Self-Enforcing Feedback Activation between BCL6 and Pre-B Cell Receptor Signaling Defines a Distinct Subtype of Acute Lymphoblastic Leukemia

Highlights

- ALL can be divided into two distinct subtypes based on pre-BCR function
- Pre-BCR-induced activation of BCL6 further increased pre-BCR signaling output
- Pre-BCR inhibitors reduced BCL6 levels and selectively killed pre-BCR⁺ ALL cells
- BCL6 represents a biomarker to identify patients with pre-BCR⁺ ALL

Accession Numbers
GSE59332
GSE59538
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http://dx.doi.org/10.1016/j.ccell.2015.02.003

SUMMARY

Studying 830 pre-B ALL cases from four clinical trials, we found that human ALL can be divided into two fundamentally distinct subtypes based on pre-BCR function. While absent in the majority of ALL cases, tonic pre-BCR signaling was found in 112 cases (13.5%). In these cases, tonic pre-BCR signaling induced activation of BCL6, which in turn increased pre-BCR signaling output at the transcriptional level. Interestingly, inhibition of pre-BCR-related tyrosine kinases reduced constitutive BCL6 expression and selectively killed patient-derived pre-BCR+ ALL cells. These findings identify a genetically and phenotypically distinct subset of human ALL that critically depends on tonic pre-BCR signaling. In vivo treatment studies suggested that pre-BCR tyrosine kinase inhibitors are useful for the treatment of patients with pre-BCR+ ALL.

Significance

Recent work successfully introduced BCR signaling inhibitors into patient care for various subtypes of mature B cell lymphoma, for example, Ibrutinib (BTK) and Idelalisib (PI3Kδ), for germinal center-derived B cell lymphoma. However, it is not known whether pre-BCR signaling represents a therapeutic target in pre-B ALL. Here we report the identification of a subset of human ALL cases that critically depends on tonic pre-BCR signaling and is selectively sensitive to small molecule inhibitors of SYK and SRC tyrosine kinases downstream of the pre-BCR.
Figure 1. Expression and Activity of the Pre-BCR Receptor in Subsets of Pre-B ALL

(A) Flow cytometry staining for cell cytoplasmic μHC.

(B) Cell surface expression of the surrogate light-chain components λ5 (IGLL1) and VpreB.

(C) Ca²⁺ mobilization in response to pre-BCR engagement using μHC-specific antibodies in different subtypes of ALL cases.

(D) Expression of TCF3-PBX1 and ETV6-PDGFRB in Kasumi2, 697, ICN12, RCH-ACV, SMS-SB, and Nel­m-5.

(E) Kinase activity and protein expression of LYN and SRC-pY416.

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INTRODUCTION

Bone marrow progenitor cells in mice produce approximately 10 million pre-B cells daily (Osmond, 1991), the vast majority of which is eliminated at the pre-B cell receptor (BCR) checkpoint (Sakaguchi and Melchers, 1986). Early pre-B cells are programmed to die unless they productively rearrange V\textsubscript{H}DJ\textsubscript{H} gene segments and are rescued by “tonic” pre-BCR signal activity into the long-lived pool of mature peripheral B cells (Rajewsky, 1996). Even in mature B cells, continuous tonic signaling from the BCR is required for B cell survival and maintenance and conditional ablation of tonic BCR signaling results in rapid B cell depletion (Kraus et al., 2004). Interestingly, however, loss of tonic BCR signaling can be rescued by activation of PI3K-AKT signaling (Srinivasan et al., 2009), identifying PI3K-AKT as a central survival pathway downstream of the (pre-)BCR. Tonic pre-BCR signaling involves constitutive activity of the proximal pre-BCR-associated SRC family kinases LYN, FYN, and BLK (Saijo et al., 2003), as well as SYK and ZAP70 (Schweighoffer et al., 2003), which then activate PI3K (Guo et al., 2000; Okada et al., 2000). Recent work highlighted the particular importance of the PI3K p110\textsubscript{δ} (PIK3CD) isoform for pre-BCR survival signaling during early B cell development (Ramadani et al., 2010). The discovery that most subtypes of B cell lymphoma critically depend on BCR signaling (Davis et al., 2010; Schmitz et al., 2012) has led to the development of new targeting strategies that focus on BCR signaling at the level of SRC kinases (Lyn, Fyn, and Blk), SYK/ZAP70 and PI3Kδ (Burger and Okkenhaug, 2014; Chen et al., 2006, 2013; Cheng et al., 2011; Ke et al., 2009; Yang et al., 2006). In addition, small molecule inhibition of BTK, which mediates “chronic active BCR signaling” in activated B cell-like (ABC) diffuse large B cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), and mantle cell lymphoma (MCL) has achieved major clinical success in the treatment of these diseases (Byrd et al., 2013a; Davis et al., 2010; Schmitz et al., 2012; Wang et al., 2013). While the role of BCR signaling in the biology and treatment has been elucidated in all major B cell lymphoma subtypes, the role of pre-BCR signaling has not been systematically studied in human pre-B acute lymphoblastic leukemia (ALL).

Goals of the present study were (1) to identify cases of human pre-B ALL with tonic or chronic active pre-BCR signaling, (2) to estimate their frequency, (3) to determine the role of pre-BCR signaling in specific pre-B ALL subtypes, (4) to identify cooperating genetic lesions, and (5) to develop a concept for therapeutic targeting of the pre-BCR pathway in human pre-B ALL.

RESULTS

Expression and Activity of the Pre-BCR Defines a Distinct Subtype of Human ALL

To elucidate pre-BCR expression and function in pre-B ALL cells, we measured expression of the immunoglobulin μ heavy chain (μHC) and the pre-BCR surrogate light-chain components λ5 (IGLL1) and VpreB on a series of 31 patient-derived pre-B ALL xenograft samples and 15 ALL cell lines by flow cytometry (Tables S1–S3). Twenty eight of the 46 pre-B ALL samples and cell lines tested lacked surface pre-BCR expression, including 5 MLL-rearranged (MLLr), 16 BCR-ABL1, 2 ETV6-RUNX1, and 5 ALL with other abnormalities. Of the 18 pre-BCR\textsuperscript{+} ALL cases, 14 harbored a PBX1 gene rearrangement (1q23), 1 carried a deletion at 6q21, 1 carried both PBX1 gene rearrangement and 6q21 deletion, and 2 harbored PDGFRB gene rearrangements (Figures 1A, 1B, and S1A–S1I). Engagement of the pre-BCR using μHC-specific antibodies resulted in strong Ca\textsuperscript{2+} mobilization from cytoplasmic stores in all 7 pre-BCR\textsuperscript{+} ALL cases tested but not in any of the 19 other cases (Figures 1C and S1A–S1I). These findings suggest that most cases of human ALL lack pre-BCR signaling (pre-BCR\textsuperscript{−}), whereas a distinct ALL subgroup (pre-BCR\textsuperscript{+}) exists that is defined by pre-BCR expression and activity. Indeed, key components of the pre-BCR signaling, including SRC family kinases (LYN, BLK), SYK, BTK, and PLCγ2 were constitutively active in six pre-BCR\textsuperscript{+} ALL samples (Figure 1D). Interestingly, phosphorylation of these molecules was sensitive to treatment of the dual ABL1/SRC-BTK inhibitor Dasatinib (Figure 1D).

Tonic Pre-BCR Signaling, Including Activation of SRC, SYK, and PI3K, in a Subset of Human ALL

To compare baseline signaling activity of pre-BCR\textsuperscript{−} and pre-BCR\textsuperscript{+} ALL cells in a large cohort of patients (MDACC 1983–2007, n = 208), we divided patient samples into two groups based on flow cytometry measurements of pre-BCR (μHC) expression and the Igα signaling chain CD79A (Figure S1J). None of the pre-BCR\textsuperscript{+} ALL cases in this cohort (n = 26) expressed the stem/progenitor cell antigen CD34 and CD25, which are both expressed on the surface of most of the 182 pre-BCR\textsuperscript{−} ALL cases (Figure S1K). Using a panel of 133 validated antibodies, we interrogated those 208 pre-B ALL samples at the time of diagnosis for expression and activity of 66 signaling molecules on reverse phase protein arrays (RPPAs) (Tibes et al., 2006). RPPA analyses revealed that pre-BCR\textsuperscript{+} ALL cells exhibit significantly higher expression and activity of SRC family kinases, including LYN, and strong constitutive activity of multiple components of the PI3K-AKT pathway, including PIK3R1 (p85α), PIK3CD (p110δ), AKT1-pS\textsuperscript{473}/T\textsuperscript{308}, MTOR-pS\textsuperscript{2448}, RPS6KB-pS\textsuperscript{377}, and RPS6-pS\textsuperscript{235}/G\textsuperscript{236} (Figures 1E and S1L). Consistent with strong PI3K-AKT signaling, expression and activity of PTEN, a negative regulator of PI3K-AKT signaling, are low in pre-BCR\textsuperscript{+} ALL cells. These findings are consistent with the established central role of SRC/LYN kinase (Saijo et al., 2003) and PI3K-AKT signaling (Srinivasan et al., 2009), in particular PIK3δ (Ramadani et al., 2010), in tonic pre-BCR signaling. Pre-BCR\textsuperscript{−} ALL cases are also distinct from pre-BCR\textsuperscript{+} ALL cases by particularly low levels of STAT5 expression and activity (Figure S1L). We conclude that pre-BCR\textsuperscript{+} ALL cells are defined by

(D) Patient-derived Pre-BCR\textsuperscript{+} ALL cells were treated with or without Dasatinib (25 μmol/l, 24 hr). Phosphorylation of SRC, BLK, SYK, BTK, and PLCγ2 was measured by western blot.

(E) Protein expression and phosphorylation levels of LYN, SRC, PIK3R1 (p85α), PIK3CD (p110δ), and AKT were measured by RPPAs in pre-BCR\textsuperscript{−} versus pre-BCR\textsuperscript{+} ALL patient samples (MDACC 1983–2007, n = 208). y axis shows log\textsubscript{10} expression values of RPPA data; p values were calculated from two-sided Wilcoxon test. See also Figure S1 and Tables S1–S3.
a distinct signaling phenotype, including constitutive SRC and PI3K-AKT signaling and lack of STAT5 activity.

**Tonic Pre-BCR Signaling Is Associated with a Distinct Gene Expression Profile in Human ALL**

Upregulation of tonic pre-BCR signaling at the expense of STAT5 in pre-B ALL is reminiscent of negative regulation of IL7R/STAT5 upon pre-BCR activation during normal early B cell development (Ochiai et al., 2012). The shift from IL7R/STAT5 to pre-BCR signaling during early B cell differentiation triggers profound gene expression changes. Therefore, we analyzed the gene expression data from three pre-B ALL clinical trials for children (COG P9906, n = 207; Harvey et al., 2010; and St. Jude Research Hospital, n = 132; Ross et al., 2003) and adults (ECOG E2993, n = 191; Geng et al., 2012). Patients in these trials were classified as pre-BCR+ or pre-BCR- based on pre-BCR expression (VHDJH gene rearrangements were negatively selected in all other PBX1 among ALL cases with and us (Bicocca et al., 2012), pre-BCR expression was frequent in 112 cases (13.5%). In agreement with a recent study by others (Bicocca et al., 2012), we collectively found evidence of pre-BCR function (Figure S1K), the STAT5 target genes (ID2, CD69, CD99, ITGA6, CCND2, SOCS2 and PRDM1 (BLIMP1) (Figures 2A–2C).

**Frequent Rearrangement of PBX1 in pre-BCR+ ALL**

We studied the coding capacity of immunoglobulin heavy-chain V\_\_D\_\_j\_\_ gene rearrangements in 152 primary pre-B ALL samples and normal B cell precursors as reference (Trageser et al., 2009). Human bone marrow pro-B, pre-B, and immature B cells from healthy donors (n = 2) were studied by single-cell PCR. In marked contrast to other pre-B ALL subtypes, most cases with TCF3-PBX1 rearrangement and/or 6q21 deletion carried productively rearranged IG\_M alleles and showed evidence of selection for expression of in-frame V\_\_D\_\_j\_\_ gene rearrangements, like normal pre-B cells (Figures 2D and 2E; Table S4). In contrast, productive V\_\_D\_\_j\_\_ gene rearrangements were negatively selected in all other ALL subtypes. Combining data from four clinical trials (n = 830; Table S5), we collectively found evidence of pre-BCR function in 112 cases (13.5%). In agreement with a recent study by others and us (Bicocca et al., 2012), pre-BCR expression was frequent among ALL cases with PBX1 rearrangement or duplication and 6q21 deletion, but rare or absent in cases with MLLr, BCR-ABL1, ET\_\_V6-RUNX1, and hyperdiploid ALL (Figure 2F; Tables S5 and S6).

**TCF3-PBX1 Binds to and Upregulates Pre-BCR Genes in Human ALL Cells**

PBX1 is a proto-oncogene with a critical role in hematopoiesis and lymphopoiesis (Sanyal et al., 2007). In pre-B ALL, PBX1 (1q23) is frequently rearranged to the B cell-specific TCF3 (19p13) locus, encoding E2A (E12 and E47) transcription factors. In these cases, the N-terminal transcriptional activation domains of TCF3 (exons 1-16, TCF3\(a\)) are fused to the C-terminal Hox cooperative motif and homeodomain of PBX1 (exons 4-9, PBX1\(b\)) (Nourse et al., 1999). To identify targets of oncogenic TCF3-PBX1 activity, we performed chromatin immunoprecipitation (ChIP), followed by massively parallel DNA sequencing (ChIPseq), using antibodies against the PBX1\(b\), the TCF3\(a\), and the TCF3-PBX1 co-activator p300 (Bayly et al., 2004) in a TCF3-PBX1 ALL cell line (697) and a primary TCF3-PBX1 ALL sample (ICN12) (Figure S2A). ChIPseq data revealed that TCF3-PBX1, in concert with its coactivator p300, strongly bound to promoter regions of genes that encode key components of the pre-BCR, including IGL\_L1, VPREB1, VPREB3, CD79A, and CD79B, as well as \(\mu\)HC enhancer regions (Figure 3A). Specific binding of TCF3-PBX1 to these six loci was confirmed by single-locus quantitative ChIP (qChIP), using the fusion-specific TCF3-PBX1-hf (Figures 3B and S2B) and the TCF3\(a\), PBX1\(b\), and p300 antibodies (Figures S2C–S2F). To identify genome-wide target genes that are directly regulated by TCF3-PBX1, we matched TCF3-PBX1 binding (ChIPseq; Figure S2D–S2F) with specific gene expression changes that distinguish TCF3-PBX1 cases from other pre-B ALL subtypes (Ross et al., 2003). Among the 1,343 TCF3-PBX1 (PBX1\(a\), TCF3\(a\) and p300) target genes in promotor regions (within 2-kb distance from transcription start site), 99 were specifically upregulated, including multiple pre-BCR components (IGL\_L1, VREB1, VREB3, IG\_M) and pre-BCR downstream signaling molecules (BLK, LCK, SYK, ZAP70, PIK3CD, PIK3R1). In addition, 111 targets were significantly downregulated in TCF3-PBX1 compared to other pre-B ALL subtypes, including the STAT5 target genes CD69 and SOCS2 (Figures 3C and 3D). A pathway analysis of TCF3-PBX1 ChIPseq/gene expression targets suggested that TCF3-PBX1 is a positive regulator of B cell development and BCR signaling and negatively regulated STAT5 (Figure 3E).

To further test whether gene rearrangement and oncogenic activation of TCF3-PBX1 induces pre-BCR activity, we studied full-blown ALL populations that had developed after latencies of 90–180 days in the bone marrow from the TCF3-PBX1- ALL, in full-blown ALL populations that had developed after latencies of 90–180 days in the bone marrow from the TCF3-PBX1- ALL, and in full-blown ALL populations that had developed after latencies of 90–180 days in the bone marrow from the TCF3-PBX1- ALL. Flow cytometry of surface \(\mu\)HC and CD79B (Ig\_l) expression confirmed that ALL cells developing in TCF3-PBX1-transgenic mouse are characterized by pre-BCR expression in contrast to pre-BCR- ALL in BCR-ABL1- and MLL-AF4-transgenic mice (Figure 3F).

**Pre-BCR Signaling Regulates the Balance between BCL6 and STAT5 Activity in Human ALL**

Studying gene expression changes in 516 ALL cases from three clinical trials (St Jude, COG P9906, ECOG E2993; Table S5), we found that pre-BCR+ ALL cells (n = 71, 13.8%) exhibit consistently higher expression levels of BCL6 than pre-BCR- cases (n = 445, 86.2%; Figures 2A–2C). Quantitative RT-PCR analyses confirmed 6- to 30-fold higher mRNA and protein levels of BCL6 compared with other ALL subsets (Figures 4A and S3A). These findings suggested that BCL6 may be a direct target of TCF3-PBX1 and other oncogenic transcription factors that drive pre-BCR+ ALL. However, ChIPseq and qChIP analysis showed no evidence of binding of TCF3-PBX1 to the BCL6 locus (Figures S3B and S3C), indicating that TCF3-PBX1 induces BCL6 expression through an indirect mechanism. Recent studies by us and others (Duy et al., 2010; Swam­nathan et al., 2013) revealed a critical role of BCL6 as a survival...
Figure 2. Pre-BCR Signaling in ALL Is Associated with a Distinct Gene Expression and Signal Transduction Phenotype

(A–C) Gene expression microarray data was analyzed from three cohorts of pre-B ALL patient samples for children (St. Jude Research Hospital; COG P9906) and adults (ECOG E2993). In each dataset, the patient samples were ranked based on their average mRNA expression levels of pre-BCR molecules (\textit{IGLL1}, \textit{IGLL3}, \textit{VPREB1}, \textit{VPREB3}, \textit{IGHM}, \textit{SYK}, and \textit{ZAP70}). The top 15\% and the bottom 25\% cases were considered as the pre-BCR + and pre-BCR \textit{}/C0 and subject to the clustering analysis. Supervised analysis on pre-BCR + versus pre-BCR \textit{}/C0 ALL revealed a 40-gene expression signature that is significantly upregulated or downregulated across all three cohorts. The color scale bar represents the relative log 2 expression changes.

(D) The configuration of the \textit{\mu}HC locus (\textit{IGHM}) was studied in human bone marrow pro-B, pre-B, and immature B cells.

(E) Different subtypes of ALL patient samples (n = 152). The y axis shows frequencies of normal B cells or ALL clones with a functional or a non-functional \textit{IGHM} gene rearrangement in these populations.

(F) Frequency of different cytogenetic subtypes of pre-B ALL cases as pre-BCR\textsuperscript{+} or pre-BCR\textsuperscript{−} based on four clinical trials (n = 830, MDACC, St. Jude, COG and ECOG).

See also Tables S4–S6.
Figure 3. TCF3-PBX1 Fusion Protein Binds to and Upregulates Genes Encoding Pre-BCR Components

(A) ChIPseq tracks for TCF3, PBX1, and p300 antibodies versus input in a TCF3-PBX1 ALL line 697 and a primary sample ICN12 on IGLL1, VPREB1, VPREB3, CD79A, and CD79B promoter regions and the IGHM enhancer regions (Eμ). Gene models were shown in UCSC genome browser hg18.

(B) QChIP validation using an HA antibody that is specific for the HA-Flag-tagged TCF3-PBX1 fusion or an EV as control. N!CAPD2 serves as a negative control gene. Data represent means ± SEM (n = 3); p values are from t test.

(C) The scatter plot shows the average gene expression values of the TCF3-PBX1-rearranged (x axis) versus the non-TCF3-PBX1-rearranged ALL patient samples (y axis) from the St. Jude dataset on the TCF3-PBX1 target genes (n = 1,343). Blue and red highlighted are the downregulated (n = 111) and upregulated (n = 99) genes (>1.5-fold change).

(D) The heat-map representation for some upregulated or downregulated genes. The color scale bar represents the log2 expression changes.
biopsies from pre-BCR + ALL cases included various degrees of TCF3-PBX1, or EV vector or full-blown ALL populations that had developed after latencies of 90–180 days in the bone marrow from the MLL-AF4-bearing mice. Likewise, Cre-mediated deletion of Stat5 in pre-BCR+ (TCF3-PBX1+) ALL cases express BCL6 at high levels (Figure S3D; Table S7). These cases carried lesions affecting IGH-BCL6 and/or gain of 1q23 encompassing TCF3-PBX1 and/or fusion events involving IGH/BCL6 rearrangement. We propose that the mutually exclusive nature of cytokine receptor/STAT5 and pre-BCR/BCL6 pathways in human ALL reflects distinct developmental origins from early (Fraction C) and late (Fraction C’) pre-B cells (Figure 4I). BCL6 ChIPseq analysis revealed that BCL6 and STAT5 binding sites were the most highly enriched DNA elements (p = 10−60 for both) associated with BCL6 binding peaks in TCF3-PBX1 ALL cells (Figure 4D), suggesting that BCL6 and STAT5 may compete for binding to those promoters, which would be consistent with mutual exclusive activity of STAT5 and BCL6 in pre-BCR− and pre-BCR+ ALL subtypes, respectively.

**SYK Tyrosine Kinase Signaling Is Required for BCL6 Activation Downstream of Tonic Pre-BCR Signaling**

Based on the finding that BCL6 is induced by pre-BCR signaling and is a potential target for drug treatment in pre-BCR+ ALL cells, we measured the effects of small molecule inhibitors of proximal pre-BCR tyrosine kinases on BCL6 expression. While 57 of 61 ALL cases with PBX1-rearrangement or duplication showed evidence of tonic pre-BCR signaling (Figure 2F; Table S5), we did not find pre-BCR activity in any of 196 cases with Ph+ ALL (BCR-ABL1). Consistent with divergent pre-BCR activity in these subsets, we found that TCF3-PBX1 but not BCR-ABL1 fusion oncogenes induced expression of BCL6 in pre-B ALL cells (Figure 4E). Treatment with small molecule tyrosine kinase inhibitors of SYK (PRT062607) and BTK (ibrutinib) reduced BCL6 expression in pre-BCR+ TCF3-PBX1 ALL cells (Figures 4F and 4G), demonstrating that SYK tyrosine kinase activity is required for BCL6 expression in pre-BCR+ ALL cells. While TCF3-PBX1 induces constitutive BCL6 expression via tonic pre-BCR signaling, BCR-ABL1 represses BCL6 expression. Ph+ ALL lack BCL6 expression in the presence of active BCR-ABL1, however, ABL1 tyrosine kinase inhibitors (Nilotinib, Dasatinib) relieve BCR-ABL1/STAT5-mediated repression of BCL6 (Figure 4H). Interestingly, the specific ABL1 kinase inhibitor Nilotinib, which does not affect pre-BCR signaling, has no effect on constitutive BCL6 expression in TCF3-PBX1 ALL cells. However, the dual ABL1/SRC kinase inhibitor Dasatinib, which inhibits the SRC kinases BLK, LYN, and FYN upstream of SYK, almost entirely abolished BCL6 expression in pre-BCR+ TCF3-PBX1 ALL cells (Figure 4H). These findings reveal that based on divergent functions of pre-BCR/BCL6 signaling human ALL can be divided into two genetically and phenotypically distinct subsets.

We propose that in the majority of ALL cases, the transforming oncogene (e.g., BCR-ABL1) mimics constitutive active cytokine signaling via activation of STAT5 and repression of BCL6. In 10%-15% of the cases, oncogenic lesions (e.g., TCF3-PBX1; Table S8) result in tonic pre-BCR signaling and activation of BCL6. These differences may reflect different cells of origins of the two subsets. BCR-ABL1 engages IL7R-dependent survival and proliferation signals via STAT5 that are active in pro-B and early pre-B cells (Fraction C); in contrast, activation of pre-BCR signaling (e.g., by TCF3-PBX1) in late pre-B cells (Fraction C’) suppresses IL7R and cytokine signaling (Ochiai et al., 2012). We propose that the mutually exclusive nature of cytokine receptor/STAT5 and pre-BCR/BCL6 pathways in human ALL reflects distinct developmental origins from early (Fraction C) and late (Fraction C’) pre-B cells (Figure 4I). BCL6 ChIPseq analysis revealed that BCL6 and STAT5 binding sites were the most highly enriched DNA elements (p = 10^{-60} for both) associated with BCL6 binding peaks in TCF3-PBX1 ALL cells (Figure 4D), suggesting that BCL6 and STAT5 may compete for binding to those promoters, which would be consistent with mutual exclusive activity of STAT5 and BCL6 in pre-BCR− and pre-BCR+ ALL subtypes, respectively.

**Genetic Mouse Model of Conditional BCL6 Ablation in Pre-BCR+ TCF3-PBX1 ALL**

BCL6 functions as a classical proto-oncogene in germlinal center-derived B cell lymphoma (Ye et al., 1993) and represents a powerful survival factor at the pre-BCR checkpoint during early development. In BCR-ABL1 ALL (pre-BCR+), inhibition of STAT5 activity by treatment with tyrosine kinase inhibitors induced strong expression of BCL6 (Duy et al., 2011). Likewise, in our western blot analysis, while pre-BCR− (TCF3-PBX1) ALL cases expressed high levels of BCL6 in the absence of STAT5 activity, we observed the converse in pre-BCR− (BCR-ABL1) ALL cells (Figure 4A). To test whether pre-BCR signaling directly affects the balance between BCL6 and STAT5, we used an inducible system for pre-BCR activation in mouse ALL cells. To this end, Rag2−/− mouse ALL cells, lacking the ability of endogenous pre-BCR expression, were engineered to express a prearranged tetracyclin-inducible μHC, which results in subsequent pre-BCR expression (Trager et al., 2009). Inducible pre-BCR activation resulted in a massive increase of BCL6 protein levels (40-fold) and concomitant silencing of STAT5 activity (p-Y694; Figure 4B). Similarly, complementation of a defective pre-BCR signaling chain in Blnk−/− or Ighm−/− mouse ALL cells by reconstitution of Blnk or μHC-expression caused dramatic upregulation of BCL6 at the expense of STAT5 activity (p-Y694; Figure 4B). Likewise, Cre-mediated deletion of Stat5α/β in early pre-B cells (Fraction C) was sufficient to increase BCL6 protein levels (Figure 4C). These experiments provide genetic evidence that the balance between BCL6 and STAT5-activity is directly influenced by pre-BCR signaling and hence suggests that high expression levels of BCL6 represent as a surrogate marker for pre-BCR activity in human ALL cells.

**BCL6 Expression Represents a Surrogate Marker for Pre-BCR Activity in Human ALL**

Immunohistochemical analyses for expression of the pre-BCR (μHC) and BCL6 were performed on bone marrow biopsies from 72 ALL patients. These analyses confirmed that all 12 pre-BCR+ (μHC+) ALL cases express BCL6 at high levels (Figure S3D; Table S7). These cases carried lesions affecting PBX1, through TCF3-PBX1 and/or gain of 1q23 encompassing PBX1. In contrast, 60 other cases lacked both BCL6 and μHC surface expression (Figure S3E). We next performed double-stainings for BCL6 and μHC on the same slides, confirming that pre-BCR+ ALL cases express neither μHC nor BCL6. In pre-BCR+ ALL cases, virtually all cells expressed both surface μHC and BCL6 at levels comparable to mature B cell lymphoma carrying IGH-BCL6 rearrangement (Figure 4D). Bone marrow biopsies from pre-BCR− ALL cases included various degrees of admixtures of normal bone marrow cells that lacked both μHC and BCL6 expression.

(E) Ingenuity pathway analysis for the upregulated and downregulated genes. The color scale indicates p values calculated from Ingenuity.

(F) Flow cytometry staining for cytoplasmic μHC, surface CD79B, and CD19 in mouse bone marrow CD19+ ALL cells transduced with the MLL-ENL, BCR-ABL1, TCF3-PBX1, or EV vector or full-blown ALL populations that had developed after latencies of 90–180 days in the bone marrow from the MLL-AF4−, BCR-ABL1−, or TCF3-PBX1− transgenic mice.

See also Figure S2.
B cell development (Duy et al., 2010). For these reasons, we developed a genetic mouse model to determine the role of BCL6 in pre-BCR+ ALL cells. In Bcl6<sup>+/mCherry</sup> mice, exons 5–10 were flanked by LoxP sites and Cre-mediated deletion of these exons results in expression of a truncated Bcl6 protein fused to mCherry (Figure 5A). Thereby, mCherry expression reflects transcriptional activity of the Bcl6 promoter and Cre-mediated deletion can be used for inducible ablation of Bcl6 function and as mCherry-based reporter of Bcl6 expression. In a genetic experiment, we transduced Bcl6<sup>+/mCherry</sup> pre-B cells with TCF3-PBX1 or an empty vector (EV) control. Cre-mediated deletion (Figure 5B) activated the Bcl6-mCherry reporter (Figure 5C). While only ~1% of pre-B cells carrying an EV control showed transcriptional activation of the Bcl6 promoter, more than 50% of TCF3-PBX1-transduced cells actively transcribed Bcl6 (Figure 5C). These findings demonstrate that TCF3-PBX1 indirectly induces Bcl6 expression through transcriptional activation of pre-BCR signaling.

**BCL6 Transcriptionally Activates Pre-BCR Components in TCF3-PBX1 ALL**

To identify BCL6 transcriptional targets in pre-BCR<sup>+</sup> ALL cells, we performed ChIPseq analysis in patient-derived TCF3-PBX1 ALL cells (Figures S4A–S4D). As in DLBCL and Ph<sup>+</sup> ALL (Duy et al., 2011), BCL6 directly binds to a number of checkpoint molecules, including RB1, CDKN2C, and CDKN1B in pre-BCR<sup>+</sup> ALL (Figure S4A). A previous study demonstrated that BCL6 increases tonic BCR signaling in DLBCL cells by transcriptional repression of the inhibitory phosphatase PTPRO (Juszczynski et al., 2009). Likewise, PTPRO was also identified as a transcriptional target of BCL6 in pre-BCR<sup>+</sup> ALL cells. In addition, BCL6 also binds to pre-BCR components (IgLL1, VPREB1) and downstream signaling molecules (BLK, BANK1, SYK) (Figure 5D). QChIP validated BCL6 binding to these loci (Figure S4B). To determine how BCL6 binding affects gene expression in PBX1-rearranged ALL cells, we measured gene expression changes in response to acute ablation of Bcl6 in a Bcl6<sup>+/mCherry</sup> mouse model for TCF3-PBX1 ALL. Upon Cre-mediated deletion of Bcl6, multiple Bcl6 target genes were de-repressed, including checkpoint molecules RB1, CDKN1A, CDKN1B, CDKN2A, and CDKN2C (Figures 5E and 5F). Also, PTPRO were among the de-repressed BCL6 target genes, suggesting that BCL6 increases tonic pre-BCR signaling by transcriptional repression of the (pre-)BCR signaling inhibitor PTPRO as described in DLBCL (Juszczynski et al., 2009). Interestingly, BCL6 targets with transcriptional activation by Bcl6 include multiple pre-BCR related molecules (Bank1, Syk; Figures 5E and 5F). To test BCL6-dependent gene regulation in ALL patient samples, we integrated BCL6 ChIPseq target genes with a BCL6-dependent signature of gene expression in 118 cases of childhood pre-B ALL (St. Jude). This analysis showed transcriptional activation of 63 BCL6 target genes, including multiple pre-BCR related molecules (IgLL1, BLK, VPREB1, PIK3CD, SYK; Figures 5G and 5H). Collectively, these findings suggest a self-enforcing activation loop between tonic pre-BCR signaling and BCL6 activity in a subset of human ALL: oncogenic activation of tonic BCR signaling (e.g., as a result of PBX1 rearrangement and duplication) induces strong activation of BCL6, which further activates pre-BCR signaling (e.g., through transcriptional repression of PTPRO and PRDM1 and activation of IgLL1, VPREB1, BLK, SYK). Interestingly, PBX1 binding to promoter regions of pre-BCR-related genes frequently co-localizes with binding of BCL6, suggesting that BCL6 and PBX1 cooperate in the regulation of these genes (Figure 5I).

**Pre-BCR<sup>+</sup> ALL Cells Are Dependent on BCL6 Activity**

Pre-BCR<sup>+</sup> ALL and BCR-dependent B cell lymphoma share constitutively high BCL6 expression levels. To determine whether high expression levels of BCL6 correlate with clinical outcome of ALL patients, we analyzed mRNA levels of BCL6 at the time of diagnosis in children with high-risk ALL (COG P9906) and adults with ALL (ECOG E2993). Only patients of known pre-BCR expression status were included in the analysis. Since BCL6 was specifically upregulated by tonic pre-BCR signaling, we performed clinical outcome analyses separately for pre-BCR<sup>+</sup> and pre-BCR<sup>+</sup> ALL cases. In both the pediatric (COG P9906, p = 0.043) and adult (ECOG E2993, p = 0.013) clinical trials, higher than median expression levels of BCL6 were predictive of poor outcome among patients with pre-BCR<sup>+</sup> but not pre-BCR<sup>+</sup> ALL (Figures 6A and 6B). These findings suggest
that high expression levels of BCL6 affect the course of disease in pre-BCR− but not pre-BCR+ ALL. To determine the potential contribution of BCL6 to survival and proliferation of pre-BCR+ ALL cells, we studied inducible ablation of Bcl6 in a mouse model (Figures 5A–5G). Bcl6fl/fl TCF3-PBX1 ALL cells were transduced with 4-hydroxy-tamoxifen (4-OHT)-inducible Cre (Cre-ERT2) or an EV control (ER T2). While 4-OHT mediated deletion of Bcl6 resulted in progressive depletion of Cre-ERT2- transduced cells, it had no measurable effects in ER T2- transduced Bcl6fl/fl TCF3-PBX1 ALL cells (Figure 6C). To verify BCL6 dependency of pre-BCR+ ALL in a patient-derived setting, we transduced xenografted TCF3-PBX1 ALL cells with an inducible dominant-negative BCL6 mutant lacking the BCL6-BTB domain (ΔBCL6-ERT2) (Shaffer et al., 2000). While the fraction of ICN12 cells carrying ER T2 EVs remained stable after 4-OHT treatment, cells transduced with ΔBCL6-ERT2 were rapidly depleted (Figure 6D). These findings collectively identify BCL6 as a potential target for the treatment of pre-BCR+ ALL.

**Pharmacological Inhibition of BCL6 in Pre-BCR+ ALL Cells**

In a complementary approach, we tested the consequences of shRNA-mediated knockdown of BCL6 in pre-BCR+ ALL cells. Upon doxycycline-inducible expression of BCL6-specific shRNAs, we observed moderate reduction of BCL6 protein expression and impaired colony formation capacity in pre-BCR+ ALL cells (Figures 5SA–5SSC). For proof-of-principle experiments, a specific retro-inverso BCL6 peptide-inhibitor (RI-BPI) was used to inhibit BCL6 function (Cerchietti et al., 2009). Treatment of patient-derived pre-BCR+ ALL cells with 5 μmol/l RI-BPI induced cell cycle arrest within 1 day of treatment (Figures 6E and S6D) and caused impaired colony formation capacity (Figure S5E). Whereas RI-BPI treatment alone did not induce significant acute toxicity, it strongly sensitized pre-BCR+ ALL cells to Vincristine (1 nmol/l, p = 0.003; Figures 6F and S5F), a mitotic spindle inhibitor that is part of the chemotherapy backbone in most current clinical trials for ALL. In conclusion, BCL6 represents a predictor of poor clinical outcome and a potential target for therapy of patients with pre-BCR+ ALL.

**Pre-BCR+ and Pre-BCR− ALL Cells Exhibit Distinct Kinase-Inhibitor Sensitivity Profiles**

Besides inhibition of BCL6 expression (Figures 4F–4H and S6A), PRT062607 (SYK), Ibrutinib (BTK), and Dasatinib (SRC) induced selective toxicity in pre-BCR+ ALL cells (Figure 7A). Pre-BCR+ ALL (n = 7), pre-BCR− ALL (n = 8), and mature B cell lymphoma kinases PRT062607 (SYK), Ibrutinib (BTK), Dasatinib (SRC), as well as inhibitors of pre-BCR downstream signaling, including GS-1101 (PI3Kδ), AZD053563 (AKT1), Rapamycin (mTOR), AZD6244 (MEK1), SCH772984 (ERK1, ERK2), Enzastaurin (PKCδ), MI2 (MALT1), and BMS345541 (IKK, NF-κB) (Figure S6B). For treatment with the dual SRC/ABL1 kinase inhibitor Dasatinib, only Ph+ ALL cells expressing mutant BCR-ABL1 (T315I) were studied to rule out effects of BCR-ABL1 tyrosine kinase inhibition. Compared with pre-BCR+ ALL and mature B cell lymphoma cells, pre-BCR+ ALL cells were selectively sensitive to inhibition of SYK and SRC (BLK, LYN) and, to some lesser degree, to inhibition of BTK (Figure 7A) and PI3Kδ (Figure S6B). On the other hand, pre-BCR− ALL cells were selectively sensitive to inhibitors of MEK1 (AZD6244) and ERK1/2 (SCH772984), suggesting that pre-BCR+ and pre-BCR− ALL cells can be distinguished based on a specific profile of kinase-inhibitor sensitivity.

To address this possibility in a formal experiment, we treated pre-BCR+ (n = 8) and pre-BCR− (n = 9) ALL cells with a diverse panel of 51 kinase inhibitors that are currently being studied for the treatment of hematological malignancies and solid tumors (Figure 7B). This analysis confirmed that pre-BCR+ ALL cells are particularly sensitive to inhibitors of SYK, SRC, and PIK3δ, whereas pre-BCR− ALL cells are more responsive to inhibition of MEK1 and ERK1/2 (Figure 7B). Integrating experimentally measured sensitivities to individual inhibitors with their known biochemical IC50 values for individual kinase targets, we established a kinase dendrogram of specific vulnerabilities of pre-BCR+ and pre-BCR− ALL (Figure 7C). While pre-BCR+ ALL cells were more dependent on SYK, ZAP70, BTK, and LYN activity, pre-BCR− ALL cells were most vulnerable to inhibition of ABL1, PDGFR, ERK1, ERK2, MET, and KIT kinase activity (Figure 7C).

**Validation of Pre-BCR-BCL6 Signaling as a Therapeutic Target in Pre-BCR+ ALL Cells**

The usefulness of pre-BCR signaling inhibitors PRT062607 (SYK), Ibrutinib (BTK), and Dasatinib (SRC, BTK) for the treatment of pre-BCR+ ALL was tested in a proof-of-principle experiment. To this end, one million patient-derived TCF3-PBX1 pre-BCR+ ALL cells (ICN12) labeled with luciferase were injected into the spleen of NOD/SCID/SQ mice (n = 8). On day 0, mice were divided equally into two groups. Group 1 was treated with 50 μmol/l RI-BPI, and Group 2 (control) was treated with 0.1% ethanol. After 7 days, luciferase expression was measured in Group 1 and Group 2. Luciferase expression was significantly higher in Group 2 compared to Group 1 (p < 0.05). These findings suggest that pre-BCR+ ALL cells are selectively sensitive to inhibition of pre-BCR-BCL6 signaling.

Figure 5. BCL6 Is a Key Regulator of the Transcriptional Program in Pre-BCR+ ALL Cells

(A) A genetic model for inducible ablation of BCL6 and a mCherry-based Bcl6 reporter (Bcl6fl/MM-mCherry). Exons 5–10 of the Bcl6 locus were targeted for inducible Cre-mediated deletion. LoxP sites are indicated as black triangles.

(B) PCR validation of the floxed, deleted, and WT Bcl6 allele from Bcl6fl/MM-mCherry mouse bone marrow pre-B cells.

(C) FACS analysis of EV+ or TCF3-PBX1+ retrowirally transduced Bcl6fl/MM-mCherry pre-B cells that were transduced with either EVGRP or CreGRP vector for Bcl6 deletion. y axis indicates mCherry expression, and x axis indicates GFP expression.

(D) BCL6 ChIPseq binding tracks of target genes in patient-derived TCF3-PBX1 ALL cells (ICN12). y axis represents the number of reads for peak summit normalized by the total number of reads per track. Gene models are shown in UCSC genome browser hg18. A control intragenic region and BCL6 were used as negative and positive controls.

(E and F) A meta-analysis of BCL6 ChIPseq target genes (n = 666) with gene expression microarray data for the EV versus Cre transduced Bcl6fl/MM TCF3-PBX1 ALL cells.

(G and H) For the Bcl6fl/MM versus Bcl6fl/MM ALL patient samples from St. Jude. The scatter or heat-map plots showed upregulated and downregulated BCL6 target genes in each dataset. The color scale bars represent relative log2 expression changes.

(i) PBX1 and BCL6 ChIPseq binding tracks versus input in ICN12 cells on pre-BCR-related genes (IGLL1, VPREB1, BLK, BANK1).

See also Figure S4.
intravenously into sublethally irradiated NOD/SCID mice. Recipient mice were then treated with vehicle (n = 11), PRT062607 (100 mg/kg, n = 7), Ibrutinib (75 mg/kg, n = 7), and Dasatinib (40 mg/kg, n = 12). All of the inhibitors were well tolerated and achieved significant reduction of leukemia burden in vivo, as determined by luciferase bioimaging (Figure 8A). These experiments, together with in vitro testing (Figures 7A and 7B), suggested that Dasatinib has the strongest anti-leukemic effect among the three pre-BCR signaling inhibitors. For this reason, we prioritized Dasatinib for detailed in vitro validation experiments for a group of 135 patient-derived ALL samples, including various ALL subtypes but not Ph+ ALL. Patient-derived ALL cells were cultured over 72 hr in the absence or presence of Dasatinib in concentrations ranging from 1 to 1,000 nmol/l to calculate Dasatinib IC50 values for these cases. Our previous kinase inhibitor screening assays (Tyner et al., 2013) showed that the median IC50 for Dasatinib of all hematology-malignancy samples received at OHSU (n > 400) was 900 nmol/l. Importantly, all 22 pre-BCR+ ALL cases were sensitive to Dasatinib (IC50 < 50 nmol/l). In contrast, only 6 of 113 pre-BCR- ALL cases had IC50 < 50 nmol/l, and the vast majority of these cases did not respond to Dasatinib at concentrations up to 1,000 nmol/l (Figure 8B). Based on a large dataset from 135 patient-derived ALL samples, we conclude that Dasatinib has strong selective antileukemia effects on pre-BCR+ ALL cases. To confirm efficacy of Dasatinib in vivo, we transplanted leukemia cells from four patients with pre-BCR+ ALL into sublethally irradiated NOD/SCID mice. Three of these cases carried a TCF3-PBX1 gene rearrangement, and one case was hypodiploid. In one case (07-112), treatment with Dasatinib alone was sufficient to eradicate ALL and to cure mouse transplant recipients, whereas vehicle-treated mice died within 130 days after injection (Figure 8C). In the three other cases, Dasatinib treatment significantly delayed leukemic expansion and prolonged overall survival of transplant recipient mice. A composite Kaplan-Meier analysis of the four cases injected into a total of 25 mice per group showed a substantial benefit of Dasatinib treatment (p = 0.0001; Figure 8C). To monitor expansion of human leukemia cells (expressing human CD19 and CD45) in the recipient mice, peripheral blood from these mice was drawn weekly and analyzed for chimerism. The Dasatinib-treated cohorts showed minimal signs of circulating human chimerism comparing to the untreated cohorts (Figure 8C). These results demonstrate feasibility and efficacy of leukemia clearance in vivo of pre-BCR+ ALL by Dasatinib based on four patient-derived samples. We expect that combinations with other pre-BCR signaling inhibitors (e.g., PRT062607, Ibrutinib) may have synergistic effects, which will be the focus of future studies by our group.

**DISCUSSION**

Recent analyses revealed that BCR-dependent B cell lymphomas can be divided based on tonic or chronic active BCR signaling (Davis et al., 2010; Rickert, 2013; Schmitz et al., 2012). Tonic BCR signaling involves activation of one single pathway, namely PI3Kδ signaling downstream of SRC (LYN, FYN, BLK) and SYK (Burger and Okkenhaug, 2014; Chen et al., 2013) and is associated with germinal center B cell-like (GCB-) DLBCL and Burkitt’s lymphoma (Young and Staudt, 2013). On the other hand, chronic active BCR signaling in ABC-DLBCL, CLL and MCL engages multiple downstream pathways including BTK (and its downstream targets PKCδ, MALT1, and NF-κB), calcineurin/NFAT, and MEK/Erk (Young and Staudt, 2013). While the majority of B cell lymphoma cases are BCR dependent and sensitive to BCR signaling antagonists, classical Hodgkin’s lymphoma (Kanzler et al., 1996), and primary medias-tinal B cell lymphoma (PMBL) (Leithäuser et al., 2001) together account for 15%–20% of human B cell lymphoma and lack BCR function. The classification of B cell lymphoma based on (1) chronic active, (2) tonic, and (3) lack of BCR signaling informed the development of new treatment strategies and has led to the successful introduction of BTK (chronic active) (Byrd et al., 2013a, 2013b; Wang et al., 2011) and PI3Kδ (tonic BCR signaling) inhibitors (Gopal et al., 2014) into patient care.

Like mature B cell lymphoma, pre-B ALL originates from B cell precursors that critically depend on survival signals emanating from a functional (pre-)BCR. In contrast to B cell lymphoma, however, a classification of human ALL based on pre-BCR function and activity is not available. Studying 830 ALL cases from four clinical trials, we found no functional equivalent of chronic active BCR signaling in B cell lymphoma. In the majority of ALL cases, ALL cells lacked pre-BCR signaling (pre-BCR−), a functional equivalent to classical Hodgkin’s lymphoma and PBML among mature B cell malignancies. However, in about 13.5% of human ALL cases, ALL cells expressed a functional pre-BCR (pre-BCR+) and were highly sensitive to inhibition of SYK and SRC kinases and relatively resistant to inhibition of PKCδ, MALT1, and IKK/NF-κB.

Unlike ABC-DLBCL (chronic active BCR signaling), GCB-DLBCL express high levels of BCL6 in the context of tonic BCR signaling (Juszczynski et al., 2009). Here we found constitutive and pre-BCR-dependent expression of BCL6 in all pre-BCR+ ALL cases studied. Conversely, as a marker in

**Figure 6. Pre-BCR+ ALL Cells Are Dependent on BCL6 Activity**
(A and B) Patients were segregated into two groups based on higher or lower than the median expression of BCL6 in pre-BCR+ and pre-BCR- ALL in two clinical trials: (A) COG P9906 and (B) ECOG E2993. Kaplan-Meier estimates were used to plot the survival probabilities; p values were calculated from the log-rank test. (C) Bcl6fl/fl TCF3-PBX1 pre-B ALL cells were transduced with 4-OHT-inducible Cre (Cre-ERT2-GFP) or EV control (ER T2-GFP). Percentage of GFP-positive cells was measured by flow cytometry at different time points following 4-OHT treatment, and time course data are depicted. (D) Patient-derived Pre-BCR+ ALL cells (ICN12) were transduced with 4-OHT-inducible dominant-negative BCL6 (ΔN BCL6-ER T2-GFP) or EV control (ER T2-GFP). Percentages of GFP-positive cells were measured by flow cytometry at different time points following 4-OHT treatment. (E) ICN12 cells were treated with vehicle or 5 μmol/l RI-BPI for 24 hr and then subjected to cell-cycle analysis (BrdU and 7-AAD staining). (F) ICN12 cells were exposed to vehicle, Vincristine (1 nmol/l), RI-BPI (5 μmol/l), or combination of Vincristine (1 nmol/l) and RI-BPI (5 μmol/l) for 3 days, followed by flow cytometry for Annexin V and 7-AAD staining. Data represent means ± SEM (n = 3); p values from t test. See also Figure S5.
immunohistochemistry staining, BCL6 reliably identified pre-BCR+ ALL cases. BCL6 expression is indeed dependent on tonic pre-BCR signaling, as small molecule inhibition of SYK and SRC family kinases abolished BCL6 expression in pre-BCR+ ALL cells. Since functional assays to measure tonic pre-BCR signaling in ALL patient samples may not be practical for diagnostic purposes, we propose that BCL6 and mHC immunostaining may be a feasible alternative to rapidly identify patients that might benefit from treatment with inhibitors of tonic pre-BCR signaling (e.g., SYK, SRC, PIK3d).

Figure 7. Validation of Pharmacological Inhibition of BCL6-Pre-BCR Signaling as Therapeutic Target in Pre-BCR+ ALL Cells

(A) Cell viability was measured using CCK-8 in presence or absence of PRT062607 (SYK), Ibrutinib (BTK), or Dasatinib (ABL1/SRC/BTK) for 72 hr with gradients of concentrations as indicated in the x axis in pre-BCR+ ALL (n = 7), pre-BCR− ALL (n = 8) and 2 Burkitt lymphoma (MN60, MHH-preB). y axis shows the percentage of viable cells with the untreated cells as control (set to 100%). Data represent means ± SD (n = 3).

(B) Pre-BCR+ (n = 8) and pre-BCR− (n = 9) patient-derived ALL samples were treated with a diverse panel of 51 kinase inhibitors as described previously (Tyner et al., 2013). The heat map represents the IC50 values for each sample relative to the observed median IC50 value for over 400 primary leukemia samples interrogated by this assay at OHSU. Red or blue colors denote higher or lower than the median sensitivity of the pre-B ALL cells tested. Pre-BCR+ ALL: 07-112, 11-064, ICN12, 697, RCH-ACV, Kasumi-2, HPB null, Nalm6. Pre-BCR− ALL: BV173, SUPB15, BLQ5, LAX2, SEM, RS4;11, REH, LAX7R, SFO3.

(C) Kinase dendrogram of pre-BCR+ and pre-BCR− ALL based on experimentally measured sensitivities to individual inhibitors and their known inhibitory profile based on biochemical IC50 values for individual kinase targets. TREESpots software was used (KINOMEscan, http://www.discoverx.com/home). See also Figure S6.

EXPERIMENTAL PROCEDURES

Primary Human Samples and Cell Lines
After informed consent from all participants, primary ALL cases were obtained with the approval of the Institutional Review Boards of the University of
Primary human ALL samples were cultured on OP9 stroma in minimum essential medium (MEM; Life Technologies) with GlutaMAX containing 20% fetal bovine serum (FBS), 100-IU ml⁻¹ penicillin, 100-μg ml⁻¹ streptomycin, and 1-mM sodium pyruvate at 37°C in a humidified incubator with 5% CO₂. The human cell lines were purchased from DSMZ. See Tables S1–S3.

**In Vivo Leukemia Cell Transplantation and Treatment**

All mouse experiments were subject to institutional approval by the University of California San Francisco Institutional Animal Care and Use Committee; 10⁶ cells from primary TCF3-PBX1 pre-BCR⁺ ALL were inoculated via intravenous injection into sublethally irradiated (250 cGy) adult female NOD/SCID mice (n = 37). After injection of leukemia cells, the mice were randomly separated into four groups and treated with (1) Dasatinib (40 mg/kg, n = 12), (2) Ibrutinib (75 mg/kg, n = 7), (3) PRT062607 (100 mg/kg, n = 7), and (4) vehicle (n = 11). Leukemic infiltration was confirmed by flow cytometry and bioimaging.

**Statistical Analysis**

The Kaplan-Meier method was used to estimate overall survival. Log-rank test was used to compare survival differences between patient groups. The R package “survival” v.2.35-8 was used for the survival analysis.

California San Francisco (UCSF) and Oregon Health and Science University (OHSU). See Tables S1–S3.
ACCESSION NUMBERS

The gene expression microarray and ChIP-seq data reported in this paper have been deposited in the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) database with the GEO accession numbers GSE59332 and GSE59538.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2015.02.003.

AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We thank Michael L. Cleary (Stanford) for critical discussions, Arthur L. Shaffer and Louis M Staudt for inducible BCL6 constructs, Mark Kamps and David B. Sykes for inducible TGF3-PBX1 vectors, Lothar Hennighausen for Statsab6/6 mice, and Julia Gaster-Foster and I-Ming Chen for fresh samples from the COG ALL Biology Bank (proposa...


