand Jagged 2 (*JAG2*) which also has a TCF3-PBX1 binding site 362.68 kb away (Table 1). Crosstalk between pre-BCR and Notch signaling is plausible since several studies have shown that Notch synergizes with mature BCR signaling[127,128,153]. Poe *et al.* recently demonstrated that NOTCH2 ligation with the DLL1 ligand increased BCR responsiveness to anti-IgM and led to persistent NOTCH2 surface expression in a chronic graft versus host disease model[128]. Therefore, one hypothesis is that TCF3-PBX1 promotes the expression of Notch receptors and ligands leading to a hyper-responsive pre-BCR (Figure 10).

Finally, the proliferation of t(1;19) B-ALL cells might also depend on a PI3K/MAPK/Rho GTPase signaling axis. Rho GTPases are thought to mediate crosstalk between multiple signaling pathways from various receptors such as G protein-coupled, T-cell and B-cell receptors[95]. For example, RhoA promotes BCR-dependent synthesis of PIP_2 which is the main substrate for PLCγ2 and PI3K. It is therefore plausible that Rho GTPases could also be important for downstream pre-BCR signaling. Consistent with this hypothesis, several genes involved in Rho signaling are downregulated upon *TCF3-PBX1* knockdown. These include *RHOA*, *RHOB*, *RHOC*, *RAC3*, *RND3*, *ARL4C* and *RASIP1* (data not shown). Furthermore, *RHOB*, *ARL4C* and *RASIP1* are also upregulated in t(1;19) B-ALL primary samples and have a TCF3-PBX1 binding site within 500 kb (Table 1). Conversely, *TCF3-PBX1* knockdown leads to a significant increase in *RHOH* expression. However, *RHOH* is considered to be an antagonist of Rho signaling since it lacks intrinsic GTPase activity[154]. The expression of a wide range of genes involved in Rho signaling seem to be dependent on the presence of TCF3-PBX1 and the directionality of these relationships suggests that *TCF3-PBX1* promotes Rho signaling. Due to the large number of TCF3-PBX1 target genes encoding GTP-binding proteins, it seems unlikely that a single gene is responsible for the consequences seen on PI3K and MAPK signaling. TCF3-PBX1 probably perturbs a large transcriptional network of genes related to Rho signaling and this might collectively increase the synthesis of PIP_2 which should ultimately result in an increase in pre-BCR-dependent phosphorylation of AKT and ERK (Figure 10).

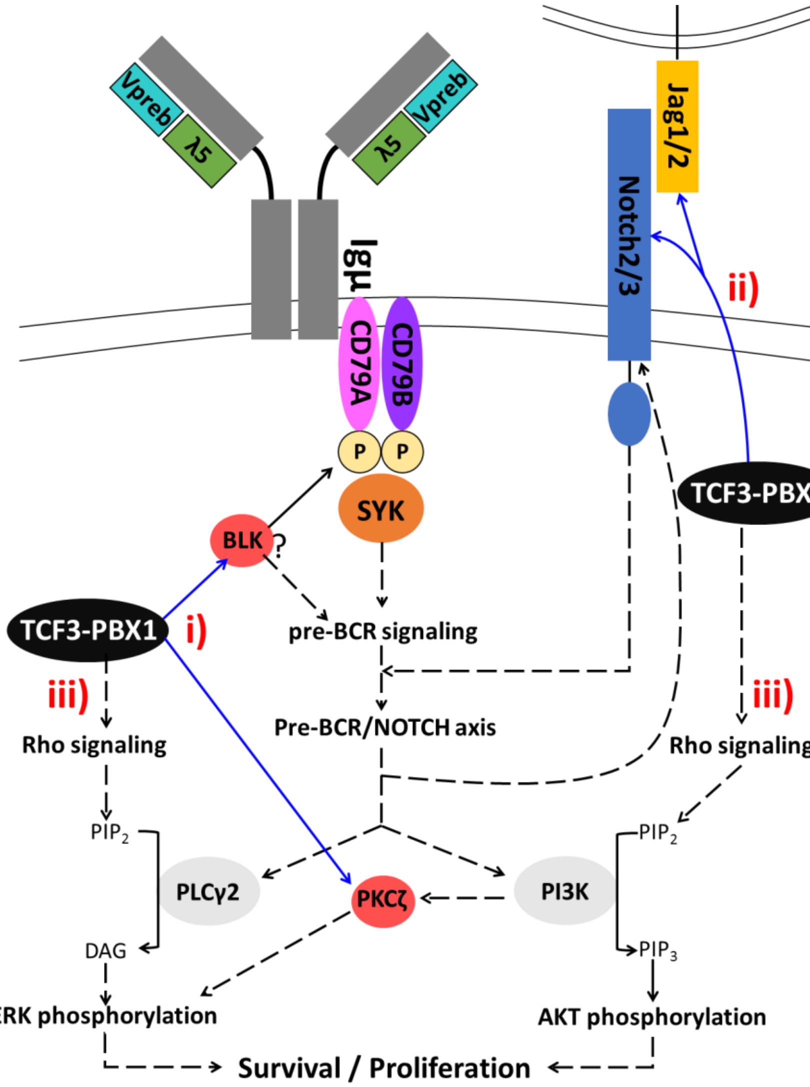


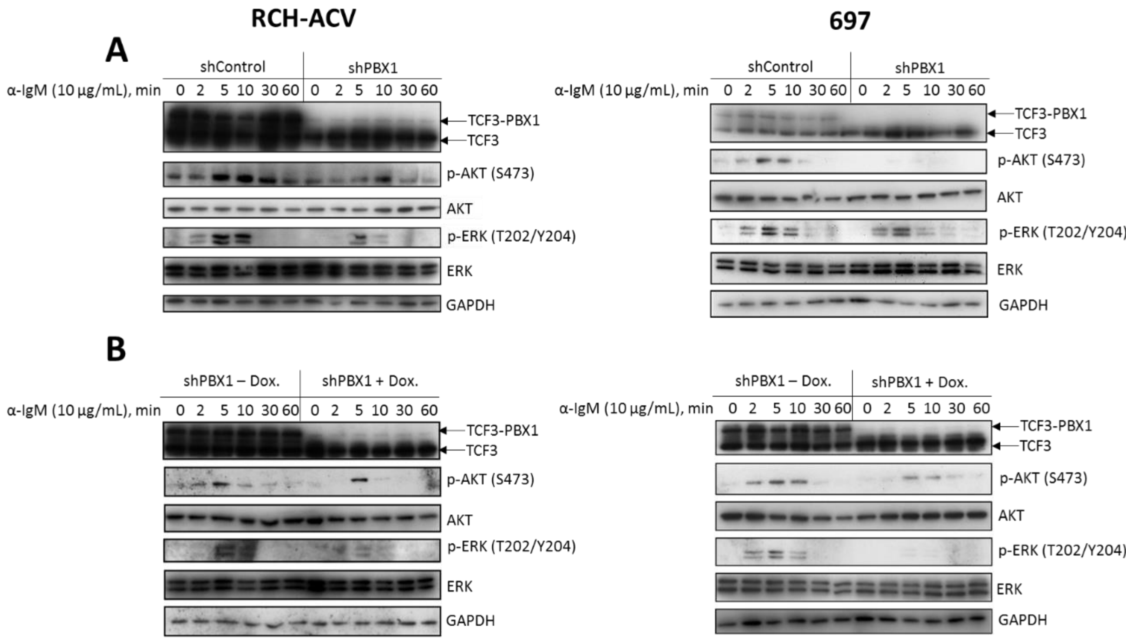
Figure 10. Hypothetical mechanisms for TCF3-PBX1-mediated activation of downstream pre-BCR signaling.

Schematic of possible mechanisms for downstream activation of pre-BCR signaling by *TCF3-PBX1*. i) TCF3-PBX1 transcriptionally activates genes encoding key downstream pre-BCR signaling molecules such as *BLK* and *PRKCZ*. The role of BLK in pre-BCR signaling is unclear since *TCF3-PBX1* knockdown fails to reduce CD79A phosphorylation and *BLK* knockout has no effect on AKT and ERK phosphorylation. ii) TCF3-PBX1 transcriptionally activates the expression of Notch signaling receptors and ligands. Crosstalk between Notch and pre-BCR signaling generates a positive feedback loop which leads to persistent surface expression of Notch2. iii) TCF3-PBX1 transcriptionally regulates a wide variety of genes which ultimately promotes Rho signaling. These include members of the Rho-family small GTPases, guanine nucleotide exchange factors, GTP-binding proteins and other proteins directly associated with this pathway. Rho signaling leads to the synthesis of PIP_2 which can be converted into DAG and PIP_3 by PLCγ2 and PKCζ, respectively. The generation of DAG activates the MAPK signaling pathway while PIP_3 leads to AKT phosphorylation. Dashed arrows represent indirect mechanisms. Blue arrows indicate transcriptional activation.

In this project, we directly investigated the role of TCF3-PBX1 in t(1;19)-associated ALL. Our results provide strong additional support to the notion that TCF3-PBX1 is an oncogenic driver in these leukemias and identify numerous putative target genes that might mediate TCF3-PBX1-driven leukemogenesis. Although the multiplicity of plausible target genes poses a challenge to deciphering their relative oncogenic importance, this project has nonetheless advanced our knowledge. Importantly, our results dispel the hypothetical notion that TCF3-PBX1 promotes growth and/or survival by directly inducing the expression of the pre-BCR. TCF3-PBX1 does, however, enhance pre-BCR-dependent activation of the PI3K and MAPK signaling pathways. We hypothesize that this is caused by aberrant Notch or Rho signaling either of which might crosstalk with the pathways that function downstream of the pre-BCR. The relationship between Notch and Rho signaling with the BCR has already been studied. Future studies should focus on determining whether these signaling pathways can crosstalk with the pre-BCR in t(1;19) ALL. *NOTCH2* and *NOTCH3* are highly expressed in t(1;19) ALL and are downregulated upon *TCF3-PBX1* knockdown (Figure 8). Therefore, the anti-Notch2/3 neutralizing monoclonal antibody OMP-59R5 (OncoMed Pharmaceuticals, Redwood City, CA, USA) might be especially effective in treating t(1;19) ALL patients[155]. Alternatively, TCF3-PBX1 could be promoting pro-survival pathways through downstream transcriptional activation of genes encoding key pre-BCR signaling molecules such as BLK or PKCζ which can be targeted by ibrutinib or a myristoylated pseudo-substrate inhibitor, respectively[96,152]. Indeed, inhibition of BLK, in combination with BTK, was recently shown to prevent pre-BCR$^+$ ALL progression[96]. But, the mechanism remains unknown. Complete comprehension of such genes could be the key to developing new therapeutic strategies for children suffering with t(1;19) B-ALL.

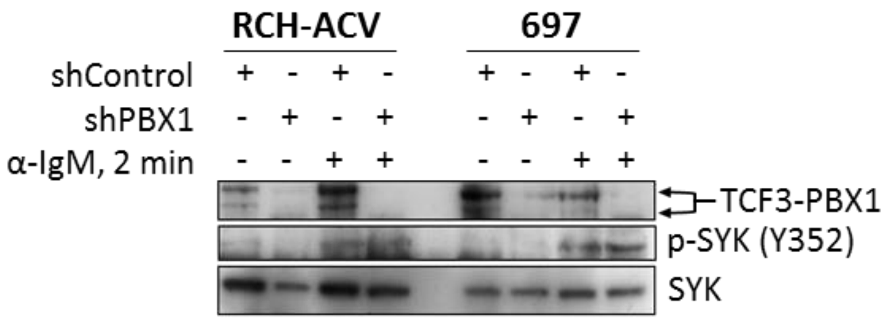
Chapter 6

Supplemental Figures



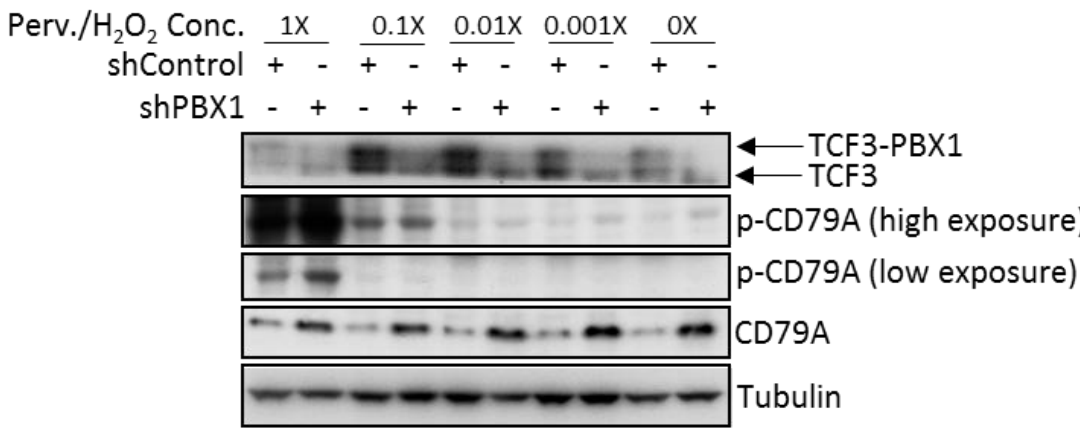
Supplemental Figure 1. ***TCF3-PBX1*** **knockdown reduces overall AKT and ERK activation following α-IgM stimulation.**

(A-B) RCH-ACV (left) and 697 (right) cells expressing shPBX1 abrogates pre-BCR-dependent activation of AKT and ERK compared to (A) cells expressing a control shRNA (shControl) or (B) uninduced shPBX1 cells, following time-course stimulation with α-IgM. Cells were induced with doxycycline (1 µg/mL) and re-plated overnight with fresh doxycycline prior to stimulation with α-IgM.



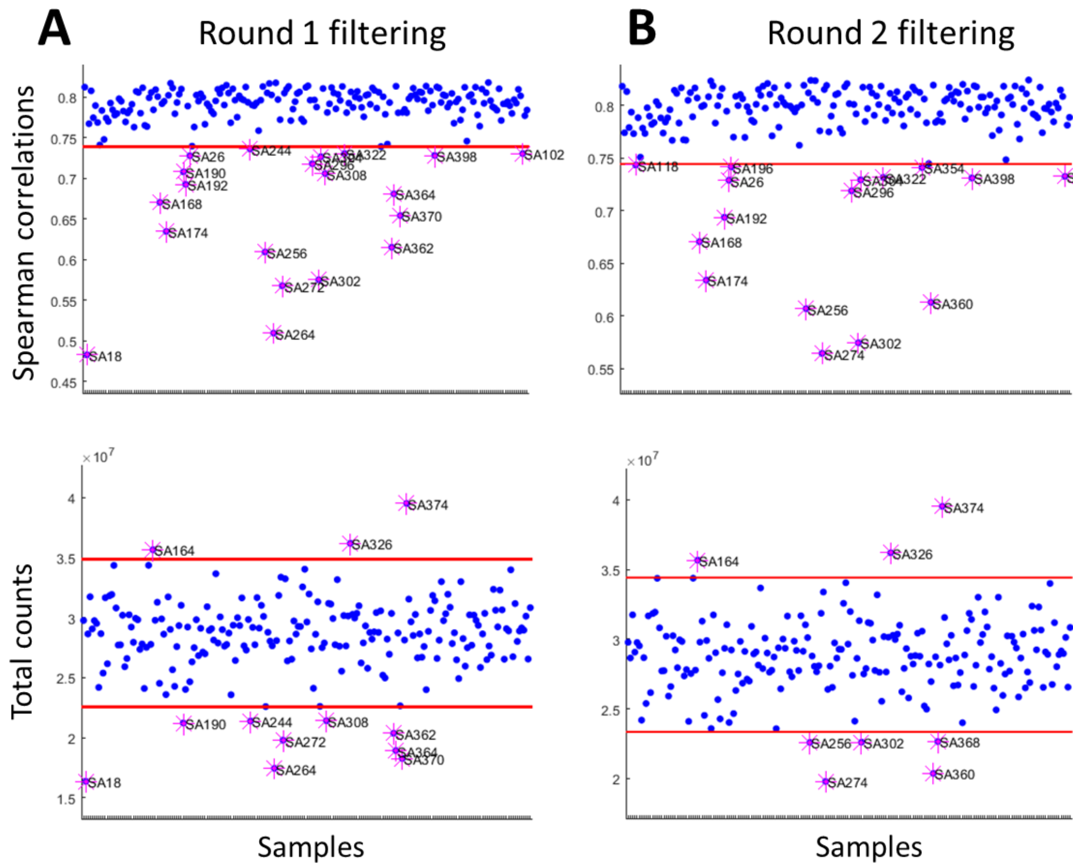
Supplemental Figure 2. *TCF3-PBX1* knockdown does not reduce overall SYK phosphorylation at tyrosine 352.

Western blot showing that *TCF3-PBX1* knockdown does not reduce α-IgM-mediated phosphorylation of SYK at tyrosine residue 352 (Y352). Cells were induced with doxycycline (1 µg/mL) for 72 hours and re-plated overnight with fresh doxycycline prior to stimulation with α-IgM.



Supplemental Figure 3. *TCF3-PBX1* does not promote CD79A phosphorylation.

TCF3-PBX1 knockdown failed to decrease the abundance of phosphorylated CD79A in RCH-ACV cells treated with various concentrations of pervanadate. Cells were induced with doxycycline (1 µg/mL) for 72 hours prior to pervanadate treatments.



Samples removed

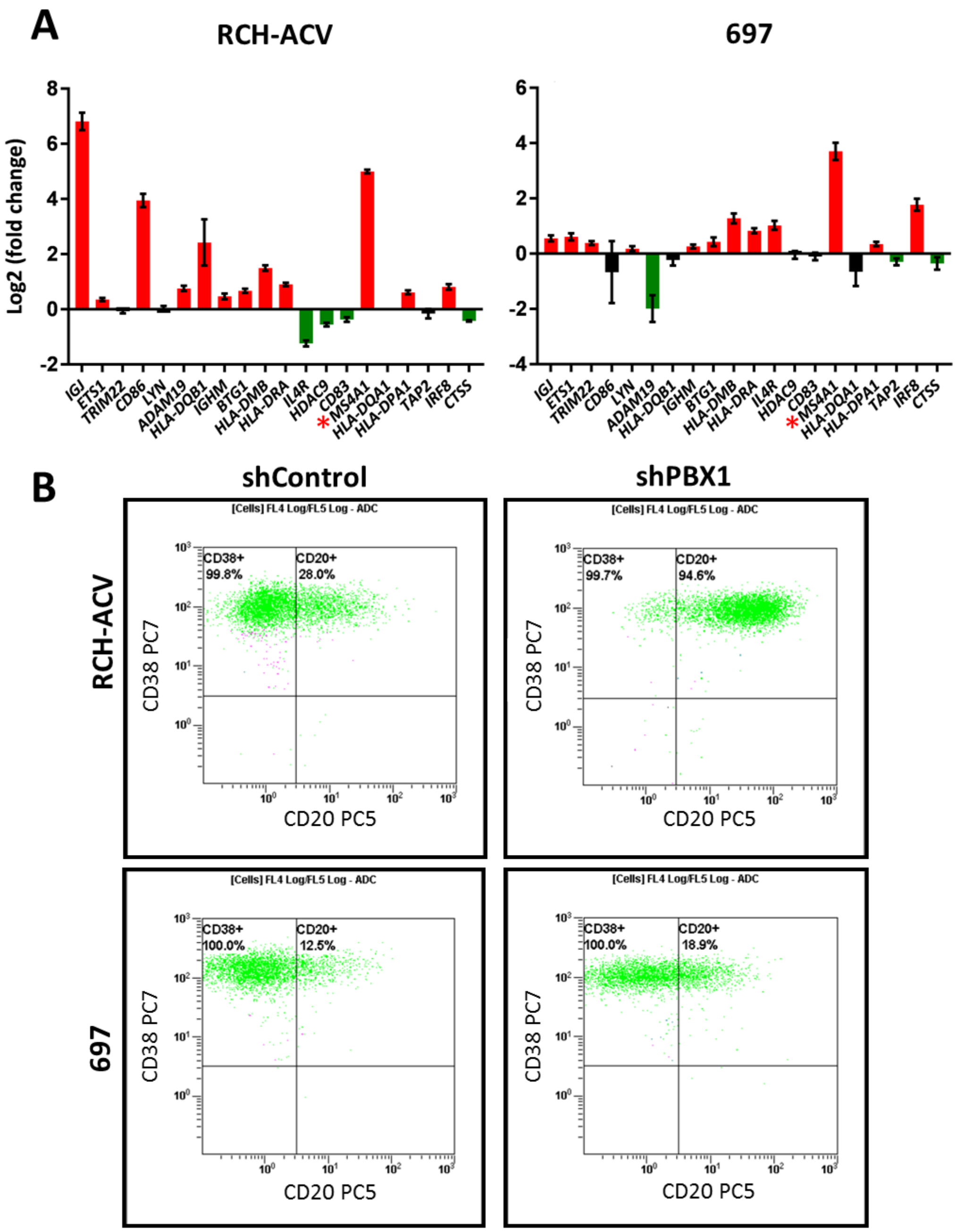
SA18
SA190
SA244
SA264
SA272
SA308
SA362
SA364
SA370

Samples removed

SA256
SA274
SA302
SA360

Supplemental Figure 4. Pre-processing of TARGET-ALL gene-array expression data.

(A-B) Spearman correlations (upper) and total counts (lower) were plotted to help identify outlier samples. The red lines represent 1.5IQR above the 75[th] percentile or 1.5IQR below the 25[th] percentile. Samples that fell outside of this threshold are labeled and indicated by pink asterisks. (A) Samples that were labeled in both plots were removed. (B) The Spearman correlation and total count plots were regenerated after the first round of filtering. Samples that were removed are indicated at the bottom.



Supplemental Figure 5. Immature B-cell-specific genes are upregulated upon *TCF3-PBX1* knockdown

(A) Results of RNA-Seq differential gene expression profiling for immature B-cell-specific transcripts in both RCH-ACV (left) and 697 (right) cells after *TCF3-PBX1* knockdown. This subset of genes was previously identified by Hystad and colleagues[156]. Red and green bars indicate genes that are differentially induced and downregulated ($p < 0.05$, two-tailed Student's *t* test) in shPBX1-expressing cells, respectively. This includes *MS4A1* which encodes the CD20 surface receptor (red asterisk). Cells were induced with doxycycline (1 µg/mL) for 72 hours prior to sequencing (B) Immunophenotypic analysis of surface CD20 in RCH-ACV (left) and 697 (right) cells after *TCF3-PBX1* knockdown.

Supplemental Table 1. Candidates for direct transcriptional regulation by TCF3-PBX1

AC023481.1	*EDEM1*	*LRRN2*	*RHOB*	*TNFRSF10B*
AC026202.1	*EML1*	*LSP1*	*RND3*	*TNFRSF10D*
AC133680.1	*EMP2*	*MAFA*	*RNU4ATAC17P*	*TNFRSF17*
ADAM11	*ERG*	*MAST1*	*RP11-1149O23.3*	*TNFRSF1B*
ADCY5	*ETV5*	*MIR1539*	*RP11-1246C19.1*	*TNFRSF8*
AMBP	*FAM124A*	*MLKL*	*RP11-12J10.3*	*TNFSF9*
AP003774.6	*FAM129C*	*MPP3*	*RP11-13K12.1*	*TNS3*
APBB1	*FAM43A*	*MS4A1*	*RP11-147L13.8*	*TOX*
ARHGEF4	*FAM92A1P1*	*MXRA7*	*RP11-148O21.4*	*TRAC*
ARL4C	*FOXO1*	*MYBPH*	*RP11-254F7.3*	*TRPV2*
ARVCF	*FUT1*	*MYO1C*	*RP11-44N11.1*	*TSPAN17*
ATP2B2	*GABRD*	*MYT1L*	*RP11-674N23.1*	*TTC28*
ATXN1	*GNG7*	*MZB1*	*RP11-674N23.4*	*UAP1L1*
B3GAT1	*GPR114*	*NEDD4L*	*RP11-744N12.3*	*VDR*
BCAR3	*GPR56*	*NFIL3*	*RP11-87G24.3*	*VPS26B*
BCL3	*HELZ2*	*NMT2*	*RP5-892K4.1*	*VPS37D*
BHLHE40	*HMGB3P32*	*NOL3*	*RRAS*	*VWA1*
BLK	*HMX2*	*NPY*	*RTN4RL1*	*ZDHHC11*
C10orf54	*HTRA3*	*PDGFRB*	*S1PR5*	
C1RL	*HYDIN*	*PFKFB4*	*SCCPDH*	
C9orf117	*ICAM1*	*PLCB2*	*SCN1B*	
CACNA1H	*ICAM4*	*PLEKHA1*	*SEMA6B*	
CACNA2D4	*IER2*	*PLEKHG3*	*SERINC2*	
CALD1	*IGLV3-1*	*PNMAL2*	*SH3BP2*	
CCNE2	*IGLV6-57*	*PPP1R14C*	*SLC29A4*	
CD52	*IL21R*	*PPP2R5B*	*SMAD7*	
CD70	*IL21R-AS1*	*PRKCH*	*SNAI1*	
CD79B	*IQCE*	*PRKCZ*	*SNCB*	
CDHR5	*IRF8*	*PRR5L*	*SOGA2*	
CMTM4	*JAG2*	*PTGFRN*	*SOX18*	
COL5A3	*KANK1*	*PTMS*	*SOX8*	
COL6A1	*KB-208E9.1*	*PTRH1*	*STC2*	
CPA6	*KCNJ12*	*RAB13*	*STEAP1B*	
CRIP2	*KIAA0513*	*RAB15*	*STIM2*	
CTC-250I14.6	*KIAA1462*	*RAB3D*	*SYNGR1*	
CTC-436K13.3	*KIF12*	*RAC3*	*SYNPO*	
CTC-510F12.6	*KLF11*	*RAPGEFL1*	*TBC1D10C*	
CTD-2369P2.8	*LDLRAD3*	*RASIP1*	*TMEM132A*	
DFNA5	*LGALS1*	*RASSF4*	*TMEM52*	
DNM1	*LINC00426*	*RCOR2*	*TNFRSF10A*	

References

1. Inaba, H., Greaves, M. & Mullighan, C. G. Acute lymphoblastic leukaemia. *Lancet* **381,** 1943–1955 (2013).

2. Pui, C.-H., Relling, M. V & Downing, J. R. Acute Lymphoblastic Leukemia. *N. Engl. J. Med.* **350,** 1535–1548 (2004).

3. Murphy, S. L., Xu, J. & Kochanek, K. D. Deaths: final data for 2010. *Natl. Vital Stat. Rep.* **61,** 1–117 (2013).

4. Faderl, S., O'Brien, S., Pui, C.-H., Stock, W., Wetzler, M., *et al.* Adult acute lymphoblastic leukemia. *Cancer* **116,** 1165–1176 (2010).

5. Cobaleda, C., Schebesta, A., Delogu, A. & Busslinger, M. Pax5: the guardian of B-cell identity and function. *Nat. Immunol.* **8,** 463–470 (2007).

6. Pongubala, J. M. R., Northrup, D. L., Lancki, D. W., Medina, K. L., Treiber, T., *et al.* Transcription factor EBF restricts alternative lineage options and promotes B-cell fate commitment independently of Pax5. *Nat. Immunol.* **9,** 203–215 (2008).

7. Bain, G., Maandag, E. C., Izon, D. J., Amsen, D., Kruisbeek, a M., *et al.* E2A proteins are required for proper B-cell development and initiation of immunoglobulin gene rearrangements. *Cell* **79,** 885–92 (1994).

8. Sigvardsson, M., Clark, D. R., Fitzsimmons, D., Doyle, M., Akerblad, P., *et al.* Early B-cell factor, E2A, and Pax-5 cooperate to activate the early B-cell-specific mb-1 promoter. *Mol. Cell. Biol.* **22,** 8539–51 (2002).

9. Busslinger, M. Transcriptional control of early B-cell development. *Annu. Rev. Immunol.* **22,** 55–79 (2004).

10. Zhu, J. & Emerson, S. G. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene* **21,** 3295–3313 (2002).

11. Orkin, S. H. & Zon, L. I. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* **132,** 631–44 (2008).

12. Igarashi, H., Gregory, S. C., Yokota, T., Sakaguchi, N. & Kincade, P. W. Transcription from the RAG1 Locus Marks the Earliest Lymphocyte Progenitors in Bone Marrow. *Immunity* **17,** 117–130 (2002).

13. Rickert, R. C. New insights into pre-BCR and BCR signalling with relevance to B-cell malignancies. *Nat. Rev. Immunol.* **13,** 578–591 (2013).

14. Pieper, K., Grimbacher, B. & Eibel, H. B-cell biology and development. *J. Allergy Clin. Immunol.* **131,** 959–971 (2013).

15. Chiaretti, S., Zini, G. & Bassan, R. Diagnosis and subclassification of acute lymphoblastic leukemia. *Mediterr. J. Hematol. Infect. Dis.* **6,** e2014073 (2014).

16. Buchner, M., Swaminathan, S., Chen, Z. & Müschen, M. Mechanisms of pre-B-cell receptor checkpoint control and its oncogenic subversion in acute lymphoblastic leukemia. *Immunol. Rev.* **263,** 192–209 (2015).

17. Mullighan, C. G. Molecular genetics of B-precursor acute lymphoblastic leukemia. *J. Clin. Invest.* **122,** 3407–15 (2012).

18. Geng, H., Hurtz, C., Lenz, K. B., Chen, Z., Baumjohann, D., *et al.* Self-enforcing feedback activation between BCL6 and pre-B-cell receptor signaling defines a distinct subtype of acute lymphoblastic leukemia. *Cancer Cell* **27,** 409–25 (2015).

19. Shurtleff, S. A., Buijs, A., Behm, F. G., Rubnitz, J. E., Raimondi, S. C., *et al.* TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. *Leukemia* **9,** 1985–9 (1995).

20. Hilden, J. M. Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group. *Blood* **108,** 441–451 (2006).

21. Jan, M. & Majeti, R. Clonal evolution of acute leukemia genomes. *Oncogene* **32,** 135–140 (2013).

22. Notta, F., Mullighan, C. G., Wang, J. C. Y., Poeppl, A., Doulatov, S., *et al.* Evolution of human BCR–ABL1 lymphoblastic leukaemia-initiating cells. *Nature* **469,** 362–367 (2011).

23. Anderson, K., Lutz, C., van Delft, F. W., Bateman, C. M., Guo, Y., *et al.* Genetic variegation of clonal architecture and propagating cells in leukaemia. *Nature* **469,** 356–361 (2011).

24. Raimondi, S. C., Behm, F. G., Roberson, P. K., Williams, D. L., Pui, C. H., *et al.* Cytogenetics of pre-B-cell acute lymphoblastic leukemia with emphasis on prognostic implications of the t(1;19). *J. Clin. Oncol.* **8,** 1380–1388 (1990).

25. Kamps, M. P., Murre, C., Sun, X. & Baltimore, D. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B all. *Cell* **60,** 547–555 (1990).

26. Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Stanbridge, E., *et al.* Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. *Cell* **60,** 535–545 (1990).

27. LeBrun, D. E2A basic helix-loop-helix transcription factors in human leukemia. *Front Biosci* **8,** 206–222 (2003).

28. Murre, C., McCaw, P. S. & Baltimore, D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* **56,** 777–783 (1989).

29. Zhuang, Y., Soriano, P. & Weintraub, H. The helix-loop-helix gene E2A is required for B-cell formation. *Cell* **79,** 875–84 (1994).

30. De Pooter, R. F. & Kee, B. L. E proteins and the regulation of early lymphocyte development. *Immunol. Rev.* **238,** 93–109 (2010).

31. Xu, W., Carr, T., Ramirez, K., McGregor, S., Sigvardsson, M., *et al.* E2A transcription factors limit expression of Gata3 to facilitate T lymphocyte lineage commitment. *Blood* **121,** 1534–1542 (2013).

32. Kee, B. L. E and ID proteins branch out. *Nat. Rev. Immunol.* **9,** 175–184 (2009).

33. Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., *et al.* Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58,** 537–544 (1989).

34. Ephrussi, A., Church, G. M., Tonegawa, S. & Gilbert, W. B lineage--specific interactions of an immunoglobulin enhancer with cellular factors in vivo. *Science* **227,** 134–40 (1985).

35. Massari, M. E. & Murre, C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol. Cell. Biol.* **20,** 429–40 (2000).

36. Porcher, C., Liao, E. C., Fujiwara, Y., Zon, L. I. & Orkin, S. H. Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding. *Development* **126,** 4603–15 (1999).

37. O'Riordan, M. & Grosschedl, R. Coordinate regulation of B-cell differentiation by the transcription factors EBF and E2A. *Immunity* **11,** 21–31 (1999).

38. Bain, G., Engel, I., Robanus Maandag, E. C., te Riele, H. P., Voland, J. R., *et al.* E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol. Cell. Biol.* **17,** 4782–91 (1997).

39. Engel, I. & Murre, C. Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. *Proc. Natl. Acad. Sci.* **96,** 996–1001 (1999).

40. Bürglin, T. R. & Affolter, M. Homeodomain proteins: an update. *Chromosoma* **125,** 497–521 (2016).

41. Bürglin, T. R. Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Res.* **25,** 4173–4180 (1997).

42. Hisa, T., Spence, S. E., Rachel, R. A., Fujita, M., Nakamura, T., *et al.* Hematopoietic, angiogenic and eye defects in Meis1 mutant animals. *EMBO J.* **23,** 450–459 (2004).

43. Fernandez-Diaz, L. C., Laurent, A., Girasoli, S., Turco, M., Longobardi, E., *et al.* The absence of Prep1 causes p53-dependent apoptosis of mouse pluripotent epiblast cells. *Development* **137,** 3393–3403 (2010).

44. Selleri, L., Depew, M. J., Jacobs, Y., Chanda, S. K., Tsang, K. Y., *et al.* Requirement for

Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation. *Development* **128,** 3543–57 (2001).

45. Schnabel, C. A., Selleri, L. & Cleary, M. L. Pbx1 is essential for adrenal development and urogenital differentiation. *Genesis* **37,** 123–30 (2003).

46. Merabet, S. & Mann, R. S. To be specific or not: The critical relationship between hox and TALE proteins. *Trends Genet.* **32,** 334–347 (2016).

47. Slattery, M., Riley, T., Liu, P., Abe, N., Gomez-Alcala, P., *et al.* Cofactor binding evokes latent differences in DNA binding specificity between Hox proteins. *Cell* **147,** 1270–82 (2011).

48. Riley, T. R., Slattery, M., Abe, N., Rastogi, C., Liu, D., *et al.* SELEX-seq: a method for characterizing the complete repertoire of binding site preferences for transcription factor complexes. *Methods Mol. Biol.* **1196,** 255–78 (2014).

49. Penkov, D., Tanaka, S., Di Rocco, G., Berthelsen, J., Blasi, F., *et al.* Cooperative interactions between PBX, PREP, and HOX proteins modulate the activity of the alpha 2(V) collagen (COL5A2) promoter. *J. Biol. Chem.* **275,** 16681–9 (2000).

50. Merabet, S. & Hudry, B. Hox transcriptional specificity despite a single class of cofactors: Are flexible interaction modes the key? *BioEssays* **35,** 88–92 (2013).

51. Wang, Y., Yates, F., Naveiras, O., Ernst, P. & Daley, G. Q. Embryonic stem cell-derived hematopoietic stem cells. *Proc. Natl. Acad. Sci.* **102,** 19081–19086 (2005).

52. Davidson, A. J., Ernst, P., Wang, Y., Dekens, M. P. S., Kingsley, P. D., *et al.* Cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. *Nature* **425,** 300–306 (2003).

53. Krivtsov, A. V., Twomey, D., Feng, Z., Stubbs, M. C., Wang, Y., *et al.* Transformation from committed progenitor to leukaemia stem cell initiated by MLL–AF9. *Nature* **442,** 818–822 (2006).

54. Yan, J., Chen, Y.-X., Desmond, A., Silva, A., Yang, Y., *et al.* Cdx4 and Menin Co-Regulate Hoxa9 Expression in Hematopoietic Cells. *PLoS One* **1,** e47 (2006).

55. Sanyal, M., Tung, J. W., Karsunky, H., Zeng, H., Selleri, L., *et al.* B-cell development fails in the absence of the Pbx1 proto-oncogene. *Blood* **109,** 4191–4199 (2007).

56. Quong, M. W., Massari, M. E., Zwart, R. & Murre, C. A new transcriptional-activation motif restricted to a class of helix-loop-helix proteins is functionally conserved in both yeast and mammalian cells. *Mol Cell Biol* **13,** 792–800 (1993).

57. Aronheim, A., Shiran, R., Rosen, A. & Walker, M. D. The E2A gene product contains two separable and functionally distinct transcription activation domains. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8063–7 (1993).

58. Bhalla, S., Spaulding, C., Brumbaugh, R. L., Zagort, D. E., Massari, M. E., *et al.* Differential roles for the E2A activation domains in B lymphocytes and macrophages. *J.*

Immunol. **180,** 1694–703 (2008).

59. Bayly, R., Chuen, L., Currie, R. A., Hyndman, B. D., Casselman, R., *et al.* E2A-PBX1 interacts directly with the KIX domain of CBP/p300 in the induction of proliferation in primary hematopoietic cells. *J. Biol. Chem.* **279,** 55362–55371 (2004).

60. Denis, C. M., Chitayat, S., Plevin, M. J., Wang, F., Thompson, P., *et al.* Structural basis of CBP/p300 recruitment in leukemia induction by E2A-PBX1. *Blood* **120,** 3968–3977 (2012).

61. Denis, C. M., Langelaan, D. N., Kirlin, A. C., Chitayat, S., Munro, K., *et al.* Functional redundancy between the transcriptional activation domains of E2A is mediated by binding to the KIX domain of CBP/p300. *Nucleic Acids Res.* **42,** 7370–7382 (2014).

62. Bayly, R., Murase, T., Hyndman, B. D., Savage, R., Nurmohamed, S., *et al.* Critical role for a single leucine residue in leukemia induction by E2A-PBX1. *Mol. Cell. Biol.* **26,** 6442–52 (2006).

63. Vo, N. & Goodman, R. H. CREB-binding protein and p300 in transcriptional regulation. *J. Biol. Chem.* **276,** 13505–8 (2001).

64. Ito, A., Lai, C.-H., Zhao, X., Saito, S., Hamilton, M. H., *et al.* p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. *EMBO J.* **20,** 1331–1340 (2001).

65. Bradney, C., Hjelmeland, M., Komatsu, Y., Yoshida, M., Yao, T.-P., *et al.* Regulation of E2A activities by histone acetyltransferases in B lymphocyte development. *J. Biol. Chem.* **278,** 2370–6 (2003).

66. Stengel, A., Schnittger, S., Weissmann, S., Kuznia, S., Kern, W., *et al.* TP53 mutations occur in 15.7% of ALL and are associated with MYC-rearrangement, low hypodiploidy, and a poor prognosis. *Blood* **124,** 251–258 (2014).

67. Müschen, M. Rationale for targeting the pre – B-cell receptor signaling pathway in acute lymphoblastic leukemia. *Blood* **125,** 3688–3694 (2015).

68. Köhrer, S., Havranek, O., Seyfried, F., Hurtz, C., Coffey, G. P., *et al.* Pre-BCR signaling in precursor B-cell acute lymphoblastic leukemia regulates PI3K/AKT, FOXO1 and MYC, and can be targeted by SYK inhibition. *Leukemia* **30,** 1246–1254 (2016).

69. Duque-Afonso, J., Lin, C.-H., Han, K., Wei, M. C., Feng, J., *et al.* E2A-PBX1 remodels oncogenic signaling networks in B-cell precursor acute lymphoid leukemia. *Cancer Res.* **76,** 6937–6949 (2016).

70. Rickert, R. C. New insights into pre-BCR and BCR signalling with relevance to B-cell malignancies. *Nat. Rev. Immunol.* **13,** 578–591 (2013).

71. Köhler, F., Hug, E., Eschbach, C., Meixlsperger, S., Hobeika, E., *et al.* Autoreactive B-cell receptors mimic autonomous pre-B-cell receptor signaling and induce proliferation of early B-cells. *Immunity* **29,** 912–21 (2008).

72. Guloglu, F. B., Bajor, E., Smith, B. P. & Roman, C. A. J. The unique region of surrogate light chain component lambda5 is a heavy chain-specific regulator of precursor B-cell receptor signaling. *J. Immunol.* **175,** 358–66 (2005).

73. Ohnishi, K. & Melchers, F. The nonimmunoglobulin portion of λ5 mediates cell-autonomous pre-B-cell receptor signaling. *Nat. Immunol.* **4,** 849–856 (2003).

74. Minegishi, Y., Coustan-Smith, E., Wang, Y. H., Cooper, M. D., Campana, D., *et al.* Mutations in the human lambda5/14.1 gene result in B-cell deficiency and agammaglobulinemia. *J. Exp. Med.* **187,** 71–7 (1998).

75. ten Boekel, E., Melchers, F. & Rolink, A. G. Precursor B-cells showing H chain allelic inclusion display allelic exclusion at the level of pre-B-cell receptor surface expression. *Immunity* **8,** 199–207 (1998).

76. Melchers, F., Rolink, A., Grawunder, U., Winkler, T. H., Karasuyama, H., *et al.* Positive and negative selection events during B lymphopoiesis. *Curr. Opin. Immunol.* **7,** 214–227 (1995).

77. Mårtensson, I.-L., Almqvist, N., Grimsholm, O. & Bernardi, A. I. The pre-B-cell receptor checkpoint. *FEBS Lett.* **584,** 2572–2579 (2010).

78. Gauthier, L., Rossi, B., Roux, F., Termine, E. & Schiff, C. Galectin-1 is a stromal cell ligand of the pre-B-cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. *Proc. Natl. Acad. Sci.* **99,** 13014–13019 (2002).

79. Monroe, J. G. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat. Rev. Immunol.* **6,** 283–294 (2006).

80. Hermiston, M. L., Xu, Z. & Weiss, A. CD45: a critical regulator of signaling thresholds in immune cells. *Annu. Rev. Immunol.* **21,** 107–37 (2003).

81. Schmitz, R., Baumann, G. & Gram, H. Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src and Syk as assessed by phage display. *J. Mol. Biol.* **260,** 664–677 (1996).

82. Burkhardt, a L., Brunswick, M., Bolen, J. B. & Mond, J. J. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7410–7414 (1991).

83. Pelanda, R., Braun, U., Hobeika, E., Nussenzweig, M. C. & Reth, M. B-cell progenitors are arrested in maturation but have intact VDJ recombination in the absence of Ig-alpha and Ig-beta. *J. Immunol.* **169,** 865–72 (2002).

84. Rowley, R. B., Burkhardt, A. L., Chao, H. G., Matsueda, G. R. & Bolen, J. B. Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig alpha/Ig beta immunoreceptor tyrosine activation motif binding and autophosphorylation. *J. Biol. Chem.* **270,** 11590–4 (1995).

85. Mócsai, A., Ruland, J. & Tybulewicz, V. L. J. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* **10,** 387–402 (2010).

86. Werner, M., Hobeika, E. & Jumaa, H. Role of PI3K in the generation and survival of B-cells. *Immunol. Rev.* **237,** 55–71 (2010).

87. Moon, K. D., Post, C. B., Durden, D. L., Zhou, Q., De, P., *et al.* Molecular basis for a direct interaction between the Syk protein-tyrosine kinase and phosphoinositide 3-kinase. *J. Biol. Chem.* **280,** 1543–51 (2005).

88. Yamazaki, T., Takeda, K., Gotoh, K., Takeshima, H., Akira, S., *et al.* Essential immunoregulatory role for BCAP in B-cell development and function. *J. Exp. Med.* **195,** 535–45 (2002).

89. Aiba, Y., Kameyama, M., Yamazaki, T., Tedder, T. F. & Kurosaki, T. Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase. *Blood* **111,** 1497–1503 (2007).

90. Otero, D. C., Omori, S. A. & Rickert, R. C. Cd19-dependent activation of Akt kinase in B-lymphocytes. *J. Biol. Chem.* **276,** 1474–8 (2001).

91. Herzog, S., Reth, M. & Jumaa, H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat. Rev. Immunol.* **9,** 195–205 (2009).

92. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., *et al.* Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96,** 857–68 (1999).

93. Fang, X., Yu, S. X., Lu, Y., Bast, R. C., Woodgett, J. R., *et al.* Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc. Natl. Acad. Sci.* **97,** 11960–11965 (2000).

94. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., *et al.* Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91,** 231–41 (1997).

95. Tybulewicz, V. L. J. & Henderson, R. B. Rho family GTPases and their regulators in lymphocytes. *Nat. Rev. Immunol.* **9,** 630–644 (2009).

96. Kim, E., Hurtz, C., Koehrer, S., Wang, Z., Balasubramanian, S., *et al.* Ibrutinib inhibits pre-BCR+B-cell acute lymphoblastic leukemia progression by targeting BTK and BLK. *Blood* **129,** 1155–1165 (2017).

97. Yasuda, T., Sanjo, H., Pagès, G., Kawano, Y., Karasuyama, H., *et al.* Erk kinases link pre-B-cell receptor signaling to transcriptional events required for early B-cell expansion. *Immunity* **28,** 499–508 (2008).

98. Bankovich, A. J., Raunser, S., Juo, Z. S., Walz, T., Davis, M. M., *et al.* Structural insight into pre-B-cell receptor function. *Science* **316,** 291–4 (2007).

99. Erasmus, M. F., Matlawska-Wasowska, K., Kinjyo, I., Mahajan, A., Winter, S. S., *et al.* Dynamic pre-BCR homodimers fine-tune autonomous survival signals in B-cell precursor acute lymphoblastic leukemia. *Sci. Signal.* **9,** ra116-ra116 (2016).

100. Carroll, A. J., Crist, W. M., Parmley, R. T., Roper, M., Cooper, M. D., *et al.* Pre-B-cell

leukemia associated with chromosome translocation 1;19. *Blood* **63,** 721–4 (1984).

101. Chen, D., Zheng, J., Gerasimcik, N., Lagerstedt, K., Sjögren, H., *et al.* The expression pattern of the pre-B-cell receptor components correlates with cellular stage and clinical outcome in acute lymphoblastic leukemia. *PLoS One* **11,** e0162638 (2016).

102. van der Veer, A., van der Velden, V. H. J., Willemse, M. E., Hoogeveen, P. G., Petricoin, E. F., *et al.* Interference with pre-B-cell receptor signaling offers a therapeutic option for TCF3-rearranged childhood acute lymphoblastic leukemia. *Blood Cancer J.* **4,** e181–e181 (2014).

103. Bicocca, V. T., Chang, B. H., Masouleh, B. K., Muschen, M., Loriaux, M. M., *et al.* Crosstalk between ROR1 and the Pre-B-cell receptor promotes survival of t(1;19) acute lymphoblastic leukemia. *Cancer Cell* **22,** 656–67 (2012).

104. Hu, Y. & Smyth, G. K. ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J. Immunol. Methods* **347,** 70–78 (2009).

105. Kuwahara, K., Kawai, T., Mitsuyoshi, S., Matsuo, Y., Kikuchi, H., *et al.* Cross-linking of B-cell antigen receptor-related structure of pre-B-cell lines induces tyrosine phosphorylation of p85 and p110 subunits and activation of phosphatidylinositol 3-kinase. *Int. Immunol.* **8,** 1273–1285 (1996).

106. Vega, F. M. & Ridley, A. J. The RhoB small GTPase in physiology and disease. *Small GTPases* **9,** 384–393 (2018).

107. Dymecki, S. M., Niederhuber, J. E. & Desiderio, S. V. Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells. *Science* **247,** 332–6 (1990).

108. Pasqualato, S., Renault, L. & Cherfils, J. Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for 'front-back' communication. *EMBO Rep.* **3,** 1035–1041 (2002).

109. Kerr, M. C., Bennetts, J. S., Simpson, F., Thomas, E. C., Flegg, C., *et al.* A novel mammalian retromer component, Vps26B. *Traffic* **6,** 991–1001 (2005).

110. Haining, E. J., Yang, J., Bailey, R. L., Khan, K., Collier, R., *et al.* The TspanC8 subgroup of tetraspanins interacts with A disintegrin and metalloprotease 10 (ADAM10) and regulates its maturation and cell surface expression. *J. Biol. Chem.* **287,** 39753–65 (2012).

111. Hirai, T. & Chida, K. Protein kinase Czeta (PKCzeta): activation mechanisms and cellular functions. *J. Biochem.* **133,** 1–7 (2003).

112. Hugnot, J. P., Pedeutour, F., Le Calvez, C., Grosgeorge, J., Passage, E., *et al.* The human inward rectifying K+ channel Kir 2.2 (KCNJ12) gene: gene structure, assignment to chromosome 17p11.1, and identification of a simple tandem repeat polymorphism. *Genomics* **39,** 113–6 (1997).

113. Kim, J. G., Armstrong, R. C., v Agoston, D., Robinsky, A., Wiese, C., *et al.* Myelin transcription factor 1 (Myt1) of the oligodendrocyte lineage, along with a closely related

CCHC zinc finger, is expressed in developing neurons in the mammalian central nervous system. *J. Neurosci. Res.* **50,** 272–90 (1997).

114. Wei, S., Segura, S., Vendrell, J., Aviles, F. X., Lanoue, E., *et al.* Identification and characterization of three members of the human metallocarboxypeptidase gene family. *J. Biol. Chem.* **277,** 14954–64 (2002).

115. Fries, E. & Blom, A. M. Bikunin — not just a plasma proteinase inhibitor. *Int. J. Biochem. Cell Biol.* **32,** 125–137 (2000).

116. Xu, K., Sacharidou, A., Fu, S., Chong, D. C., Skaug, B., *et al.* Blood vessel tubulogenesis requires Rasip1 regulation of GTPase signaling. *Dev. Cell* **20,** 526–39 (2011).

117. Sarkar, S., Roy, B. C., Hatano, N., Aoyagi, T., Gohji, K., *et al.* A novel ankyrin repeat-containing gene (Kank) located at 9p24 is a growth suppressor of renal cell carcinoma. *J. Biol. Chem.* **277,** 36585–91 (2002).

118. Mundel, P., Heid, H. W., Mundel, T. M., Krüger, M., Reiser, J., *et al.* Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. *J. Cell Biol.* **139,** 193–204 (1997).

119. Radtke, F. & Raj, K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat. Rev. Cancer* **3,** 756–767 (2003).

120. Wadehra, M., Goodglick, L. & Braun, J. The tetraspan protein EMP2 modulates the surface expression of caveolins and glycosylphosphatidyl inositol-linked proteins. *Mol. Biol. Cell* **15,** 2073–83 (2004).

121. Smith, C. W., Pritchard, K. & Marston, S. B. The mechanism of Ca2+ regulation of vascular smooth muscle thin filaments by caldesmon and calmodulin. *J Biol Chem* (1987).

122. Liu, Q.-R., Zhang, P.-W., Zhen, Q., Walther, D., Wang, X.-B., *et al.* KEPI, a PKC-dependent protein phosphatase 1 inhibitor regulated by morphine. *J. Biol. Chem.* **277,** 13312–20 (2002).

123. Schepers, G. E., Bullejos, M., Hosking, B. M. & Koopman, P. Cloning and characterisation of the Sry-related transcription factor gene Sox8. *Nucleic Acids Res.* **28,** 1473–80 (2000).

124. Vaughan, K. T., Weber, F. E., Ried, T., Ward, D. C., Reinach, F. C., *et al.* Human myosin-binding protein H (MyBP-H): complete primary sequence, genomic organization, and chromospinal localization. *Genomics* **16,** 34–40 (1993).

125. Schmitz, R., Baumann, G. & Gram, H. Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src and Syk as assessed by phage display. *J. Mol. Biol.* **260,** 664–77 (1996).

126. Parsons, S. J. & Parsons, J. T. Src family kinases, key regulators of signal transduction. *Oncogene* **23,** 7906–7909 (2004).

127. He, F., Wang, L., Hu, X.-B., Yin, D.-D., Zhang, P., *et al.* Notch and BCR signaling synergistically promote the proliferation of Raji B-lymphoma cells. *Leuk. Res.* **33,** 798–

802 (2009).

128. Poe, J. C., Jia, W., Su, H., Anand, S., Rose, J. J., *et al.* An aberrant NOTCH2-BCR signaling axis in B-cells from patients with chronic GVHD. *Blood* **130,** 2131–2145 (2017).

129. Woodcroft, M. W., Nanan, K., Thompson, P., Tyryshkin, K., Smith, S. P., *et al.* Retrovirus-mediated expression of E2A-PBX1 blocks lymphoid fate but permits retention of myeloid potential in early hematopoietic progenitors. *PLoS One* **10,** e0130495 (2015).

130. Casagrande, G., te Kronnie, G. & Basso, G. The effects of siRNA-mediated inhibition of E2A-PBX1 on EB-1 and Wnt16b expression in the 697 pre-B leukemia cell line. *Haematologica* **91,** 765–71 (2006).

131. Diakos, C., Xiao, Y., Zheng, S., Kager, L., Dworzak, M., *et al.* Direct and indirect targets of the E2A-PBX1 leukemia-specific fusion protein. *PLoS One* **9,** e87602 (2014).

132. Holmlund, T., Lindberg, M. J., Grander, D. & Wallberg, A. E. GCN5 acetylates and regulates the stability of the oncoprotein E2A-PBX1 in acute lymphoblastic leukemia. *Leukemia* **27,** 578–585 (2013).

133. Jackson, A. L. & Linsley, P. S. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat. Rev. Drug Discov.* **9,** 57–67 (2010).

134. Turner, B. M. Epigenetic responses to environmental change and their evolutionary implications. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **364,** 3403–18 (2009).

135. Eldfors, S., Kuusanmäki, H., Kontro, M., Majumder, M. M., Parsons, A., *et al.* Idelalisib sensitivity and mechanisms of disease progression in relapsed TCF3-PBX1 acute lymphoblastic leukemia. *Leukemia* **31,** 51–57 (2017).

136. Forbes, S. A., Beare, D., Gunasekaran, P., Leung, K., Bindal, N., *et al.* COSMIC: Exploring the world’s knowledge of somatic mutations in human cancer. *Nucleic Acids Res.* **43,** D805–D811 (2015).

137. Geng, H., Brennan, S., Milne, T. A., Chen, W.-Y., Li, Y., *et al.* Integrative epigenomic analysis identifies biomarkers and therapeutic targets in adult B-acute lymphoblastic leukemia. *Cancer Discov.* **2,** 1004–23 (2012).

138. Harvey, R. C., Mullighan, C. G., Wang, X., Dobbin, K. K., Davidson, G. S., *et al.* Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. *Blood* **116,** 4874–4884 (2010).

139. Hartsink-Segers, S. A., Beaudoin, J. J., Luijendijk, M. W. J., Exalto, C., Pieters, R., *et al.* PKCζ and PKMζ are overexpressed in TCF3-rearranged paediatric acute lymphoblastic leukaemia and are associated with increased thiopurine sensitivity. *Leukemia* **29,** 304–311 (2015).

140. Lin, C.-H., Wong, S. H.-K., Kurzer, J. H., Schneidawind, C., Wei, M. C., *et al.* SETDB2

links E2A-PBX1 to cell-cycle dysregulation in acute leukemia through CDKN2C repression. *Cell Rep.* **23,** 1166–1177 (2018).

141. Fu, X., McGrath, S., Pasillas, M., Nakazawa, S. & Kamps, M. P. EB-1, a tyrosine kinase signal transduction gene, is transcriptionally activated in the t(1;19) subset of pre-B ALL, which express oncoprotein E2a-Pbx1. *Oncogene* **18,** 4920–4929 (1999).

142. de Bock, C. E., Ardjmand, A., Molloy, T. J., Bone, S. M., Johnstone, D., *et al.* The Fat1 cadherin is overexpressed and an independent prognostic factor for survival in paired diagnosis–relapse samples of precursor B-cell acute lymphoblastic leukemia. *Leukemia* **26,** 918–926 (2012).

143. McWhirter, J. R., Neuteboom, S. T. C., Wancewicz, E. V, Monia, B. P., Downing, J. R., *et al.* Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. *Proc. Natl. Acad. Sci.* **96,** 11464–11469 (1999).

144. Wicks, I. P., Wilkinson, D., Salvaris, E. & Boyd, a W. Molecular cloning of HEK, the gene encoding a receptor tyrosine kinase expressed by human lymphoid tumor cell lines. *Proc. Natl. Acad. Sci.* **89,** 1611–1615 (1992).

145. Charmsaz, S., Al-Ejeh, F., Yeadon, T. M., Miller, K. J., Smith, F. M., *et al.* EphA3 as a target for antibody immunotherapy in acute lymphoblastic leukemia. *Leukemia* **31,** 1779–1787 (2017).

146. Mazieres, J., You, L., He, B., Xu, Z., Lee, A. Y., *et al.* Inhibition of Wnt16 in human acute lymphoblastoid leukemia cells containing the t(1;19) translocation induces apoptosis. *Oncogene* **24,** 5396–5400 (2005).

147. Hauser, J., Wallenius, A., Sveshnikova, N., Saarikettu, J. & Grundström, T. Calmodulin inhibition of E2A stops expression of surrogate light chains of the pre-B-cell receptor and CD19. *Mol. Immunol.* **47,** 1031–1038 (2010).

148. Verma-Gaur, J., Hauser, J. & Grundström, T. Negative feedback regulation of antigen receptors through calmodulin inhibition of E2A. *J. Immunol.* **188,** 6175–83 (2012).

149. Hauser, J., Verma-Gaur, J. & Grundström, T. Broad feedback inhibition of pre-B-cell receptor signaling components. *Mol. Immunol.* **54,** 247–253 (2013).

150. Corneliussen, B., Holm, M., Waltersson, Y., Onions, J., Hallberg, B., *et al.* Calcium/calmodulin inhibition of basic-helix-loop-helix transcription factor domains. *Nature* **368,** 760–764 (1994).

151. Tretter, T., Ross, A. E., Dordai, D. I. & Desiderio, S. Mimicry of pre-B-cell receptor signaling by activation of the tyrosine kinase Blk. *J. Exp. Med.* **198,** 1863–73 (2003).

152. Petit, I., Goichberg, P., Spiegel, A., Peled, A., Brodie, C., *et al.* Atypical PKC-ζ regulates SDF-1–mediated migration and development of human CD34+ progenitor cells. *J. Clin. Invest.* **115,** 168–176 (2005).

153. Thomas, M., Calamito, M., Srivastava, B., Maillard, I., Pear, W. S., *et al.* Notch activity synergizes with B-cell-receptor and CD40 signaling to enhance B-cell activation. *Blood*

109, 3342–3350 (2007).

154. Li, X., Bu, X., Lu, B., Avraham, H., Flavell, R. A., *et al.* The hematopoiesis-specific GTP-binding protein RhoH is GTPase deficient and modulates activities of other Rho GTPases by an inhibitory function. *Mol. Cell. Biol.* **22,** 1158–71 (2002).

155. Espinoza, I. & Miele, L. Notch inhibitors for cancer treatment. *Pharmacol. Ther.* **139,** 95–110 (2013).

156. Hystad, M. E., Myklebust, J. H., Bø, T. H., Sivertsen, E. a, Rian, E., *et al.* Characterization of early stages of human B-cell development by gene expression profiling. *J. Immunol.* **179,** 3662–3671 (2007).