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

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Abstract

The B cell-specific transcription factor BACH2 is required for affinity maturation of mature 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-DJ_H gene segments. Upon productive V_H-DJ_H gene rearrangement, pre-B cell receptor signaling ends negative selection through BCL6-mediated repression of p53. In patients with pre-B ALL, BACH2-mediated checkpoint control is frequently compromised. Low levels of BACH2 expression represent a strong independent predictor of poor clinical outcome. *Bach2*$^{+/+}$ pre-B cells resist leukemic transformation by Myc through Bach2-dependent upregulation of p53, and fail to initiate fatal leukemia in transplant recipient mice. ChIP-seq and gene expression analyses reveal that BACH2

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competes with BCL6 for promoter binding and reverses BCL6-mediated repression of p53 and other checkpoint control genes. These findings identify Bach2 as a critical mediator negative selection at the pre-B cell receptor checkpoint and a safeguard against leukemogenesis.

Introduction

In mice, bone marrow progenitor cells produce approximately 10 million pre-B cells daily[3]. Newly formed pre-B cells, however, are destined to die unless they productively rearrange V_H-DJ_H gene segments and are rescued by pre-B cell receptor signals into the long-lived peripheral B cell pool[4-5]. We recently identified the transcriptional repressor BCL6 as critical survival factor that rescues pre-B cells that productively rearranged V_H-DJ_H gene segments and emerged from the pre-B cell receptor checkpoint[6-7]. However, the mechanisms leading to clearance of other pre-B cells that failed to productively rearrange V_H-DJ_H gene segments and thus lack pre-B cell receptor expression are poorly understood.

Results

Bach2 induces Arf/p53 downstream of Pax5 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 to pre-B cell transition[8]. We identified 18 genes with specific upregulation at the pre-B cell receptor checkpoint including components of the pre-B cell receptor itself (*IGLL1, VPREB1*), effectors of V_H-DJ_H recombination (*RAG1, RAG2, DNTT*), and mediators of survival signaling (Supplementary Fig. 1). We next studied gene expression changes at the pre-B cell receptor checkpoint under two experimental conditions, namely (i) initiation of V(D)J recombination upon inducible activation of Pax5[9] and, (ii) inducible expression of a productively rearranged V_H-DJ_H encoding a functional immunoglobulin μ heavy chain (μHC), which initiates pre-B cell receptor signaling[7] (Fig. 1a). We reason that pre-B cell receptor checkpoint control should occur between these two events. As predicted, Pax5-mediated initiation of V(D)J recombination is reflected by strong upregulation of Rag1, Rag2 and caused upregulation of Arf/p53 and Bach2. By contrast, inducible activation of μHC expression induced expression of Bcl6, a transcriptional repressor of Arf/p53 (Figs. 1a-c). Bcl6-mediated repression of Arf/p53 is needed to rescue μHC^+ pre-B cells once they have passed the pre-B cell receptor checkpoint[6-7]. Consistent with findings in human B cell differentiation (Supplementary Fig. 1), we found high levels of Bach2 prior to (Pro-B and pre-BI cells) and high levels of Bcl6 subsequent to (pre-BII cells) passage of the pre-B cell receptor checkpoint in mice (Fig. 1d). These findings imply that Bach2 potentially contributes to negative selection of pre-B cells that failed to productively rearrange V_H-DJ_H gene segments at the pre-B cell receptor checkpoint. To test this hypothesis, we assayed pre-B cell receptor checkpoint control in pro-B and pre-BI cells from *Bach2*$^{-/-}$ mice[2] and wildtype controls under experimental conditions. Activation of GFP-tagged Pax5 induces accumulation of Arf/p53 and results in rapid elimination of *Bach2*$^{+/+}$ but not *Bach2*$^{-/-}$ pro-B and pre-BI cells (Figs. 1e-g).

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BCL6 reverses Bach2-induced transcriptional activation of Arf/p53

Previous studies by our group identified Bcl6 as a strong transcriptional repressor of Arf/p53 in normal pre-B cells[6] and pre-B ALL[10]. Of note, ChIP-seq analysis and QChIP validation revealed that both BCL6 and BACH2 bind to overlapping promoter regions of *CDKN2A* (Arf) and *TP53* (*p53)* and other checkpoint regulators (*CDKN1A* (p21), *CDKN1B* (p27), *GADD45A, 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 determines negative (Bach2>Bcl6) and positive (Bach2<Bcl6) selection events at the pre-B cell receptor checkpoint (Fig. 2b). 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 strongly increased in the absence of Bcl6 (Figs. 2d, 2f and Supplementary Fig. 6) or upon inducible overexpression of Bach2 (Fig. 2c and Supplementary Figure 4). Likewise, mRNA levels of the p53-dependent tumor suppressor Btg2 were reduced by >20-fold in the absence of Bach2 but increased by 3-fold in the absence of Bcl6 (Fig. 2f and Supplementary Fig. 4). To test whether Bach2 negatively regulates the ability of Bcl6 to bind to *Cdkn2a* (*Arf*) and *Tp53* (*p53*) promoters, we performed Bcl6-ChIP experiments with Bcl6$^{-/-}$ (negative control), *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ cells. Binding of Bcl6 to *Cdkn2a* (*Arf*) and *Tp53* (*p53*) was significantly increased in *Bach2*$^{-/-}$ compared to *Bach2*$^{+/+}$ cells (Fig. 2e and Supplementary Fig. 5). Gene expression analysis for a subset of common Bach2- and Bcl6-target genes revealed that Bcl6 and Bach2 affect gene expression levels of checkpoint regulators including *Cdkn2a (Arf), Tp53, Gadd45b, Btg1* and *Btg2*, in opposite directions (Figs. 2d, 2f and Supplementary Fig. 6). These findings collectively indicate that the balance between Bcl6 and Bach2 determines repression or transcriptional activation of *Cdkn2a (Arf), Tp53 (p53)* and related checkpoint molecules.

Bach2 mediates expression of Rag1, Rag2 and activates V(D)J recombination

We measured functional consequences of Bach2-deficiency at the pre-B cell receptor checkpoint in *Bach2*$^{-/-}$ and wildtype mice. Using a classical pre-B cell differentiation model based on tyrosine kinase inhibition (Imatinib; IM) of *BCR-ABL1*-transformed pre-B cells[12-13], we studied gene expression changes upon differentiation of *Bach2*$^{-/-}$ and *Bach2*$^{+/+}$ pre-BI cells (Fig. 3a). In this analysis, Bach2-deficiency was associated with increased expression of the early progenitor antigen Ly6f (Sca-1) and reduced expression of the pre-B cell antigen Il2ra (CD25; Figs. 3a-b). Importantly, mRNA levels of Rag1 and Rag2, critical effectors of V(D)J recombination, were reduced by ~20-fold. Likewise, Imatinib (IM)-induced differentiation induced strong upregulation of Rag1/Rag2 expression in *Bach2*$^{+/+}$ pre-BI cells as previously described[6,12-13], but failed to upregulate Rag1/Rag2 beyond baseline levels in *Bach2*$^{-/-}$cells (Fig. 3c). Inducible overexpression of Bach2 was sufficient to drive Rag1/Rag2 upregulation (Fig. 3d) and single-locus ChIP revealed that BACH2 binds *RAG1* and *RAG2* promoters, which was enhanced by IM-treatment (Fig. 3e).

To test if defective expression of Rag1/Rag2 results in impaired V(D)J recombination activity, we transduced *Bach2*$^{-/-}$ and *Bach2*$^{+/+}$ pre-BI cells with a V(D)J recombination RSS substrate. Consistent with the massively increased Rag1/Rag2 expression, IM-treatment

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resulted in a 6-fold increase of baseline recombination of the RSS substrate in *Bach2*$^{+/+}$ pre-B cells. By contrast, IM-induced V(D)J recombination activity was reduced by 20-fold in *Bach2*$^{-/-}$ pre-BI cells as compared to wildtype counterparts (Fig. 3f and Supplementary Fig. 7).

Bach2 is required for clearance of pre-BI cell clones that carry non-functional V_H-DJ_H gene rearrangements

We next studied the composition of B cell progenitor populations in bone marrows and spleens of *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ mice. Total numbers of CD19$^+$ B220$^+$ B cells were only slightly reduced in the bone marrow and spleen of *Bach2*$^{-/-}$ mice (Fig. 3g and Supplementary Fig. 8). While pre-B cells from *Bach2*$^{+/+}$ bone marrow were stringently selected for productive in-frame V_H-DJ_H gene rearrangements, pre-B cells from *Bach2*$^{-/-}$ mice lacked selection for V_H-DJ_H junctions with coding capacity (Fig. 3g and Supplementary Fig. 8). Based on random V(D)J recombination, only one of three pre-B cell clones achieve in-frame rearrangements and coding capacity for a functional pre-B cell receptor. Effective negative pre-B cell selection causes clearance of cells with non-functional out-of-frame V_H-DJ_H rearrangements and, hence, results in a length distribution of V_H-DJ_H junctions with size peaks that are spaced by three nucleotides[14]. Fragment length analysis revealed a peak size distribution of V_H-DJ_H junctions that were selected for multiples of 3 bp among *Bach2*$^{+/+}$ as compared to random distribution in *Bach2*$^{-/-}$ counterparts (Fig. 3g and Supplementary Fig. 8). Sequence analysis of V_H-DJ_H junctions confirmed that out-of-frame V_H-DJ_H rearrangements were stringently cleared from the repertoire in *Bach2*$^{+/+}$ pre-B cells as opposed to accumulation of a large fraction of clones with non-functional rearrangements among *Bach2*$^{-/-}$ pre-B cells (Fig. 3g and Supplementary Fig. 8).

To test if activation of Bach2 downstream of Pax5 is sufficient to clear the pre-B cell repertoire from non-functional V_H-DJ_H rearrangements, we developed a vector system for inducible expression of Bach2. IL7-dependent *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pre-B cells were transduced with tamoxifen (4-OHT)-inducible Bach2-ER^{T2} and ER^{T2} empty vector controls. Cells were treated with 4-OHT for 24 hours and sequence analysis of V_H-DJ_H junctions was performed (Fig. 3h and Supplementary Figs. 9 and 10). While inducible overexpression of Bach2 had no significant effect on the repertoire composition of *Bach2*$^{+/+}$ pre-B cells, inducible Bach2-reconstitution in *Bach2*$^{-/-}$ pre-B cells resulted in rapid elimination of clones carrying non-functional V_H-DJ_H rearrangements (gray shaded in Supplementary Fig. 10). We conclude that Bach2 is required and sufficient for stringent clearance of pre-B cells that failed to productively rearrange V_H-DJ_H segments at the pre-B cell receptor checkpoint.

Bach2 mediates PAX5-dependent tumor suppression in pre-B ALL through activation of p53

In a high throughput screen for retroviral integrations in mouse pre-B cell leukemia (RTCGD), we found *Bach2* introns 1-3 as common integration sites (Supplementary Fig. 11), raising the possibility that Bach2 functions as tumor suppressor in human pre-B ALL.

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We therefore, tested whether Bach2 affects survival and proliferation of human pre-B ALL cells. To this end, we transduced xenografts from clinically-derived Ph^+ ALL cells with vectors for either Bach2 or empty vector controls (EV). Primary Ph^+ ALL cells carrying GFP-tagged Bach2 were rapidly depleted (Figs 4a-b and Supplementary Fig. 12). While Ph^+ ALL cells expressed p53 protein in all cases studied, in 6 of 10 cases Ph^+ ALL cells had low expression of ARF (Fig. 4c). Despite low ARF expression, the Ph^+ ALL cells underwent cell death upon Bach2 overexpression (Fig. 4b). While Bach2 is required for upregulation of both ARF and TP53, these findings indicate BACH2 induces cell death in Ph^+ ALL cells independently of ARF.

To test the requirements of Arf/p53 downstream of Bach2 in a genetic experiment, *Cdkn2a*$^{-/-}$ and *Tp53*$^{-/-}$ pre-B ALL cells were transduced with GFP-tagged Bach2 or empty vector controls (Figs. 4d-e). While Bach2 caused rapid elimination of *Cdkn2a*$^{-/-}$ and wildtype leukemia cells, the effect of Bach2 on *Tp53*$^{-/-}$ leukemia cells was delayed and 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. Our Arf/p53 Western blot analysis (Fig. 4c) agrees with previous findings that defects of p53 are rare in Ph^+ ALL, whereas deletions of *ARF* are frequent (52%)[15].

To identify potential defects in Bach2 function in human leukemia, leukemic blasts from 83 cases of Ph^+ ALL (ECOG 2993) and pre-B cells from 12 normal bone marrow samples were analyzed with the HELP assay for promoter methylation (Log HpaII/MspI)[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; P=0.0026). In addition, sequence analysis of the *BACH2* coding region of primary Ph^+ ALL samples revealed *BACH2* BTB domain missense mutations in 2 of 10 cases studied (Supplementary Fig. 14). Given that p53 is intact in most Ph^+ ALLs, we conclude that inactivation of Bach2 upstream of p53 represents an important and non-redundant lesion.

Genetic lesions affecting PAX5 result in defective BACH2 expression in B cell lineage ALL

Our earlier experiments showed that forced expression of Pax5 induces upregulation of Bach2 at the pre-B cell receptor checkpoint (Fig. 1a-b). Recent studies demonstrated that deletions and rearrangements of the *PAX5* gene resulting in dominant-negative fusion molecules occur in ~20% of cases of pre-B ALL[15,19]. We therefore tested whether loss of PAX5 function results in transcriptional inactivation of BACH2 in human pre-B ALL cells. While overexpression of wildtype PAX5 increases transcriptional activity of the *BACH2* promoter as measured by a luciferase reporter construct, overexpression of the dominant-negative PAX5-ETV6 fusion suppressed *BACH2* promoter activity (Fig. 4f). Likewise, mRNA levels of *BACH2* were increased by expression of wildtype PAX5 but decreased by dominant-negative *PAX5-C20orf112* and *PAX5-ETV6* fusion genes[19] (Figs. 4g-i). Next, we studied gene expression values for three BACH2 probe sets in 160 cases of childhood pre-B ALL. Compared to 54 cases carrying either *PAX5* deletions or somatic mutations of *PAX5*, BACH2 mRNA levels were significantly higher in 106 wildtype *PAX5* pre-B ALL cases (Fig. 4i). We conclude that BACH2 links PAX5 to activation of p53 and, hence, represents an important tumor suppressor in human pre-B ALL.

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Loss of BACH2 expression represents an independent predictor of poor outcome in ALL patients

BACH2 levels in ALLs carrying *E2A-PBX1* and *TEL-AML1* gene rearrangements were similar to those in normal pre-B cells from healthy bone marrow donors. However, Bach2 mRNA levels were significantly lower in *MLL-AF4*- and Ph^+ ALL subtypes (Supplementary Fig. 14). We also studied BACH2 mRNA levels in matched sample pairs from 49 patients that had a relapse of leukemia after initial successful treatment. Comparing matched sample pairs from the time of diagnosis and subsequent relapse of leukemia, BACH2 mRNA levels were substantially reduced in relapse samples (Fig. 5a and Supplementary Fig. 15). In addition, BACH2 expression levels at the time of diagnosis predicted detection of minimal residual disease (MRD) in a bone marrow biopsy taken on day 29 of treatment (Fig. 5b). Importantly, loss of BACH2 expression strongly correlated with poor overall outcome among 207 patients with childhood ALL (P9906 Children's Oncology Group). Patients with higher than median expression levels of BACH2 had a relapse-free survival (RFS) of 77% whereas those with lower than median BACH2 expression levels had a RFS of 47% (Supplementary Fig. 16b).

To study whether Bach2 mRNA levels represent an independent predictor of clinical outcome, we performed multivariate analyses using three established predictors of poor clinical outcome as reference, namely MRD, high white blood cell counts (WBC>100,000/μl) and deletion of the *IKZF1* tumor suppressor[17]. 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 even worse clinical outcome. Lower than median expression levels of BACH2 at diagnosis predicted inferior clinical outcome for patients with positive MRD on day 29, patients with WBC>100,000/μl (Figs. 5c-e and Supplementary Fig. 16), and for patients with *IKZF1*-deletion (Supplementary Fig. 17).

Inverse relationship between BACH2 and BCL6 in clinical outcome prediction

Our data in Figs. 1 and 2 demonstrated that both Bach2 and Bcl6 bind *Cdkn2a* and *Tp53* promoters but inversely regulate protein levels of Arf and p53 mRNA. Interestingly, 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. While higher than median mRNA levels of BACH2 at the time of diagnosis predict favorable clinical outcome, the opposite is true for ALL patients with BCL6 mRNA at higher than median levels (COG P9906). Studying BACH2 and BCL6 mRNA levels in a bivariate analysis identified subgroups of patients with particularly favorable ($BACH2^{High}$-$BCL6^{Low}$) and particularly poor ($BACH2^{Low}$-$BCL6^{High}$) outcome based on the ratio of BACH2:BCL6 mRNA levels (Fig. 5f).

BACH2 defines a region of minimal deletion at chromosome 6q15 and BACH2 deletion often occurs at the time of leukemia relapse

BACH2 is located at chromosome 6q15 and deletions of 6q encompassing 6q15 were found in 32% of cases of B cell lineage ALL[18]. We therefore studied B cell lineage ALL cell lines (n=9) and primary cases (n=4) with 6q deletions by high-resolution SNP analysis and found

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that *BACH2* lies within a region of minimal deletion (RMD) on 6q (Fig. 5g). Studying bone marrow samples from primary ALL cases at the time of (i) diagnosis, (ii) clinical remission and (iii) relapse of leukemia, we found that deletion of the *BACH2* locus was acquired at the time of leukemia relapse in 3 of 4 cases studied and was pre-existing in the 4th case (Figs. 5g-j).

Bach2-dependent gene expression changes overlap with Stat5- and Myc-regulated genes in Ph$^+$ ALL

Ph$^+$ ALL cells critically depend on tyrosine kinase signaling from (i) the *BCR-ABL1* oncogene, (ii) downstream phosphorylation of Stat5 and (iii) transcriptional activation of Myc[20]. Interestingly, a global analysis of Bach2-dependent gene expression changes revealed that these changes significantly overlap with gene expression changes caused by (i) *BCR-ABL1* kinase inhibition, inducible deletion of (ii) *Stat5a/b* and (iii) *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 *BCRABL1* in Ph$^+$ ALL cells.

Consistent with this scenario, *BCR-ABL1*-transformed *Bach2*$^{-/-}$ pre-B ALL cells are significantly less sensitive to *BCR-ABL1* tyrosine kinase inhibition (TKI; IM) than their wildtype counterparts (Figs. 6a-b). In the absence of *Bach2,* IM-induced apoptosis was reduced by 3-fold (Fig. 6a) and the fraction of cells that remained in cell cycle despite IM-treatment was increased by 2-fold (Fig. 6b). While *BCR-ABL1*-transformed *Bach2*$^{+/+}$ cells are able to form colonies only after a lag phase of ~10 days, colony formation of *Bach2*$^{-/-}$ pre-B ALL cells was significantly accelerated (Fig. 6c).

Expression of Bach2 and Myc are mutually exclusive during oncogenic transformation of pre-B cells

Overexpression of the Myc proto-oncogene frequently leads to increased proliferation and malignant transformation. However, Myc-driven proliferation can only be increased 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 critical to terminate pre-malignant clones and, hence, represent a powerful safeguard against malignant transformation. Studying progressive transformation of pre-B cells in *BCR-ABL1*-tg mice *in vivo*, we found that mRNA levels of Bach2 and Myc are inversely regulated. In wildtype and young (<30 days, non-leukemic) *BCR-ABL1*-tg mice, mRNA levels of Bach2 are high whereas mRNA levels of Myc are low. Between ages of 30 and 120 days, *BCR-ABL1*-tg mice undergo progressive leukemic transformation[24]. Fully transformed leukemia cells express high levels of Myc and low levels of Bach2, whereas treatment of mice with TKI (Nilotinib) restored normal expression levels for Bach2 (high) and Myc (low; Fig. 6d). We conclude that expression of Bach2 and Myc are mutually exclusive during oncogenic transformation of pre-B cells.

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Bach2-dependent activation of p53 represents a critical failsafe mechanism in Myc-mediated 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, excessively high levels of Myc result in activation of Arf/p53 and oncogene-induced senescence[21-23]. Retroviral overexpression of Myc was inefficient, induced cell death and caused significant toxicity in *Bach2*$^{+/+}$ pre-B cells but not in *Bach2*$^{-/-}$ pre-B cells (Fig. 6e-f and Supplementary Figs 19 and 20). These findings indicate that Bach2 strongly reduces susceptibility of pre-B cells to malignant transformation by Myc.

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 an increase of proliferation beyond this limit with about 70% of pre-B cells 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 levels were very low in *Bach2*$^{-/-}$ pre-B cells and not increased by Myc-overexpression (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.

Loss of Bach2 is sufficient to enable leukemic transformation by Myc

To confirm a critical role of Bach2 in Myc-induced failsafe control in an *in vivo* setting, IL7-dependent *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pre-B cells were transduced with retroviral Myc-GFP. Myc-GFP^{+} pre-B cells (Fig. 6e) were then injected into sublethally irradiated NSG recipient mice. While *Bach2*$^{-/-}$ Myc^{GFP} pre-B cells readily initiated fatal leukemia that killed the NSG recipient mice within 25 days, all NSG mice receiving *Bach2*$^{+/+}$ Myc^{GFP} pre-B cells were still healthy at 70 days after injection (Fig. 6g). After 70 days, the mice in the surviving *Bach2*$^{+/+}$ Myc^{GFP} cohort were sacrificed and analyzed together with mice receiving *Bach2*$^{-/-}$ Myc^{GFP} pre-B cells. In contrast to mice injected with *Bach2*$^{-/-}$ Myc^{GFP} pre-B cells, no CD19^{+} GFP^{+} cells (i.e. leukemia) were found in mice receiving *Bach2*$^{+/+}$ Myc^{GFP} pre-B cells (Fig. 6h and Supplementary Fig. 21). Minimal residual disease (MRD) PCR for GFP revealed small numbers of persisting leukemia cells in most of the surviving mice at 70 days after injection. However, MRD levels were 4-5 $\log_{10}$ orders lower in the wildtype group as compared to mice injected with *Bach2*$^{-/-}$ Myc^{GFP} pre-B cells (Fig. 6i).

Discussion

This study identifies Bach2-mediated activation of p53 as a critical mediator of pre-B cell receptor checkpoint control. During normal B cell development, Bach2 determines stringency of negative selection of pre-B cell clones that failed to productively rearrange productively rearrange V_H-DJ_H gene segments. In addition, Bach2-mediated activation of p53 represents a critical failsafe mechanism to terminate pre-malignant pre-B cell clones before they can complete malignant transformation. Thereby, Bach2 limits pre-B cell proliferation and oncogene signaling through activation of p53. Both in normal pre-B cells and in pre-B ALL, Bach2-mediated activation of p53 is opposed by Bcl6, a potent

transcriptional repressor of p53. While 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) and BCL6 (poor) expression levels also represents a strong predictor of clinical outcome in patients with ALL (Fig. 5f). While BACH2 function cannot be reinstated in patients with ALL carrying *BACH2* deletions, we propose that pharmacological inhibition of BCL6 (e.g. with RI-BPI) will restore the balance of BACH2/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 this paper. Sequence data are available from EMBL/GenBank under accession numbers provided in Supplementary Table 7. Microarray data are available from GEO under accession numbers provided in Supplementary Table 8.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ChIP	chromatin immunoprecipitation
RFS	relapse-free survival
EV	empty vector
HR	hazard ratio
IL	interleukin
OIS	oncogene-induced senescence
ROS	reactive oxygen species
RSS	recombination signal sequence
ALL	acute lymphoblastic leukemia

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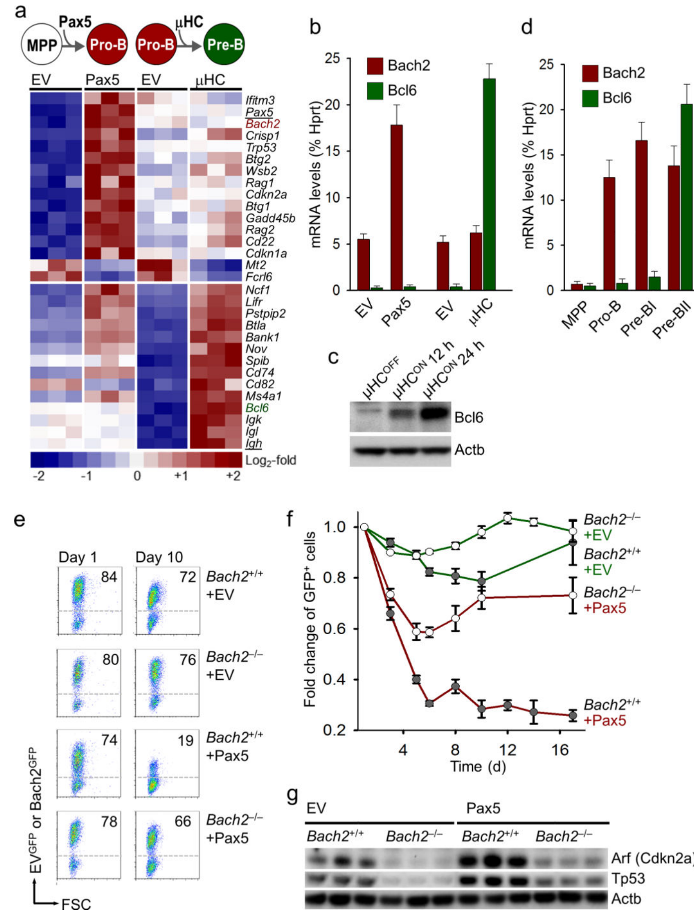

Figure 1. Bach2 and Bcl6 maintain balance between negative selection and survival of early B cells at the pre-B cell receptor checkpoint

(**a**) Gene expression changes at the pre-B cell receptor checkpoint were studied under two experimental conditions, namely (i) initiation of V(D)J recombination upon inducible activation of Pax5 compared to empty vector control (EV) and (ii) inducible expression of a functional immunoglobulin μ heavy chain (μHC). (**b**) Quantitative RT-PCR and (**c**) Western blot depicting regulation of Bach2 and Bcl6 by Pax5 and μHC-induction, respectively. (**d**) Quantitative RT-PCR for *Bach2* and *Bcl6* in multi-lineage progenitor cells (MPP), Pro-B, Pre-BI and pre-BII cells sorted from bone marrow of C57BL/6 mice. (**e-f**) GFP-tagged Pax5 or empty vector controls (EV) were transduced in transformed pro-B and pre-BI cells from *Bach2*$^{-/-}$ and wildtype mice and GFP^{+} cells were monitored (day 1 and day 10) by flow cytometry. (**g**) *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pro-B and pre-BI cells transduced with Pax5-GFP or EV were sorted and studied for protein expression of Arf and p53 by Western blotting, using β-actin as loading control.

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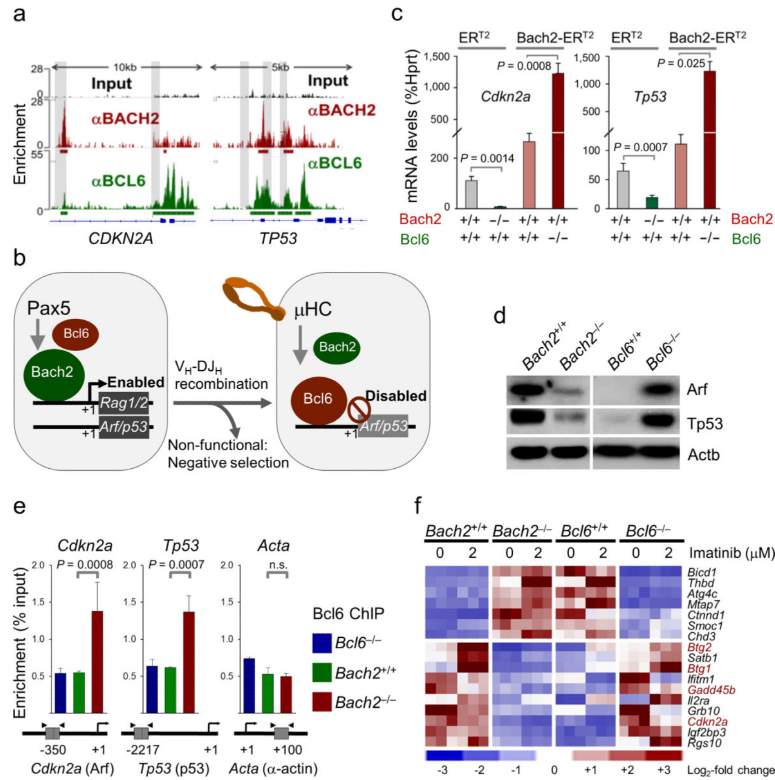

Figure 2. Bach2-dependent activation of Arf/p53 is reversed by Bcl6 upon expression of a functional μ-heavy chain

(**a**) ChIP-seq analysis was performed in a lymphoma cell line using antibodies against BACH2 (red) and BCL6 (green) and peaks with significant enrichment relative to background (input; black) are annotated by ChIP-seeqer with bold underlines. Overlapping peaks between BACH2 and BCL6 binding are indicated by shades. (**b**) The proposed scenario for BACH2-BCL6 interactions at the pre-B cell receptor checkpoint. (**c**) Effects of presence or absence of Bach2, presence or absence of Bcl6 and inducible overexpression of Bach2 (tamoxifen-inducible Bach2-ER^{T2} vector) on mRNA levels of *Arf* and *p53* measured by qRT-PCR. (**d**) Effects of presence or absence of Bach2 and presence or absence of Bcl6 on protein levels of Arf and p53 measured by Western blot using β-actin as loading control. (**e**) To directly test the hypothesis that Bach2 negatively regulates the ability of Bcl6 to bind to *Cdkn2a* (Arf) and T*p53* (p53) promoters, Bcl6-ChIP experiments with *Bcl6*$^{-/-}$ (negative control), *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ cells were performed. (**f**) A systematic ChIP-seq analysis revealed that 134 of the 541 BCL6- and 565 Bach2-target genes are shared (not shown). 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 like *Cdkn2a (Arf), Tp53, Gadd45b, Btg1 and Btg2*, in opposite directions.

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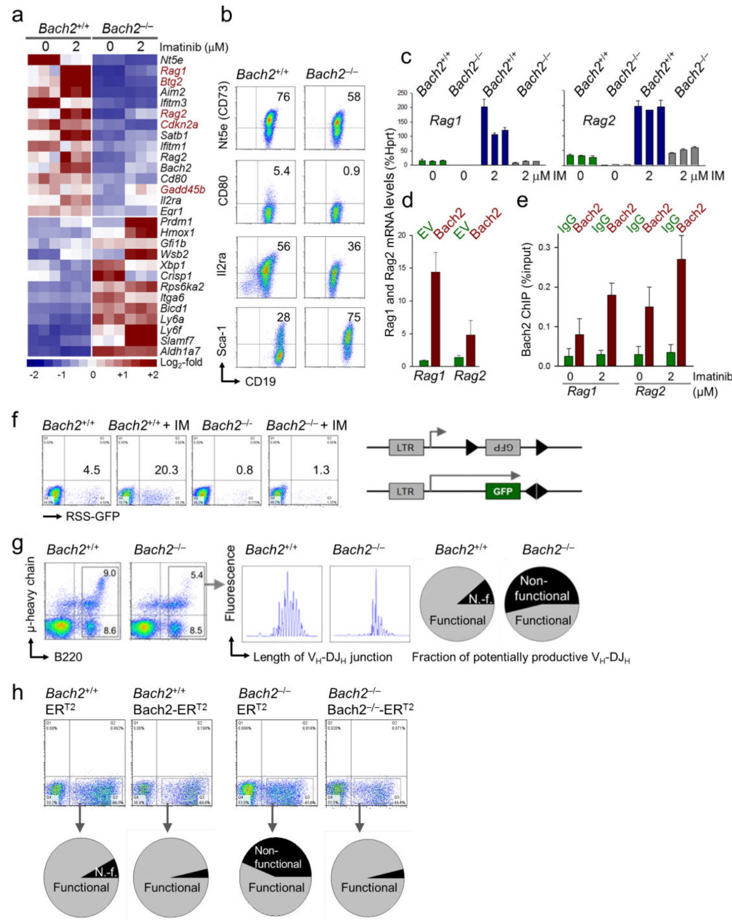

Figure 3. Bach2 mediates V(D)J recombination and μ-heavy chain checkpoint control during early B cell development

(**a**) Using a classical pre-B cell differentiation model based on tyrosine kinase inhibition (Imatinib; IM) of *BCR-ABL1*-transformed pre-B cells[12,13], gene expression changes upon inducible differentiation of Bach2$^{-/-}$ and *Bach2*$^{+/+}$ pre-BI cells were measured by microarray analysis. (**b**) Increased expression of the early progenitor antigen Ly6f (Sca-1) and reduced expression of the pre-B cell antigen Il2rα (CD25) in Bach2-deficient Pre-BI cells was validated by flow cytometry. (**c**) Reduced mRNA levels of Rag1 and Rag2 in Bach2-deficient Pre-BI ALL (*BCR-ABL1*) cells in the presence and absence of IM were validated by qRTPCR. (**d**) The effect of inducible overexpression of Bach2 on *Rag1* and *Rag2* mRNA levels was measured by qRT-PCR. **(e)** Single-locus ChIP analysis using IgG and BACH2-specific antibodies depicting the binding of BACH2 to *Rag1* and *Rag2* promoters, which is further enhanced by IM-treatment.

(**f**) To test functional consequences of defective expression of Rag1/Rag2, *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pre-BI cells were transduced with a puromycin-selectable V(D)J recombination substrate carrying an inverted GFP flanked by recombination signal sequences (RSS). Recombination activity of the RSS substrate in *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pre-BI cells was measured by flow cytometry (inversion of the GFP cassette in the correct orientation; percentages of GFP^{+} cells are given) in the presence and absence of IM treatment. (**g**) Comparison of the composition of B cell progenitor populations in the bone marrow of

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Bach2$^{+/+}$ and *Bach2*$^{-/-}$ mice by flow cytometry. Fragment length analysis revealed a size peak distribution of V_H-DJ_H junctions that were indeed selected for multiples of 3 bp among *Bach2*$^{+/+}$ compared to random distribution in *Bach2*$^{-/-}$ pre-B cells.

(**h-i**) IL7-dependent *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pre-B cells were transduced with tamoxifen (4-OHT)-inducible Bach2-ER^{T2} and ER^{T2} empty vector controls. Cells were treated with 4-OHT for 24 hours and sequence analysis of V_H-DJ_H junctions was performed. (**i**) Amino acid sequences of V_H-DJ_H junctions with non-functional V_H-DJ_H rearrangements shaded in gray.

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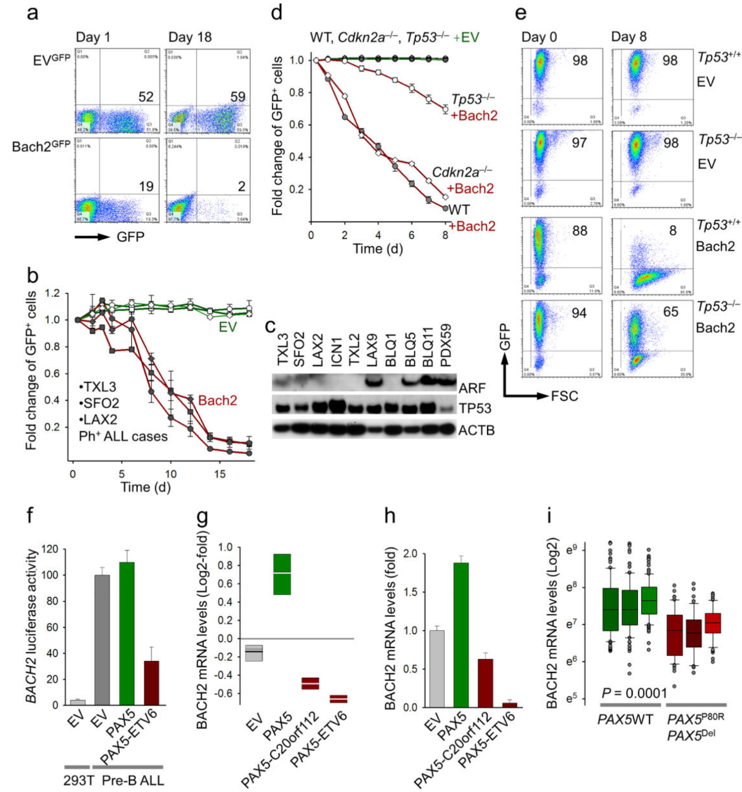

Figure 4. BACH2 mediates PAX5-dependent tumor suppression in pre-B ALL through activation of p53

(**a**) Patient-derived Ph^{+} ALL cells were propagated on OP9 stroma and transduced with vectors for Bach2^{GFP} or GFP empty vectors (EV; a), and (**b**) GFP^{+} cells were monitored by flow cytometry. (**c**) Ph^{+} ALL cases were assayed for ARF and TP53 protein expression by Western blot using β-actin as loading control. (**d-e**) Flow cytometry for GFP in *Cdkn2a*$^{-/-}$ and *Tp53*$^{-/-}$ pre-B ALL cells (*BCR-ABL1*) transduced with Bach2^{GFP} or EV^{GFP}. (**f**) Transcriptional regulation of *BACH2* by PAX5 assayed by the overexpression of dominant-negative *PAX5-ETV6* fusion in 293T cells and Nalm6 pre-B ALL cells transduced with a *BACH2* promoter luciferase reporter construct. (**g-h**) mRNA levels of *BACH2* were increased by expression of wildtype PAX5 but decreased by dominant-negative *PAX5-C20orf112* and *PAX5-ETV6* fusion genes. (**i**) Gene expression values for three *BACH2* probe sets in 160 cases of childhood pre-B ALL, 54 of which carry *PAX5* deletions or somatic mutations (221234_s_at, 227173_s_at, 215907_at; COG P9906).

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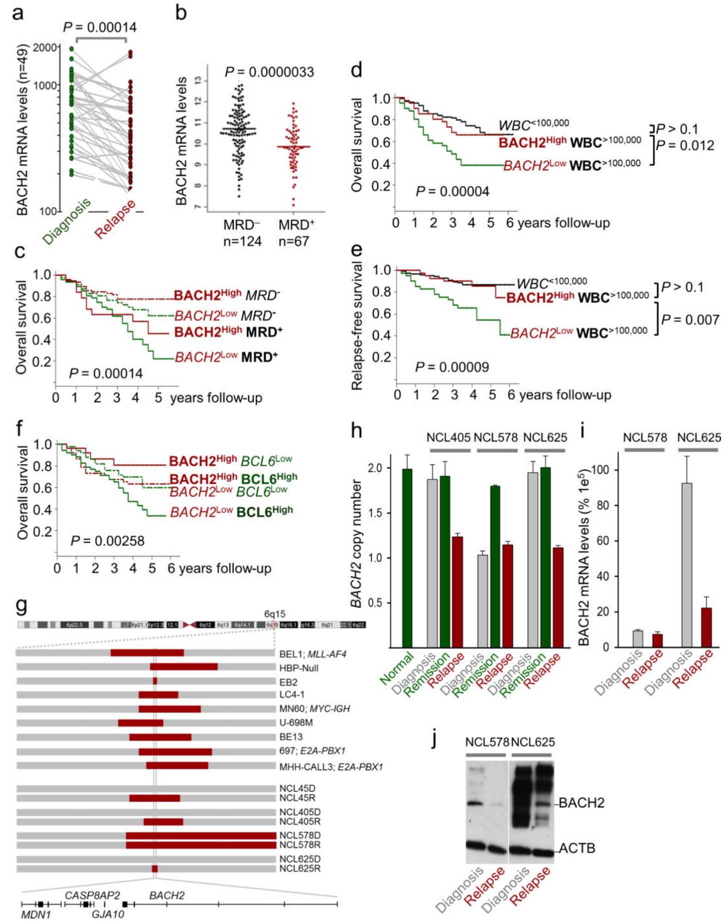

Figure 5. *BACH2* is an independent predictor of poor clinical outcome in ALL patients

(**a**) Comparison of *BACH2* mRNA levels in matched sample pairs from 49 patients at the time of diagnosis and relapse of leukemia (pair-wise t-test). (**b**) Comparison of *BACH2* mRNA levels in MRD^- and MRD^+ patients (MRD^+; n=67; COG P9906 trial). Multivariate analyses for *BACH2* mRNA levels as independent predictors of clinical outcome performed using established predictors of poor clinical outcome, namely, (**c**) detection of MRD and (**d-e**) high white blood cell counts (WBC>100,000/μl). (**f**) Studying BACH2 and BCL6 mRNA levels in a bivariate analysis identified subgroups of patients with particularly favorable ($BACH2^{High}$-$BCL6^{Low}$) and particularly poor ($BACH2^{Low}$-$BCL6^{High}$) outcome. P values are from logrank test. (**g**) High resolution SNP analysis identified *BACH2* as a region of minimum deletion (RMD) in ALL cell lines (n=9) and primary cases (n=4) with 6q deletions. Longitudinal bone marrow samples at the time of diagnosis, remission and relapse of ALL were studied by (**h**) genomic PCR using the Taqman probe Hs01527432_cn, (**i**) qRT-PCR using the Taqman probe Hs00222364_m1 and (**j**) Western blot to assay allelic status and expression of *BACH2*.

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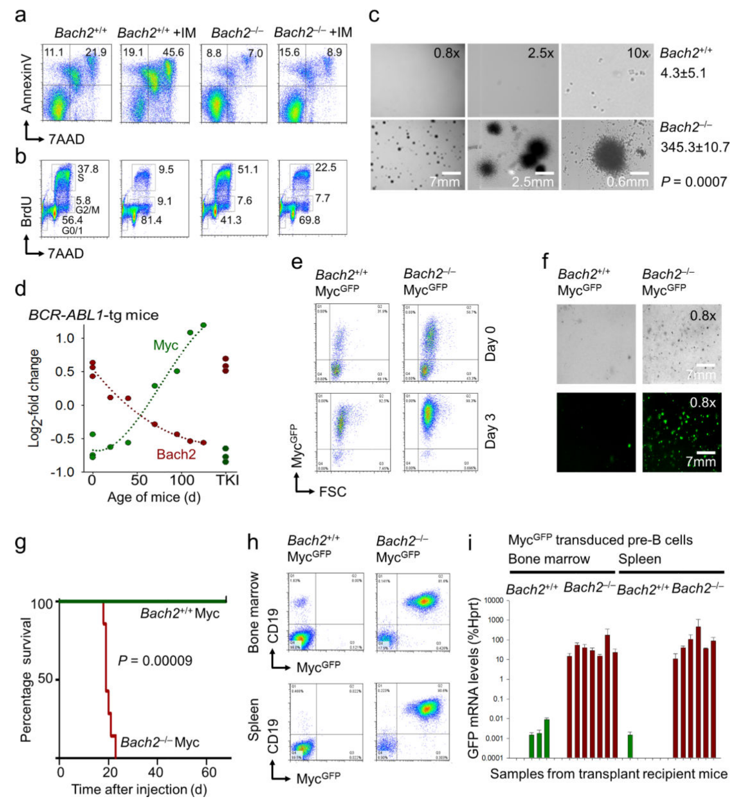

Figure 6. Bach2 prevents leukemic transformation by Myc

(**a**) Induction of cell death (Annexin V/7AAD staining) and (**b**) cell cycle arrest (BrdU staining; G0/1, S and G2/M phase distribution) in *BCR-ABL1* transformed *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pre-B ALL cells before and after imatinib treatment. (**c**) Comparison of colony forming abilities of *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ pre-B ALL cells plated in methylcellulose on day 7. *Bach2*$^{+/+}$ pre-B ALL cells were able to form colonies after a lag phase of ~10 days (not shown). (**d**) To study progressive leukemic transformation in vivo, bone marrow pre-B cells were isolated from wildtype mice and *BCR-ABL1*-tg mice of various ages reflecting different stages of transformation. mRNA levels of *Bach2* and *Myc* were plotted against age of mice [days], TKI denotes *in vivo* treatment of leukemic mice with the tyrosine kinase inhibitor Nilotinib. (**e**) *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ IL-7-dependent pre-B cells were retrovirally transduced with Myc^{GFP} or EV^{GFP} and transduction efficiency was monitored by flow cytometry on days 0 and 3. (**f**) Plating efficiency of Myc^{GFP}-transduced *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ IL-7-dependent pre-B cells in methylcellulose was monitored by fluorescence microscopy. (**g**) Sorted Myc^{GFP}-transduced *Bach2*$^{+/+}$ and *Bach2*$^{-/-}$ IL-7-dependent pre-B cells were also tested for their capacity to initiate fatal leukemia in NOD/SCID recipient mice and Kaplan-Meier analysis was performed to compare overall survival of transplant recipient mice in the two groups. (**h**) After 70 days, the mice in the surviving *Bach2*$^{+/+}$ Myc^{GFP} cohort were sacrificed and analyzed together with mice receiving *Bach2*$^{-/-}$ Myc^{GFP} pre-B cells for CD19^{+} GFP^{+} cells in bone marrow and spleen (i.e. leukemia) by flow cytometry. (**i**) Minimal residual disease (MRD) qRT-PCR for leukemic cells infiltrating

bone marrow or spleens was performed in triplicates for mice in each group using GFP-specific primers.

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