BACH2 mediates negative selection and p53-dependent tumor suppression at the pre-B cell receptor checkpoint

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The B cell–specific transcription factor BACH2 is required for affinity maturation of B cells. Here we show that Bach2-mediated activation of p53 is required for stringent elimination of pre-B cells that failed to productively rearrange immunoglobulin V_{H}-D_{JH} gene segments. After productive V_{H}-D_{JH} gene rearrangement, pre-B cell receptor signaling ends BACH2-mediated negative selection through B cell lymphoma 6 (BCL6)-mediated repression of p53. In patients with pre-B acute lymphoblastic leukemia, the BACH2-mediated checkpoint control is compromised by deletions, rare somatic mutations and loss of its upstream activator, PAX5. Low levels of BACH2 expression in these patients represent a strong independent predictor of poor clinical outcome. In this study, we demonstrate that Bach2+/- pre-B cells resist leukemic transformation by Myc through Bach2-dependent upregulation of p53 and do not initiate fatal leukemia in transplant-recipient mice. Chromatin immunoprecipitation sequencing and gene expression analyses carried out by us revealed that BACH2 competes with BCL6 for promoter binding and reverses BCL6-mediated repression of p53 and other cell cycle checkpoint–control genes. These findings identify BACH2 as a crucial mediator of negative selection at the pre-B cell receptor checkpoint and a safeguard against leukemogenesis.

RESULTS

Bach2 induces Arf and p53 during early B cell development

To identify factors that mediate negative selection at the pre-B cell receptor checkpoint in humans, we studied gene expression changes during human B cell development at the pro-B cell to pre-B cell transition2. We identified 18 genes with specific upregulation at the pre-B cell receptor checkpoint, including components of the pre-B cell receptor itself (IGLL1 and VPREB1), effectors of V_{H}-D_{JH} recombination (RAG1, RAG2 and DNTT) and mediators of survival signaling (Supplementary Fig. 1). We next studied gene expression changes at the pre-B cell receptor checkpoint in mice under two experimental conditions: initiation of V(D)J recombination after inducible activation of Pax5 (ref. 7) and inducible expression of productively rearranged V_{H}-D_{JH} encoding a functional immunoglobulin μ heavy chain (μHC), which initiates pre-B cell receptor signaling7 (Fig. 1a). We reasoned that pre-B cell receptor checkpoint control should occur between these two events. Indeed, we found that Pax5-mediated initiation of V(D)J recombination was coincident with strong upregulation of recombination activating gene 1 (Rag1) and Rag2 and caused upregulation of Arf and p53 and Bach2 (refs. 8,9). By contrast, inducible expression of μHC expression induced expression of Bcl6, a transcriptional repressor of Arf and p53 (Fig. 1a–c).

Bcl6-mediated repression of Arf and p53 is needed to rescue μHC+ pre-B cells once they have passed the pre-B cell receptor checkpoint4,5. Consistent with our findings in human B cell...
Bach2 and Bcl6 maintain a balance between negative selection and survival of early B cells at the pre-B cell receptor checkpoint. 

(a) Gene expression changes at the pre-B cell receptor checkpoint that were studied under two experimental conditions: initiation of V(D)J recombination after inducible activation of Pax5 compared to empty vector control (EV) and inducible expression of functional µHC. MPP, multipotent progenitor cells. Underlining indicates genes used to induce differentiation of early B cells from one stage to the next; the colors of Bach2 and Bcl6 imply their opposing functions. 

(b) Quantitative RT-PCR (qRT-PCR) (c) western blot analysis (d) showing the regulation of Bach2 and Bcl6 by Pax5 and µHC induction, respectively. OFF indicates that expression of µHC is not turned on, and ON indicates that µHC expression has been induced. 

(d) qRT-PCR for Bach2 and Bcl6 in MPP, pro-B cells, pre-BI cells and pre-BII cells sorted from the bone marrow of C57BL/6 mice. (e,f) GFP+ cells monitored (on days 1 and 10) by flow cytometry in transformed pro-B and pre-BI cells that were transduced with Pax5-GFP or the empty vector control.

To test this hypothesis, we assayed pre-B cell receptor checkpoint control in pro-B and pre-BI cells from Bach2−/− mice and wild-type control mice under the two experimental conditions described above. Activation of GFP-tagged Pax5 induced the accumulation of Arf and p53, determined by western blotting with β-actin (Actb) as the loading control, in Bach2+/+ and Bach2−/− pro-B and pre-BI cells that were transduced with Pax5-GFP or the empty vector control. 

Fig. 1e–g. Bcl6 reverses Bach2-induced activation of Arf and p53

Previous studies by our group identified Bcl6 as a strong transcriptional repressor of Arf and p53 in normal pre-B cells and in pre-B acute lymphoblastic leukemia (ALL)10. Of note, ChIP-seq analysis and quantitative ChIP (qChIP) validation revealed that both Bcl6 and Bach2 bind to overlapping promoter regions of CDKN2A (ARF), TP53 (P53) and other checkpoint regulators (CDKN1A (P21), CDKN1B (P27), GADD45A and GADD45B) (Fig. 2a and Supplementary Figs. 2 and 3). We therefore tested the hypothesis that BACH2 and BCL6 compete for binding to promoter regions of checkpoint regulator genes and that the ratio between the two proteins determines negative (more BACH2 than BCL6) and positive (less BACH2 than BCL6) selection events at the pre-B cell receptor checkpoint (Fig. 2 and Supplementary Figs. 4 and 5). Binding of either BCL6 or BACH2 to CDKN2A and TP53 promoters affects gene expression in opposite directions: mRNA and protein levels of Arf and p53 are significantly reduced in the absence of Bach2 but are strongly increased in the absence of Bcl6 (Fig. 2c,d,f and Supplementary Fig. 6) or after inducible overexpression of Bach2 (Fig. 2c and Supplementary Fig. 4). Likewise, mRNA levels of the p53-dependent tumor suppressor Btg2 (ref. 11) were reduced by >20-fold in the absence of Bach2 but were increased by threefold in the absence of Bcl6 (Fig. 2c,d,f and Supplementary Fig. 6) or after inducible overexpression of Bach2 (Fig. 2c and Supplementary Fig. 4). 

These findings collectively indicate that the balance between Bcl6
and Bach2 determines the repression or transcriptional activation of Cdkn2a, Tp53 and related checkpoint molecules.

**Bach2 mediates V-DJ recombination**

We measured the functional consequences of Bach2 deficiency at the pre-B cell receptor checkpoint in Bach2<sup>−/−</sup> and wild-type mice. Using a classical pre-B cell differentiation model based on tyrosine kinase inhibition (with imatinib) of BCR-ABL1-transformed pre-B cells, we studied gene expression changes after differentiation of Bach2<sup>−/−</sup> and Bach2<sup>−/+</sup> pre-B1 cells (Fig. 3a). In this analysis, Bach2 deficiency was associated with increased expression of the early progenitor antigen Ly6a (also known as Sca-1) and reduced expression of the pre-B cell antigen interleukin-2 receptor α (Il-2ra, also known as Cdkn2a, Tp53 and related checkpoint molecules.

**Figure 2** Bach2-dependent activation of Arf and p53 is reversed by Bcl6 as a result of expression of functional μHC. (a) ChIP-seq analysis performed in a lymphoma cell line using antibodies to BACH2 (red) or BCL6 (green). Peaks with significant enrichment relative to background (input; black) are underlined and were annotated using ChiPseqer. Overlapping peaks between BACH2 and BCL6 binding are indicated with shading. (b) The proposed scenario for BACH2-BCL6 interactions at the pre-B cell receptor checkpoint. Rag1/2, Rag1 and Rag2, Arf/p53, Arf and p53. (c) Effect of the presence or absence of Bach2, the presence or absence of Bcl6 and inducible overexpression of Bach2 (using the 4-OHT–inducible Bach2-ERT<sup>2</sup> vector) on mRNA levels of Arf and p53 measured by qRT-PCR. All samples were analyzed in triplicate, and *P* values were calculated using Student’s t test. (d) Effect of the presence or absence of Bach2 and the presence or absence of Bcl6 on protein amounts of Arf and p53 measured by western blot analysis using β-actin (Actb) as the loading control. (e) CHIP experiments with Bcl6<sup>−/−</sup> (nega tive control), Bach2<sup>−/−</sup> and Bach2<sup>−/+</sup> cells to directly test the hypothesis that Bach2 negatively regulates the ability of Bcl6 to bind to Cdkn2a and Tp53 promoters. All samples were analyzed in triplicate, and *P* values were calculated using Student’s t test. NS, not significant. (f) Gene expression analysis (Affymetrix GeneChip) for a subset of common Bach2 and Bcl6 target genes showing that Bcl6 and Bach2 affect gene expression levels of checkpoint regulators such as Cdkn2a, Gadd45b, Btg1 and Btg2 in opposite directions.

**Bach2 is required for clearance of nonfunctional pre-B cells**

We next studied the composition of B cell–progenitor populations in bone marrows and spleens of Bach2<sup>−/−</sup> and Bach2<sup>−/+</sup> mice. The total numbers of CD19<sup>+</sup>B220<sup>+</sup> B cells were only slightly reduced in the bone marrow and spleens of Bach2<sup>−/−</sup> mice (Fig. 3g and Supplementary Fig. 8). Whereas pre-B cells from Bach2<sup>−/+</sup> bone marrow were stringently selected for productive in-frame V(D)J rearrangements, pre-B cells from Bach2<sup>−/−</sup> bone marrow lacked selection for V<sub>Η</sub>-D<sub>Η</sub> junctions with coding capacity (Fig. 3g and Supplementary Fig. 8). On the basis of random V(D)J recombination, only one in three pre-B cell clones should achieve in-frame rearrangements and coding capacity for a functional pre-B cell receptor. Effective negative pre-B cell selection causes elimination of cells with nonfunctional out-of-frame V<sub>Η</sub>-D<sub>Η</sub> rearrangements and thus results in a length distribution of V<sub>Η</sub>-D<sub>Η</sub> junctions with size peaks that are spaced by three nucleotides. Fragment length analysis revealed a peak-size distribution of V<sub>Η</sub>-D<sub>Η</sub> junctions that were selected for multiples of 3 bp among Bach2<sup>−/−</sup> cells as compared to the random distribution in Bach2<sup>−/+</sup> cells (Fig. 3g and Supplementary Fig. 8). Sequence analysis of V<sub>Η</sub>-D<sub>Η</sub> junctions confirmed that out-of-frame V<sub>Η</sub>-D<sub>Η</sub> rearrangements were stringently cleared from the repertoire in Bach2<sup>−/−</sup> pre-B cells, as compared to wild-type cells (Fig. 3f and Supplementary Fig. 7).
opposed to the accumulation of a large fraction of clones with non-functional rearrangements seen in Bach2<sup>−/−</sup> pre-B cells (Fig. 3g and Supplementary Fig. 8).

To test whether activation of Bach2 downstream of Pax5 is sufficient to clear the pre-B cell repertoire of nonfunctional V<sub>H</sub>-DJ<sub>H</sub> rearrangements, we developed a vector system for inducible...
expression of Bach2. We transduced Il7−dependent Bach2+/+ and Bach2−/− pre-B cells with a 4-hydroxytamoxifen (4-OHT)-inducible Bach2-ERG17 vector and an empty vector control, ERG. We then treated the cells with 4-OHT for 24 h and performed a sequence analysis of V_{H}-D_{H}J_{H} junctions (Fig. 3h and Supplementary Figs. 9 and 10). Although inducible overexpression of Bach2 had no statistically significant effect on the repertoire composition of Bach2+/+ pre-B cells, inducible Bach2 reconstitution in Bach2−/− pre-B cells resulted in the rapid elimination of clones carrying nonfunctional V_{H}-D_{H}J_{H} rearrangements (Fig. 3h and Supplementary Fig. 10). We conclude that Bach2 is required and sufficient for the stringent clearance of pre-B cells that failed to productively rearrange V_{H}-D_{H}J_{H} segments at the pre-B cell receptor checkpoint.

**Bach2 mediates tumor suppression in pre-B ALL**

In a high-throughput screen for retroviral integrations in mouse pre-B cell leukemia (using the Retroviral Tagged Cancer Gene Database, http://variation.osu.edu/rtcgdl/), we found Bach2 introns 1–3 as common integration sites (Supplementary Fig. 11), raising the possibility that Bach2 functions as a tumor suppressor in pre-B ALL.

We therefore tested whether BACH2 affects the survival and proliferation of human pre-B ALL cells. To this end we transduced xenografts from clinically derived Philadelphia chromosome (Ph)+ ALL cells with either a vector for Bach2 or an empty vector control. Primary Ph+ ALL cells carrying GFP-tagged Bach2 were rapidly depleted (Fig. 4a,b and Supplementary Fig. 12). Although all Ph+ ALL cells that we examined expressed P53 protein, in six of ten patient-derived xenografts, the Ph+ ALL cells had low basal expression of ARF (Fig. 4c). Despite low ARF expression, the Ph+ ALL cells underwent cell death as a result of Bach2 overexpression (Fig. 4b). Although BACH2 is required for the upregulation of both ARF and TP53, these findings indicate that BACH2 induces cell death in Ph+ ALL cells independently of ARF.

To test the requirements of Arf and p53 downstream of Bach2 in a genetic experiment, we transduced Cdkn2a−/+ Tp53−/+ (WT), Cdkn2a−/− and Tp53−/− pre-B ALL cells with GFP-tagged Bach2 or an empty vector control (Fig. 4d,e). Whereas Bach2 caused rapid elimination of Cdkn2a−/− and wild-type leukemia cells, the effect of Bach2 on Tp53−/− leukemia cells was delayed and was substantially diminished (Fig. 4e and Supplementary Fig. 13). We conclude that p53 is an important effector of Bach2-mediated cell death, whereas Arf is dispensable. The results of our Arf and p53 western blot analysis (Fig. 4c) agree with previous findings that defects of p53 are rare in Ph+ ALL cells (52%).

To identify potential defects in Bach2 function in human leukemia, we analyzed leukemic blasts from 83 individuals with Ph+ ALL (Eastern Cooperative Oncology Group [ECOG] 2993) and pre-B cells from 12 normal bone marrow samples with the HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) assay for promoter methylation (log(HpaII/MspI)) in 16 of the BACH2 gene. The BACH2 promoter was more hypermethylated in Ph+ ALL cells as compared to normal human bone marrow pre-B cells (n = 83 Ph+ ALL cells, P = 0.0026). In addition, sequence analysis of the BACH2 coding region in primary Ph+ ALL samples revealed BACH2 BTB domain missense mutations in two of the ten individuals studied (Supplementary Fig. 14). Given that p53 is intact in most Ph+ ALLs, we conclude that inactivation of Bach2 upstream of p53 is an important and nonredundant lesion.
PAX5 lesions result in defective BACH2 expression in pre-B ALL

Our experiments described above showed that forced expression of PAX5 induces upregulation of Bach2 at the pre-B cell receptor checkpoint (Fig. 1a,b). Recent studies have demonstrated that deletions and rearrangements of the PAX5 gene that result in dominant-negative fusion molecules occur in ~20% of cases of pre-B ALL.15,17. We therefore tested whether loss of PAX5 function results in transcriptional inactivation of BACH2 in human pre-B ALL cells. Whereas overexpression of wild-type PAX5 increased transcriptional activity of the BACH2 promoter, as measured by a luciferase reporter construct, overexpression of the dominant-negative PAX5-ETV6 fusion gene suppressed BACH2 promoter activity (Fig. 4f). Likewise, mRNA levels of BACH2 were increased by expression of wild-type PAX5 but were decreased by the dominant-negative PAX5-C20orf112 and PAX5-ETV6 fusion genes17 (Fig. 4g,h).

We next studied gene expression values for three BACH2 probe sets in 160 individuals with childhood pre-B ALL. Compared to the 54 individuals carrying either PAX5 deletions or somatic mutations of PAX5, BACH2 mRNA levels were significantly higher in the 106 individuals with pre-B ALL carrying wild-type PAX5 (Fig. 4i). We conclude that BACH2 links PAX5 to activation of p53 and is therefore an important tumor suppressor in human pre-B ALL.

BACH2 expression as a predictor of ALL patient outcome

BACH2 expression in ALLs carrying E2A (also called TCF3)-PBX1 and TEL (also called ETV6)-AML1 (also called RUNX1) gene rearrangements was similar to that in normal pre-B cells from healthy bone marrow donors. However, BACH2 mRNA levels were significantly lower in MLL (also called KMT2A)-AF4 (also called AFF1) (P = 0.01106) and Ph+ ALL subtypes (P = 0.00203; Supplementary Fig. 14). We also studied BACH2 mRNA levels in matched sample pairs from 49 patients that had a relapse of leukemia after an initial successful treatment. Comparing matched sample pairs from the time of diagnosis and the subsequent relapse of leukemia, we found that BACH2 mRNA levels were substantially reduced in the relapse samples (Fig. 5a and Supplementary Fig. 15). In addition, BACH2 expression levels at the time of diagnosis predicted minimal residual disease (MRD) in bone marrow biopsies taken on day 29 of treatment (Fig. 5b). Notably, loss of BACH2 expression correlated strongly with poor overall outcome among 207 patients with childhood ALL (Children’s Oncology Group (COG) P9906). Patients with expression levels of BACH2 higher than the median level had a relapse-free survival rate of 77%, whereas patients with BACH2 expression levels lower than the median level had a relapse-free survival rate of 47% (Supplementary Fig. 16b).

To study whether Bach2 mRNA levels are an independent predictor of clinical outcome, we performed multivariate analyses using three established predictors of poor clinical outcome as a reference: MRD, high white blood cell (WBC) counts (>100,000 µl⁻¹) and deletion of the IKZF1 tumor suppressor18. Studying high-risk groups of patients on the basis of these three factors, we found that lower-than-median expression levels of BACH2 defined subgroups of patients with worse clinical outcome. Lower-than-median expression levels of BACH2 at diagnosis predicted inferior clinical outcome for patients with positive MRD on day 29, for patients with WBC counts >100,000 µl⁻¹ (Fig. 5c–e and Supplementary Fig. 16) and for patients with deletion of IKZF1 (Supplementary Fig. 17).

Inverse relationship of BACH2 and BCL6 in ALL patient outcome

Our data described above (Figs. 1 and 2) demonstrated that both Bach2 and Bcl6 bind Cdkn2a and Tp53 promoters but inversely regulate mRNA levels of Arf and p53. Notably, the antagonistic function of BACH2 relative to BCL6 in the regulation of ARF, P53 and multiple other checkpoint genes is mirrored by their opposite association with clinical outcome. Whereas higher-than-median mRNA levels of BACH2 at the time of diagnosis predict favorable clinical outcome, the opposite is true for patients with ALL with higher-than-median BCL6 mRNA levels (COG P9906). Studying BACH2 and BCL6 mRNA levels in a bivariate analysis identified subgroups of patients with particularly favorable (BACH2highBCL6low) and particularly poor (BACH2lowBCL6high) outcomes on the basis of the ratio of BACH2-to-BCL6 mRNA levels (Fig. 5f).

BACH2 is often deleted in B cell malignancies

BACH2 is located at chromosome 6q15, and deletions of 6q encompassing 6q15 have been found in 32% of individuals with B cell-lineage ALL.19. We therefore studied B cell-lineage ALL cell lines (n = 9) and individuals with primary ALL (n = 4) with 6q deletions by high-resolution SNP analysis and found that BACH2 lies within a region of minimal deletion on 6q (Fig. 5g).

Studying bone marrow samples from individuals with primary ALL at three time points, diagnosis, clinical remission and relapse of leukemia, we found that deletion of the BACH2 locus was acquired at the time of leukemia relapse in 3 of the 4 individuals studied and was pre-existing in the remaining individual (Fig. 5g–j).

Bach2 opposes Stat5 and Myc programs in Ph+ ALL

Ph+ ALL cells depend on tyrosine kinase signaling from (i) the BCR-ABL1 oncogene, (ii) downstream phosphorylation of Stat5 and (iii) transcriptional activation of Myc.20. Notably, a global analysis of BACH2-dependent gene expression changes in BCR-ABL1–transformed mouse pro-B cells revealed that these changes showed a substantial overlap with gene expression changes caused by (i) BCR-ABL1 kinase inhibition, (ii) inducible deletion of Stat5a and Stat5b and (iii) inducible deletion of Myc (Supplementary Fig. 18). These findings suggest that Bach2-mediated gene expression changes oppose a transcriptional program of survival (Stat5) and proliferation (Myc) downstream of BCR-ABL1 in Ph+ ALL cells.

Consistent with this scenario, BCR-ABL1–transformed Bach2−/− pre-B ALL cells are markedly less sensitive to inhibition of BCR-ABL1 using a tyrosine kinase inhibitor (TKI; imatinib) than their wild-type counterparts (Figs. 6a,b). In the absence of Bach2, imatinib-induced apoptosis was reduced by threefold (Fig. 6a), and the fraction of proliferating cells despite treatment with imatinib was increased by twofold (Fig. 6b). Whereas BCR-ABL1–transformed Bach2−/+ cells were able to form colonies only after a lag phase of ~10 d, colony formation of Bach2−/− pre-B ALL cells was significantly accelerated (Fig. 6c).

Bach2 and Myc are mutually exclusive during transformation

Overexpression of the Myc proto-oncogene frequently leads to increased proliferation and malignant transformation. However, Myc-driven proliferation can be increased only within certain limits, and Myc alone is not sufficient to cause malignant growth.21,22. A number of failsafe mechanisms restrain Myc-induced proliferation and tumorigenesis.23. These failsafe mechanisms are crucial for the termination
Bach2 is a barrier against Myc-induced pre-B cell transformation

We next tested the hypothesis that Bach2 negatively regulates the susceptibility of pre-B cells to Myc-mediated transformation. In normal pre-B cells, high expression of Myc results in activation of Arf and p53 and oncogene-induced senescence. Retroviral overexpression of Myc induced cell death and caused substantial toxicity in Bach2−/− pre-B cells (Fig. 6c,d and Supplementary Figs. 19 and 20). These findings indicate that Bach2 strongly reduces the susceptibility of pre-B cells to malignant transformation by Myc.

A cell-cycle analysis revealed that overexpression of Myc in Bach2−/− pre-B cells increased the fraction of cells in S phase to a maximum threshold of about 50%. However, Myc overexpression in Bach2+/− pre-B cells caused a further increase in proliferating cells, with about 70% of pre-B cells being in S phase (Supplementary Fig. 19b). Consistent with these findings, overexpression of Myc in Bach2−/− pre-B cells resulted in activation of both Arf and p53. By contrast, p53 expression was very low in Bach2+/− pre-B cells and was not increased by overexpression of Myc (Supplementary Fig. 19b). We conclude that in the absence of Bach2, p53-dependent failsafe mechanisms are not activated, obviating the requirement of additional genetic lesions for leukemic transformation.

Bach2 loss facilitates leukemic transformation by Myc

To confirm a crucial role of Bach2 in Myc-induced failsafe control in an in vivo setting, we transduced Il-7−dependent Bach2+/− and...
**Figure 6** Bach2 prevents leukemic transformation by Myc. (a,b) Induction of cell death (determined by annexin V and 7-aminoactinomycin D (7-AAD) staining; the numbers in the top right quadrants show the percentages of annexin V and 7-AAD double-positive apoptotic cells; a) and cell-cycle arrest (determined by BrdU staining and G0/1, S and G2/M phase distribution; all values are percentages of total acquired cells; b) in BCR-ABL1-transformed Bach2+/+ and Bach2−/− pre-B ALL cells before and after imatinib treatment. The percentages of total live cells in each phase of the cell cycle are indicated. (c) Comparison of the colony-forming abilities of Bach2+/+ and Bach2−/− pre-B ALL cells plated in methylcellulose on day 7 (the P value was calculated using Student’s t-test; n = 3; error bars, s.d.). (d) mRNA levels of Bach2 and Myc plotted against mouse age (d) in bone marrow pre-B cells that were isolated from wild-type and BCR-ABL1 transgenic (tg) mice of various ages. TKI denotes in vivo treatment of leukemic mice with the tyrosine kinase inhibitor nilotinib. (e) Transduction efficiency, monitored by flow cytometry on days 0 and 3, of Bach2+/+ and Bach2−/− II-7-dependent pre-B cells that were retrovirally transduced with Myc-GFP or the GFP empty vector. FSC, forward scatter. The numbers in each quadrant indicate the percentages of total live cells. (f) Plating efficiency of Myc-GFP-transduced Bach2+/+ and Bach2−/− II-7-dependent pre-B cells in methylcellulose monitored by fluorescence microscopy. (g) Kaplan–Meier survival analysis of nonobese diabetic severe combined immunodeficient (NOD-SCID IL2rg−/−) mice transplanted with sorted Myc-GFP–transduced Bach2+/+ and Bach2−/− II-7–dependent pre-B cells (n = 7 mice per group). (h) Analysis of CD19+GFP+ cells in the bone marrow and spleens, determined by flow cytometry, of the surviving mice from the Bach2+/+ Myc-GFP–transplanted cohort analyzed together with those of mice receiving Bach2−/− Myc-GFP pre-B cells at 70 d after transplant. Percentages of CD19+GFP+ cells are indicated as percentages of total live cells in the top right quadrant. (i) MRD qRT-PCR for leukemic cells infiltrating the bone marrow or spleens of the indicated groups of mice performed in triplicates for each group using GFP-specific primers. Error bars, s.d.

**DISCUSSION**

We identified Bach2-mediated activation of p53 as a crucial mediator of pre-B cell receptor checkpoint control. During normal B cell development, Bach2 determines the stringency of negative selection of pre-B cell clones that failed to productively rearrange V_{H}D_{H} gene segments. In addition, Bach2-mediated activation of p53 is an important failsafe mechanism in the termination of pre-malignant pre-B cell clones before they can complete malignant transformation. Thus, Bach2 limits pre-B cell proliferation and oncogene signaling through activation of p53. In both normal pre-B cells and pre-B ALL, Bach2-mediated activation of p53 is opposed by Bcl6, which is a potent transcriptional repressor of p53. Whereas inactivation of TP53 in ALL is rare, Bach2 is frequently inactivated in established pre-B ALL clones or at the time of leukemia relapse. Consistent with their opposing role in TP53 regulation and checkpoint control, the ratio of BACH2 (favorable) to BCL6 (poor) expression levels is also a strong predictor of clinical outcome in patients with ALL. Although BACH2 function cannot be reinstated in patients with ALL who carry BACH2 deletions, we propose that pharmacological inhibition of BCL6 (for example, with retroinverso BCL6 peptide inhibitor (RI-BPI)) will restore the balance of BACH2- to BCL6-mediated checkpoint control and relieve BCL6-mediated transcriptional repression of p53 (Supplementary Fig. 22).

**METHODS**

Methods and any associated references are available in the online version of the paper.
Accession codes. Sequence data are available from EMBL/GenBank under the accession codes HE578168, HE578164, HE578169, HE578170, HE578165, HE578163, HE578173, HE578166 and HE578176. Microarray data are available from the Gene Expression Omnibus (GEO) under the accession codes GSE30883, GSE30928, GSE24814, GSE20987, GSE30889, GSE31027, GSE28460, GSE41042, GSE44420 and GSE34941.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.S. and M.M. designed experiments and interpreted data. M.M. and S.S. also conceived the study and wrote the paper. S.S., C. Huang, H.G., Z.C., C.N., B.T., C. Hurtz, M.F.S., D.N., G.B.T. and V.R. performed experiments and interpreted data. H.G., R.H., H.K., V.R., H.P.K., W.L.C., C.L.W., A.G.H. and A.M. provided and characterized patient samples and clinical outcome data. T.G.G., H.P.K., K.I. and A.M. provided important reagents and mouse samples.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Primary human samples and human cell lines. We obtained bone marrow from individuals with primary ALL and human cell lines from DSMZ, Germany (Supplementary Tables 1 and 2) in compliance with the Institutional Review Boards of the University of California, San Francisco and the University of Southern California. We obtained informed consent from all participants. We cultured all primary human ALL samples on OP9 stromal cells in minimum essential medium (MEMx; Life Technologies) with GlutaMAX containing 20% 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% CO\(_2\).

Extraction of bone marrow cells from mice. We extracted bone marrow cells from female young age-matched Bach2\(^{+/+}\) and Bach2\(^{-/-}\) mice (less than 6 weeks of age) (Supplementary Table 3). We obtained the bone marrow cells by flushing the cavities of the femurs and tibias with PBS. After filtration through a 70-µm filter and depletion of erythrocytes using a lysis buffer (BD PharmLyse, BD Biosciences), washed cells were either cryopreserved or used for further experiments.

In vivo model for Myc-driven leukemia. We first transformed IL-7−dependent pre-B cells from Bach2\(^{+/+}\) and Bach2\(^{-/-}\) mice with a retroviral vector encoding Myc−internal ribosome entry site (IRES)-GFP (Myc-GFP). We then injected these cells intrafemurally into seven sublethally irradiated (250 Gy) female NOD/SCID/IL-2Rnull (NOG) mice per group. We monitored the transplant-recipients for weight loss, hunched posture and inability to move as indicators of leukemia progression. For the group that showed signs of leukemia, we euthanized the mice after they became terminally ill. We euthanized the healthy group at day 70 after injection. We then stained the bone marrows and spleens isolated from all mice for B cell markers to ascertain that leukemia was the cause of death. In addition, we measured GFP expression levels in the bone marrow and spleen of each mouse to detect any minimal residual disease. All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California and the University of California, San Francisco.

Bach2\(^{+/+}\), Bach2\(^{-/-}\), Myc\(^{fl/fl}\), Stat5\(^{fl/fl}\), Tp53\(^{-/-}\) and Arf\(^{-/-}\) mice. We collected bone marrow cells from the above-mentioned groups of mice and retrovirally transformed them using BCR-ABL1 (1) in the presence of 10 ng ml\(^{-1}\) IL-7 (Peprotech) in RetroNectin (Takara)-coated Petri dishes as described below. We maintained all BCR-ABL1-transformed ALL cells derived from the bone marrow of the mice in Ivosev’s modified Dulbecco’s medium (IMDM; Invitrogen) with GlutaMAX containing 20% FBS, 100 IU ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin and 50 mM 2-mercaptoethanol. BCR-ABL1-transformed ALL cells were propagated for short periods of time only, usually not longer than 2 months, to avoid acquisition of additional genetic lesions during long-term cell culture. All mice used in the study are listed in Supplementary Table 3.

BCR-ABL1 TKIs. We obtained the TKIs imatinib and nilotinib from Novartis Pharmaceuticals and LC Laboratories, respectively. TKIs were dissolved in sterile distilled water and stored at −20 °C.

Western blotting. We lysed cells in Celllytic buffer (Sigma) supplemented with 1% protease inhibitor cocktail (Pierce). Protein samples were subsequently separated on NuPAGE (Invitrogen) 4–12% Bis-Tris gradient gels and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore). For the detection of mouse and human proteins by western blot analysis, we used primary antibodies together with the WesternBlaze immunodetection system (Invitrogen). All antibodies used for western blotting and their corresponding dilutions are listed in Supplementary Table 4.

Flow cytometry. We purchased all antibodies for flow cytometry measurements, as well as their respective isotype controls, from BD Biosciences (Supplementary Table 4). Surface-staining antibodies that were phycoerythrin (PE) conjugated were used at a dilution of 1:10, and the antibodies conjugated with FITC were used at a dilution of 1:5. For apoptosis analyses, we used annexin V, propidium iodide and 7-AAD (BD Biosciences).

Clonality analysis and spectratyping of B cell populations. We amplified the entire repertoire of VH-DJH gene rearrangements from B cell populations using PCR primers specific for the J558 VH1region gene together with a primer specific for the C\(_\alpha\) constant region. Next, using a FAM-conjugated C\(_\alpha\) constant region primer along with the J558 VH1 primer used in the previous step, we labeled our PCR products in a run-off reaction and subsequently analyzed them on a capillary sequencer (ABI3100, Applied Biosystems) by fragment-length analysis. Sequences of the primers used are given in Supplementary Table 5.

Cloning of the murine stem cell virus (MSCV)–Bach2-ERT2–IRES–GFP vector. We amplified Bach2 cDNA from the K562 cell line using the primers listed in Supplementary Table 5. We then digested the cDNA using BamH1 and XhoI and inserted it into the backbone obtained after digestion of the MSCV-Cre-ERT2–IRES puromycin vector with BamH1 and XhoI. The vector obtained after this ligation was digested using BamH1 and EcoRI to obtain the Bach2-ERT2 fragment. Finally, we ligated the Bach2-ERT2 fragment into a BgIII- and EcoRI-digested MSCV-IRES-GFP vector.

 Colony-forming assays. The methylcellulose colony-forming assays were performed with 10,000 BCR-ABL1–transformed Bach2\(^{+/+}\) and Bach2\(^{-/-}\) cells or 10,000 c-Myc-BCR-ABL1–transformed Bach2\(^{+/+}\) and Bach2\(^{-/-}\) IL-7−dependent pre-B cells per group. For Myc-transduced IL-7−dependent cells, we performed the assay on OP9 stromal cells. Cells were resuspended in murine MethoCult medium ( StemCell Technologies) and cultured on dishes (3 cm in diameter) with an extra water supply dish to prevent evaporation. We counted the colonies 7–14 d after plating.

Retroviral transduction. We performed transfections of retroviral constructs and their corresponding empty vector controls (Supplementary Table 6) using Lipofectamine 2000 (Invitrogen) with Opti-MEM medium (Invitrogen). We produced retroviral supernatants to infect mouse cells by cotransfecting HEK293FT cells with the plasmids pHIT60 (gag-pol) and pHIT123 (ecotropic envelope, provided by D.B. Kohn, University of California, Los Angeles, Los Angeles, CA). To infect human cells, we replaced pHIT123 with pHIT456 (amphotropic envelope, provided by D.B. Kohn). We cultivated the cells in high-glucose DMEM (Invitrogen) with GlutaMAX containing 10% FBS, 100 IU ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin, 25 mM (2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate and 0.1 mM of nonessential amino acids. We replaced the serum-free medium after 16 h with growth medium containing 10 mM sodium butyrate. After 8 h of incubation, we changed the medium back to growth medium. Twenty-four hours later, we harvested the viral supernatants, passing them through a 0.45-µm filter, and loaded them for centrifugation (2,000 g for 90 min at 32 °C) twice on 50 µg ml\(^{-1}\) RetroNectin-coated nontissue culture–treated six-well plates. Two- to three-million pre-B cells were transduced per well by centrifugation at 600 g for 30 min and maintained overnight at 37 °C with 5% CO\(_2\) before transfer into culture flasks.

Cell-cycle analysis. For cell-cycle analysis of BCR-ABL1 ALL cells, we used the BrdU flow cytometry kit (BD Biosciences) according to the manufacturer’s instructions. We measured BrdU incorporation (allophycocyanin (APC)- labeled (552598) or GFP-labeled (559619) antibodies to BrdU (http://wwwbdbiosciences.com/home.jsp), along with DNA content by 7-AAD, in fixed and permeabilized cells. We then gated the analysis on viable cells that were identified on the basis of scatter morphology.

Single-focus qChIP. ChIP assays were performed as previously described. Before ChIP, we briefly treated 1 × 10\(^6\) BCR-ABL1–transformed mouse cells (Bach2\(^{+/+}\), Bach2\(^{-/-}\) or Bcl6\(^{-/-}\)) with or without 2 µmol l\(^{-1}\) imatinib for 16 h. For BACH2 ChIP, primary human Ph\(^+\) ALL cells (ICN1) were left untreated or were treated with 10 µmol l\(^{-1}\) imatinib for 24 h. Then the cells were crosslinked with 1% formaldehyde. After sonication by a bioruptor (Diagenode), we performed immunoprecipitations using 5 µg Bcl6 (C19, Santa Cruz Biotechnology, http://www.scbt.com/), 5 µg BACH2 (a gift from the lab of A.M.) or control IgG antibody (Santa Cruz Biotechnology, http://www.scbt.com/). We washed the complexes with low- and high-salt buffers, eluted them, reverse crosslinked them and precipitated the DNA. We finally analyzed the immunoprecipitated DNA.
sequences by qPCR. The primer sequences used for the ChIP analyses are listed in Supplementary Table 5.

PCR amplification and sequencing of the BACH2 coding region. We isolated total RNA from ten individuals with primary Ph+ ALL (Supplementary Table 1) using RNeasy (Qiagen) purification. cDNA was then generated from 5 μg of total RNA using a poly(dT) oligonucleotide that contains a T7 RNA polymerase initiation site and SuperScript III Reverse Transcriptase (Invitrogen). For ease of sequencing, we divided the entire translated region of BACH2 into five separate regions (labeled A–E) and sequenced them after PCR amplification. We performed RT-PCR using Phusion polymerase (New England BioLabs) for 35 cycles on the template cDNA derived from the ten individuals. The PCR and sequencing primer sequences used are listed in Supplementary Table 5. Amplification conditions are available by request.

Sequence alignment to identify mutations. Sequencing was carried out at the University of Southern California Norris Comprehensive Cancer Center DNA core facility. We aligned all sequences to the wild-type BACH2 cDNA sequence (obtained from the Ensembl genome browser) in Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and compared them for mutations. In addition, we identified mutations by carefully reading through the sequencing chromatograms to identify double peaks at a particular position in the trace. All sequence data are available from EMBL/GenBank under the accession codes provided in Supplementary Table 7.

Sequence analysis of VH–DJH gene rearrangements. We isolated B cells from Bach2+/+ and Bach2−/− bone marrow and spleens by MACS using anti-CD19 immunomagnetic beads. MACS was carried out using the protocol provided by Miltenyi Biotec. Subsequently, we isolated RNA from the MACS-sorted B cells and reverse transcribed it to cDNA. We then amplified VH–DJH gene rearrangements from B cell populations using primers specific for the J558 VH-region gene together with a primer specific for the Cμ constant region for 35 cycles. The primer sequences used are listed in Supplementary Table 5. We cloned the PCR fragments using a Topo TA cloning kit (Invitrogen). The PCR products were subsequently sequenced unidirectionally in a 96-well format at Genewiz Inc. We then carried out a comprehensive sequence analysis to determine whether the rearrangement was productive or nonproductive using IMGT-V Quest (http://www.imgt.org/IMGTVquest/share/textes/).

Affymetrix GeneChip analysis. We isolated total cellular RNA for both microarray and RT-PCR analyses using RNeasy (Qiagen) purification. We first checked RNA quality using an Agilent Bioanalyzer (Agilent Technologies). We then generated cDNA from 5 μg of total RNA using a poly(dT) oligonucleotide that contains a T7 RNA polymerase initiation site and SuperScript III Reverse Transcriptase (Invitrogen). The cDNA sequence was then amplified and fragmented according to the Affymetrix protocol and hybridized to U133A 2.0 Human or Mouse Gene 1.0 ST microarrays (Affymetrix). After scanning the GeneChip microarrays (with a scanner from Affymetrix), we imported the generated CEL files to the BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) and processed them using the Robust Multiarray Average (RMA) algorithm for normalization and summarization. We compared the relative signal intensities of the probe sets by comparing the intensity of the treated or untreated cells to the average value of the cell line or a control group of cells. We exported the ratios in Gene Cluster and visualized them as a heat map using Java Treeview (http://jtreeview.sourceforge.net/). Microarray data are available from GEO under the accession codes listed in Supplementary Table 8.

BACH2 gene expression data and clinical outcome. We obtained RNA data for the 207 patients in the COG F9906 trial from the TARGET web site (http://target.cancer.gov/dataportal/data/); ALL RNA probe set–level expression. Three probe sets were available for BACH2 (221234_s_at, 227173_s_at and 236796_s_at). For various analyses, we assessed each probe set separately. Alternatively, because the expression patterns for the multiple probe sets were very similar, we calculated the mean value of the intensities for each gene and used this for subsequent analysis.

Comparison of Bach2 gene signatures. For each cellular perturbation, we defined a gene signature as a gene list ranked by a metric of differential expression as a result of cellular perturbation. We calculated a perturbation score for each gene and calculated each perturbation as the differential expression metric score. This perturbation score combines the fold change and t test between the perturbed samples and controls and corresponds to the projection of the (log2, average) fold change and (signed log2) t test values onto their first principal component axis. To identify consistent expression changes, we ranked the genes by their average perturbation score (over the four perturbations) and visualized the resulting ranked matrix of expression values as a heat map. For the heat map representation, each gene was mean centered and scaled by its s.d. within each experimental batch of control and perturbation-matched samples. For a more detailed analysis of the signature overlaps, we generated scatter plots and rank-rank hypergeometric overlap (RRHO) maps. RRHO is a threshold-free method for the comparison of ranked gene lists, which calculates the significance of gene overlap using the hypergeometric distribution at all possible pairwise rank threshold combinations and thus allows for a detailed analysis of how the overlap is structured, for example, stronger overlap between consistently downregulated compared to upregulated genes. RRHO results are visualized in a heat map as the signed log hypergeometric P value for enrichment (positive) or de-enrichment (negative) compared to random expectations. In the heat map, the axes coordinates correspond to the rank thresholds used in the hypergeometric calculation. RRHO can be thought of as a two-dimensional analog of the Gene Set Enrichment Algorithm. Microarray data are available from GEO under accession numbers listed in Supplementary Table 8.

Statistical methods. Data are presented as the mean ± s.d. The comparisons for Bach2 expression levels between two groups were made by one-tailed t test or Wilcoxon's rank-sum test using R software (R Development Core Team 2009; http://www.r-project.org). The Kaplan-Meier method was used to estimate overall survival and relapse-free survival. Log-rank test was used to compare survival differences between patient groups. The R package ‘survival’ version 2.35-8 was used for the survival analysis. The level of significance was set at P < 0.05.

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