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BACH2-BCL6 balance regulates selection at the pre-BCR checkpoint

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Abstract

At the pre-B cell receptor (pre-BCR) checkpoint, developing pre-B cells are selected for successful rearrangement of V_H-DJ_H gene segments and expression of a pre-BCR. Reduced stringency at this checkpoint may obstruct the B cell repertoire with non-functional B cell clones. Earlier studies described that activation of BCL6 by a functional pre-BCR mediates positive selection of pre-B cells that have passed the checkpoint. This concept is now further elaborated by the recent finding that the BACH2 transcription factor induces negative selection and opposes BCL6-function prior to the pre-BCR checkpoint. Here we discuss the antagonism between BCL6 and BACH2 during early B cell development, as well as its implications in both repertoire selection and counter-selection of pre-malignant clones for leukemia-suppression.

Keywords

BACH2; BCL6; negative selection; pre-B cell receptor checkpoint; leukemogenesis

Pre-B cell receptor checkpoint as a safeguard against leukemogenesis

Early B cell development is a tightly regulated process with numerous checkpoints in place to control the quality of cells generated at every stage. These checkpoints ensure that early B cells do not go astray and undergo malignant transformation. Alterations in key checkpoint control signaling molecules is deleterious to a pre-B cell and increases the probability of leukemia initiation [1,2].

The first instance of checkpoint control signaling during B cell development occurs when multi-lineage progenitors commit to B-lineage by activating a complex cell signaling network composed of E2A, EBF, PAX5 and FoxO1 [3–9]. The master regulators of B cell differentiation, namely E2a and Ebf, activate the expression of Pax5 (Paired Box Protein 5) and the Rag recombinases [6] to trigger DNA rearrangement (V_H-DJ_H recombination) at the immunoglobulin heavy chain locus. While the RAG1/RAG2 enzymes execute the

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rearrangement, PAX5 remodels the chromatin at the heavy chain locus to increase the proximity of the different V segments to the rearranged D-J joint [10]. By regulating V-DJ recombination in the above described manner, PAX5 both serves as a checkpoint itself and, also activates subsequent checkpoints during early B cell development.

The μ heavy chain of the pre-B cell receptor represents the checkpoint subsequent to PAX5 activation [1,2]. At this checkpoint, two opposing processes occur- one serves to eliminate deleterious pre-B cells with non-functional V_H .DJ $_H$ rearrangements (negative selection), while the other allows the survival and proliferation of pre-B cells carrying functional heavy chain rearrangements [2]. A disruption of the balance between the two processes leads to leukemic transformation of the pre-B cell.

Previous studies highlighted the requirement of BCL6 in the survival of early B cells that emerge following successful completion of the pre-B cell receptor checkpoint [11]. Further investigations revealed that this BCL6-mediated survival is essential to trigger the subsequent step in early B cell development, namely, immunoglobulin light chain recombination [11]. Recently, BACH2, a protein directly transactivated by PAX5 [12,13], was shown to be required for the negative selection of pre-B cells that had failed to productively rearrange their immunoglobulin heavy chains [14]. Here we review how BACH2 and BCL6 play central but opposing roles during early B cell differentiation, and thereby, maintain balance between negative selection and survival at the pre-B cell receptor checkpoint. In addition, we examine the disruption of this balance during leukemic transformation of a pre-B cell. Finally, we discuss how this knowledge can be exploited to predict the clinical outcome of patients with pre-B acute lymphoblastic leukemia (pre-B ALL), so as to design improved treatment strategies.

Diverse functions of BCL6 and BACH2 in the immune system

Transcriptional regulation by BCL6

BCL6 (B cell lymphoma/leukemia gene 6) was identified on the basis of its frequent translocations involving the 3q27 region in B cell non-Hodgkin's lymphoma [15–18] (B-NHL). BCL6 is a member of the ZBTB (zinc finger and BTB) family that is comprised of an N-terminal BTB/POZ (Broad-complex, Tramtrack, Bric-a-brac/Poxvirus and zinc fingers) domain and *Krüppel*-type zinc fingers at the C-terminus. The zinc finger domain of BCL6 is required for DNA binding at its consensus sequence TTCCT(A/C)GAA [19,20]. Initial studies using reporter assays characterized BCL6 as a transcriptional repressor [21–24].

The mechanism of BCL6-mediated gene repression requires the physical interaction of corepressor proteins such as BCOR (BCL6-interacting corepressor), NCOR (nuclear receptor corepressor) or NCOR2 (SMRT) with BCL6 at target loci [25–28]. A second repressor domain (often called RD2 domain) has been found in the middle region of BCL6 that functions independently of the BTB domain by recruiting other co-repressors such as NuRD (nucleosome remodeling and deacetylase) and CtBP [29,30] (C-terminal binding protein). An integrative approach that combines ChIP-on-chip data with gene expression profiles revealed that recruitment of BCL6 to gene control regions does not necessarily cause gene repression, as only a fraction of the bound genes are repressed [31]. Regulatory variation might depend on distinct corepressor complexes at specific promoter sequences and lineage-specific mechanisms [32,33].

BCL6 is a pre-requisite for the germinal center reaction

Early studies with BCL6-deficient mice demonstrated that BCL6 is indispensable for the formation of germinal centers (GC) and, therefore, for the production of high-affinity antibodies [34–36]. This is attributed to the function of BCL6 in mediating lineage

commitment of CD4⁺ T cells to T follicular helper (T_{FH}) cells, which are required for GC formation, and to the negative regulatory effect of BCL6 on DNA damage response genes in GC B cells [37–41]. Bcl6 also acts as a switch in lymphoid cell fate decision by repressing the expression of *Blimp-1* (*Prdm1*), a transcription factor that promotes plasma cell differentiation of GC B cells and blocks the T_{FH} differentiation of CD4⁺ T cells [39] [42,43]. It is of interest to note that in mice in which Bcl6 is targeted in all tissues (germline knockout), as well as mice with targeted deletion of *Bcl6* exclusively in the B-lymphoid compartment, exhibit a normal phenotype - in terms of absolute B cell numbers - at every stage of development [35] [44]. *Bcl6* deficient mice, however, show highly restricted clonality despite the presence of a normal sized B cell pool [11]. Consistent with previous findings, conditional *Bcl6* ablation has been shown to result in a normal sized post GC pool [44]. It will be of interest to determine if the post-GC memory B cells in the *Bcl6* conditional knockout model developed by Takemori and colleagues show a pattern of clonal restriction similar to that observed in *Bcl6* deficient early B cells. Additionally, it would be interesting to test whether conditional ablation of *Bcl6* in this model affects survival of pre-B cells at the time of Ig light chain gene rearrangement in a manner similar to that demonstrated by Duy *et al.* [11] in the *Bcl6* deficient mice.

BCL6 in mature B cell malignancies and inflammation

Deregulation of *BCL6* is primarily associated with the pathogenesis of B cell lymphomas. Constitutive expression of *BCL6* is required for survival of lymphoma cell lines and induces formation of diffuse large B cell lymphoma (DLBCL) in transgenic mice [37] [45–48]. Translocations of *BCL6* that block p53-dependent apoptosis occur in 20 to 40 percent of diffuse large B cell lymphoma patients (DLBCLs) [37] [49,50].

BCL6 deficiency has also been associated with the development of autoimmune disease, with a recent study pointing to an important role for BCL6 in the function of regulatory T cells (T_{regs}) [51]. *Bcl6*-deficient mice present severe myocarditis and pulmonary vasculitis. Sawant *et al* showed that T_{regs} deficient in BCL6 are unable to prevent Th2-type inflammatory allergic responses *in vivo*. These symptoms were attributed to the requirement of Bcl6 for the repression of the Th2 program in T_{regs}, comprised of *Gata3*, *cmaf*, *Ccr8*, and the Th2 cytokines *Il4*, *Il5*, and *Il13*. BCL6 therefore helps maintain immune homeostasis and keeps inflammatory responses at bay by repressing both Th2 cell differentiation and inhibiting Th2 cytokine expression in T_{regs} [51].

Transcriptional regulation by BACH2

Another B-lymphoid transcription factor that is critical for somatic hypermutation and class switch recombination is BACH2. BTB And CNC Homology 1 Basic Leucine Zipper Transcription Factor 2, abbreviated as *BACH2*, was identified from a yeast 2-hybrid screen for MafK [52]. *BACH2* maps to chromosome 6q15 [53], and has a telomere to centromere transcriptional orientation [54]. As the name suggests, BACH2 possesses 2 domains: BTB and bZip. The BTB domain, located at the amino terminus is required for protein-protein interaction [52]. By virtue of its bZip domain, which is rich in basic amino acids like arginine and lysine, BACH2 mediates direct DNA binding [55]. The bZip domain of BACH2 also possesses the nuclear localization signal [56], which is essential for BACH2 to function as a transcription factor.

BACH2 binds to the TPA (12 O-Tetra decanoylphorbol-13-acetate) response element (TRE) on the DNA. In addition to TRE, BACH2 also binds to the related MARE (MAF Response Element) and ARE (Antioxidant Response Element) as homodimers or in combination with MAF proteins [55], and results in transcriptional repression. TRE, MARE and ARE elements share the same consensus sequence (TGAG/CTCA) and bind proteins belonging to

the MAF family [55]. Although widely characterized as a repressor, BACH2 can activate transcription at selected loci. One such situation occurs when BACH2 binds to MAZR to activate *fgf4* transcription [57].

BACH2 is indispensable for germinal center reaction

A comparison of stage specific expression of BACH2 during B cell development revealed that it was present at every stage except in plasma cells [58]. Further studies conducted to understand the reason behind the loss of BACH2 expression at the plasma cell stage led to the identification of the crucial role played by BACH2 in germinal center formation after antigen encounter [59]. Experiments carried out in a *Bach2* deficient mouse model revealed that Bach2 delays the process of plasma cell differentiation by direct transcriptional repression of plasma cell factor *Blimp1* [59–63]. Pax5, a crucial regulator of B cell differentiation, activates *Bach2* and *Aid* and initiates the germinal center reaction. The Pax5-Bach2-Bcl6 axis delays plasma cell differentiation and induces the production of high affinity antibodies by germinal center B cells upon immunization [59]. More recently, it has been observed that antigen exposure lowers Bach2 expression levels in class-switched IgG1 memory B cells thereby facilitating their differentiation into plasma cells [64].

BACH2 in the pathogenesis of immunological malignancies and autoimmunity

As observed in the case of *BCL6*, deregulated *BACH2* expression has been associated with lymphoid malignancies. Loss of heterozygosity of *BACH2* has been reported to occur at a frequency of 20 percent in human B cell lymphomas [53]. Deletions of 6q15 that include the *BACH2* locus occur in approximately 30 percent of pre-B ALL cases [65]. In a number of leukemias and lymphomas, disruption of wildtype *BACH2* expression has been attributed to viral integrations [66–69]. Insertional mutagenesis studies using experimental murine retroviral models such as MLV (Murine Leukemia Virus) demonstrate that the *BACH2* locus is a hotspot for viral integrations and transpositions [67,68]. Additionally, there is evidence to suggest that viral integrations at the *BACH2* locus can also occur in human disease. For example, Epstein Barr Virus (EBV) was found inserted into the *BACH2* locus in the human derived Burkitt Lymphoma cell line Raji [66]. Deletions and translocations of chromosome 6q spanning the *BACH2* locus occur frequently in high-grade B cell lymphomas [14] [54] [70]. In multiple studies, high BACH2 level has been identified as an independent prognostic factor deciding favorable clinical outcome in DLBCL patients [71,72].

Although largely restricted to the B-lymphoid lineage within the hematopoietic system, BACH2 expression has been recently identified to be crucial for the development of T_{regs} and the maintenance of immune homeostasis. Although *Bach2*-deficient mice appear normal at birth, they subsequently develop progressive inflammation and autoimmune disease, similar to that observed in *Bcl6*-deficient mice. By stabilizing both T_{reg} development through modulation of *Foxp3* expression [73], and, by also repressing the differentiation of T cells to its effector lineages (Th1, Th2 and Th17), Bach2 constrains excessive immune activation [73,74]. These biochemical findings are backed by the presence of genetic polymorphisms in the *BACH2* gene in multiple human autoimmune disorders, thus further underscoring the importance of BACH2 in immune tolerance.

BACH2 and BCL6 induce opposite outcomes during genotoxic stress

Rapidly proliferating GC B cells undergo a high level of cellular stress and DNA damage that is caused by somatic hypermutation and DNA double-strand break (DSB) events from class-switch recombination [75,76]. BCL6 allows these physiological processes to occur by repressing the expression of DNA damage response and checkpoint genes (*CHEK1*, *ARF*,

CDKN1A, *TP53*) as well as the DNA damage sensor *ATR*. This allows GC B cell proliferation and survival even in the presence of genotoxic stress [37] [38] [42] [77] [78].

In contrast to *BCL6*, binding of *BACH2* to its consensus sequence downregulates antioxidant response genes and triggers a program of oxidative stress induced cell death [55] [56] [79–81]. The majority of *BACH2* protein is present in the cytoplasm in the absence of oxidative stress. Oxidative stress triggers nuclear localization of *BACH2* by activating its nuclear localization signal and blocking its cytoplasmic localization [56].

BACH2 and BCL6 compete for binding to promoter at checkpoint-regulator genes

Our recent studies demonstrated that *Bach2* deficient pre-B cells had lower expression of cell death inducers like *Arf*, *p53* and *Btg2*. This observation raised the possibility that *Bach2* acts as a transactivator of these cell cycle checkpoint genes, and triggers an apoptotic program upon genotoxic stress [14]. The concomitant expression of both *BACH2* and *BCL6* in the germinal center and their opposing roles on survival indicated the regulation of a set of common genes. Consistent with this hypothesis, a systematic ChIP-seq analysis revealed that 134 of the 541 *BCL6*- and 565 *BACH2*-target genes are shared. Interestingly, a number of cell cycle checkpoint genes such as *TP53*, *ARF*, *GADD45A* and *GADD45B*, that emerged from this analysis showed opposite transcriptional outcomes upon *BACH2* and *BCL6* binding. Although *BCL6* and *BACH2* bind different consensus sequences on the DNA, the binding sites for *BACH2* and *BCL6* on the promoters of the above mentioned genes were found to be overlapping. Unlike *BCL6*, *BACH2* functions as a transcriptional activator at these loci, thus activating a program of cell death as opposed to survival in the case of *BCL6* [14]. In light of these findings, we discuss in the following sections the interplay between *BACH2* and *BCL6* in early B cell development.

BACH2 and BCL6 maintain early B cell homeostasis

Gene expression changes during early human B cell development show that *BACH2* expression is upregulated much earlier than that of *BCL6* [82]. *Bach2* is upregulated in a Pax5-dependent fashion at the multipotent progenitor (MPP) to the pro-B transition, before the completion of the heavy chain checkpoint; in contrast, *Bcl6* levels remain fairly low until the completion of the pre-B cell receptor checkpoint, and increase at the pro-B to pre-B transition [11] [14] [82].

At the pre-B cell receptor checkpoint, *Bach2* executes negative selection of early B cells with failed V_H -DJ_H rearrangements. Additionally, it modulates heavy chain rearrangement by increasing the expression and activity of the RAG recombinases [14]. We propose that the above two events are interconnected: By activating the RAG recombinases, *BACH2* increases the probability of a pre-B cell to undergo successful heavy chain rearrangement. Simultaneously, the *BACH2*-dependent rise in RAG recombinase levels would initiate a DNA damage response pathway in non-functional pre-B cells thereby aiding their negative selection. Further work will be required to test these hypotheses. *BCL6* is turned upregulated subsequent to the completion of the pre-B cell receptor checkpoint, and protects cells undergoing light chain rearrangement from the genotoxic stress caused by RAG1 and RAG2 [11].

Collectively, these observations are in agreement with the functional roles played by *Bach2* and *Bcl6* at the μ heavy chain checkpoint. While *Bach2* is required for negative selection that occurs prior to the completion of the checkpoint [14], *Bcl6* protects cells that emerge after successful completion of the checkpoint [11]. *Bach2* and *Bcl6* may thus represent a paradigm of how early B cell development is a perfectly equilibrated process with an interplay of several factors maintaining the integrity of the B cell repertoire (Figure 1). Any

deviation from this balance could result in leukemic transformation of the pre-B cell (Figure 2).

BACH2-BCL6 equilibrium is upset during leukemic transformation

Defects in pre-BCR checkpoint control have been attributed to leukemia [1,2]. Similar to its role in DLBCL, BCL6 acts as a protooncogene in the pathogenesis of *BCR-ABL1*-driven ALLs. Furthermore, BCL6 enables survival of Philadelphia chromosome-positive ALL (Ph⁺ ALL) cells upon BCR-ABL1- kinase inhibition through repression of p53 expression [83].

In contrast, as discussed earlier, BACH2 triggers oxidative stress-induced cell death, by binding and repressing the expression of antioxidant response genes. Treatment of cells with oxidative stress-inducing chemotherapeutic agents results in nuclear accumulation of BACH2 [79] and subsequently, cell death. Studies with the BCR-ABL1 inhibitor, imatinib show that BACH2 is upregulated upon imatinib treatment in Ph⁺ ALL cell lines [13] [84]. BCR-ABL1 signaling lowers BACH2 expression and activity, the former by inactivation of PAX5 [13], and, the latter by preventing nuclear translocation of BACH2 [80].

We have recently shown that BACH2 is required downstream of PAX5 to prevent the accumulation of B cells with non-functional μ HC rearrangements, providing an example of how defects in pre-BCR checkpoint control can result in pre-B ALL [14]. BACH2 expression halted or slowed down the process of pre-B leukemogenesis induced by oncogenes Myc and *BCR-ABL1* by activating apoptosis [14]. In both normal pre-B cells and pre-B ALL, BACH2-induced activation of *TP53* is opposed by BCL6. Thus, loss of *BACH2* or any of its upstream activators, such as *PAX5*, results in unopposed BCL6-mediated suppression of *TP53* at the pre-BCR checkpoint, leading to leukemia.

Concluding remarks and future perspectives

Using the pre-BCR checkpoint as an example, we have discussed how deregulated checkpoint control signaling can lead to B cell precursor ALL. B cell development is a tightly controlled process with diverse proteins maintaining the balance between negative selection and survival, and preserving the quality of the repertoire. By modulating the two opposing cellular processes at the pre-BCR checkpoint, namely apoptosis and survival, BACH2 and BCL6 ensure that the vast majority of the B cell repertoire is made up of cells carrying productive V_H-DJ_H rearrangements.

Activation of BACH2 prior to BCL6 during early B cell development represents a critical failsafe mechanism to terminate pre-malignant pre-B cell clones before they can complete malignant transformation. *BACH2* is frequently inactivated in established pre-B ALL clones or at the time of leukemia relapse [14] [85]. Consistent with their opposing role in pre-BCR checkpoint control, the ratio of BACH2 and BCL6 expression levels represent a statistically significant predictor of clinical outcome in patients with ALL [14]. While BACH2 function cannot be easily reinstated in patients with ALL carrying *BACH2* deletions or somatic mutations, we propose that pharmacological inhibition of BCL6 (with BCL6 inhibitors like RI-BPI) will restore the balance of BACH2/BCL6-mediated checkpoint control and relieve BCL6-mediated transcriptional repression of *ARF/TP53* (Figure 3). Treatment studies like these however need to be verified using *in vitro* approaches and *in vivo* models before extending them to the clinic. Additionally, it would be interesting to test experimentally if a balance between BACH2 and BCL6 is required to maintain the stringency of the germinal center B cell repertoire. It would also be imperative to test if BACH2 is involved in RAG1/2-mediated receptor editing. Such an understanding would shed new light on BACH2's role in the elimination of mature B cells with improper receptor editing. It would also be of interest to know if BACH2-BCL6 balance determines the elimination of B cells

that are irreparably damaged by AID in the germinal center during somatic hypermutation and class switch recombination. Answering these questions would pave the way to better understanding the pathogenesis of Hodgkin's B cell lymphomas that often carry crippling somatic mutations in their heavy chain loci.

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Highlights

1. BACH2 and BCL6 regulate selection events at the pre-BCR checkpoint
2. Regulation of negative and positive selection by BACH2 and BCL6 maintain homeostasis
3. Pre-B cell susceptibility to leukomogenesis depends on relative BACH2 and BCL6 levels

Figure 1. BACH2 and BCL6 maintain homeostasis at the pre-B cell receptor checkpoint
(a) BACH2 and BCL6 ensure high fidelity of the B cell repertoire by equilibrating two opposing processes, namely, negative selection and survival respectively, at the immunoglobulin heavy chain checkpoint. BACH2/BCL6 expression ratios remain high before the heavy chain checkpoint. This ensures that BACH2 can eliminate nonfunctional pre-B cells by negative selection, uninhibited by BCL6. Subsequently, a functional heavy chain upregulates BCL6 and tilts the balance to BCL6-mediated survival of pre-B cells emerging from the checkpoint. **(b)** Diagrammatic representation of the molecular mechanism behind the balance of negative selection and survival at the pre-B cell receptor checkpoint. In the absence of BCL6, BACH2 induces transactivation of *ARF/TP53* to induce negative selection by apoptosis. Once negative selection is completed, BCL6 levels increase and cause displacement of BACH2 from promoters of *ARF* and *TP53*. This triggers a survival program by BCL6-mediated repression of *ARF/TP53*.

Figure 2. BACH2 and BCL6 maintain balance between checkpoint control and leukemic transformation of early B cells

Upon challenge of early B cells with oncogenes like *cMYC*, BACH2 and BCL6 maintain the balance between apoptosis and leukemic transformation at the pre-B cell receptor checkpoint. BACH2 and BCL6 compete for promoters of *ARF* and *TP53* in B cell precursor leukemia. Upon oncogene challenge, BACH2 (boldface, left) being downstream of PAX5, ensures that deleterious pre-B cells are eliminated by negative selection through activation of the ARF/TP53- mediated tumor surveillance mechanism (left, checkpoint control). If BACH2 expression is compromised (right, dashed arrow), BCL6-mediated repression of ARF/TP53 is unopposed leading to leukemia. Boldface indicates high level of a particular protein

Figure 3. Knowledge of relative levels of BACH2 and BCL6 in Ph⁺ ALL patients can be exploited to design effective treatment strategies

(a) *BACH2* expression levels can be lowered in Ph⁺ ALL patients by 6q15 deletions encompassing its locus, by somatic mutations or hypermethylation of its promoter or by loss of its upstream activator *PAX5*. This will lower the *BACH2/BCL6* expression levels thus, leading to loss of *ARF/TP53*- dependent tumor surveillance. Such patients may not respond effectively to single agent (TKI) treatment. (b) A potential therapeutic strategy that may be employed to treat patients described in (a) is the combination of *BCL6* inhibitors (e.g. RIBPI) and TKIs to block *BCL6* mediated repression of *ARF/TP53*. This treatment design will relieve *BCL6*-induced resistance of leukemia cells to TKIs and thereby, aid in their elimination.