



The mTOR-Bach2 Cascade Controls Cell Cycle and Class Switch Recombination during B Cell Differentiation

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ABSTRACT The transcription factor Bach2 regulates both acquired and innate immunity at multiple steps, including antibody class switching and regulatory T cell development in activated B and T cells, respectively. However, little is known about the molecular mechanisms of Bach2 regulation in response to signaling of cytokines and antigen. We show here that mammalian target of rapamycin (mTOR) controls Bach2 along B cell differentiation with two distinct mechanisms in pre-B cells. First, mTOR complex 1 (mTORC1) inhibited accumulation of Bach2 protein in nuclei and reduced its stability. Second, mTOR complex 2 (mTORC2) inhibited FoxO1 to reduce *Bach2* mRNA expression. Using expression profiling and chromatin immunoprecipitation assay, the *Ccnd3* gene, encoding cyclin D3, was identified as a new direct target of Bach2. A proper cell cycle was lost at pre-B and mature B cell stages in *Bach2*-deficient mice. Furthermore, AZD8055, an mTOR inhibitor, increased class switch recombination in wild-type mature B cells but not in *Bach2*-deficient cells. These results suggest that the mTOR-Bach2 cascade regulates proper cell cycle arrest in B cells as well as immunoglobulin gene rearrangement.

KEYWORDS B cells, cell cycle, immunoglobulin gene rearrangement, mTOR, transcription factor

B cells play critical roles for humoral immunity by generating antibody-secreting plasma cells. B cells undergo multiple stages of discrete intermediates. During their differentiation, signaling from cell-stage-specific receptors controls developmental events, such as DNA rearrangement of immunoglobulin genes. The interleukin-7 receptor (IL-7R) is required for the proliferation and survival of B cell progenitors as well as their differentiation to pro-B cells in part by activating phosphatidylinositol-3-OH kinase (PI3K)-AKT (1). In pro-B cells, a fluctuation of IL-7R signaling controls immunoglobulin heavy (IgH) chain rearrangement to produce the pre-B cell receptor (pre-BCR), which is composed of IgH, surrogate light chain, Ig α , and Ig β . Upon successful generation of pre-BCR, pro-B cells become large pre-B cells. When IL-7R signaling is attenuated in large pre-B cells, cells proceed to small pre-B cells, in which Ig light (IgL) chain rearrangement is dramatically induced by the activation of the SYK-BLNK-p38 pathway under pre-BCR signaling (2). Small pre-B cells become immature B cells upon successful rearrangement of IgL genes, and therefore expression of nonautoreactive BCR occurs, which then terminates further IgL rearrangement by a strong input into the PI3K-AKT cascade (3). The PI3K-AKT cascade further regulates responses of mature B cells. It represses class switch recombination (CSR), which produces the diversity of immunoglobulin genes in germinal center B cells (4). Thus, cell surface receptors and

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their downstream signaling cascades are essential for organizing differentiation along the entire B cell development. Considering that many of the cell surface receptors ultimately elicit cell responses by changing gene expression, it is critically important to understand transcription factors (TFs), the activities of which are regulated by the signaling pathways.

Among TFs required for B cell differentiation, FoxO1 has been well studied in terms of its regulation linking to signaling regulators. AKT directly phosphorylates FoxO1, resulting in its protein degradation (5). Importantly, PI3K-mTORC2-AKT mediates inactivation of FoxO1 protein by nuclear exclusion, contributing to cell proliferation in endothelial cells (6). In contrast to the negative regulation of FoxO1 by PI3K-AKT, FoxO1 phosphorylation by p38 kinase promotes the transcriptional activity of FoxO1 (7). The attenuation of IL-7R signaling in pro-B and pre-B cells activates FoxO1, resulting in the induction of *Rag1/2* mRNA, encoding enzymes required for the rearrangement of IgH and IgL genes (2, 8, 9). Other than FoxO1, little is known about TFs in B cells that are regulated by the PI3K-AKT pathway and control cell proliferation and/or differentiation.

The transcription factor Bach2 is required for efficient commitment of common lymphoid progenitor cells to the B cell lineage as well as CSR and somatic hypermutation of Ig genes in mature B cells. Bach2 promotes lymphoid lineage differentiation by repressing myeloid cell-related genes at the divergence of myeloid and lymphoid progenitors (10, 11). After being committed to lymphoid cells, Bach2 plays essential roles for differentiation of both T- and B-lymphoid cells. In T-lymphoid cells, Bach2 controls the differentiation into CD4- and CD8-positive effector lymphocytes (12, 13). In B-lymphoid differentiation, Bach2 is required for germinal center (GC) formation, CSR, and somatic hypermutation (14). Bach2 also inhibits plasma cell (PC) differentiation by repressing *Prdm1* expression (15, 16). These observations raise the possibility that activation and/or inactivation of surface receptors modulates Bach2 to alter gene expression and therefore the responses of B cells at these various differentiation stages. Supporting this possibility, we have recently revealed that the PI3K-AKT-mammalian target of rapamycin (mTOR) cascade phosphorylates Bach2 to reduce its activity. Among multiple phosphorylation sites of Bach2, phosphorylation at serine 535 (S535) prevents the nuclear localization of Bach2 protein (17). In addition, Bach2 has been shown to regulate the expressions of *Rag1* and *Rag2*, which are repressed by PI3K-AKT signaling in the context of the pre-BCR checkpoint (2, 18). These observations suggest that signaling-mediated regulation of Bach2 function might be used in both early B cell and PC differentiation. However, the molecular mechanism and significance of receptor-mediated regulation of Bach2 are still unclear. This study reports that signaling from stage-specific receptors of B cells (i.e., IL-7R and BCR) controls Bach2 at both transcript and protein levels, regulating immunoglobulin gene recombination in both early and late stages of B cell differentiation. The mTOR-Bach2 cassette is suggested to coordinate cell cycle arrest and DNA recombination reactions in pre-B and mature B cells.

RESULTS

Regulation of Bach2 level under receptor signaling associates with *Rag* gene expression in early B cell development. To clarify the response of Bach2 to the receptor signaling during early B cell development, we first examined the effect of IL-7R signaling upon Bach2. For this purpose, we used *Rag2*^{-/-} pro-B and *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells in which the strength of IL-7R signaling can be modified by changing the concentration of IL-7 in culture medium in the absence or presence of pre-BCR signaling (pro-B and pre-B cells, respectively) (2, 19–21). As reported previously, the withdrawal of IL-7 in *Rag2*^{-/-} pro-B cells induced the expression of *Rag1*, and it was immediately cancelled by restimulation with IL-7 for 2 h (Fig. 1A). The expression of *Bach2* was induced by IL-7 withdrawal, but it was not reduced with the restimulation with IL-7. At the protein level, IL-7 withdrawal did not affect the accumulation of Bach2, which was present mainly in a phosphorylated form, or FoxO1 (Fig. 1B). Whereas IL-7

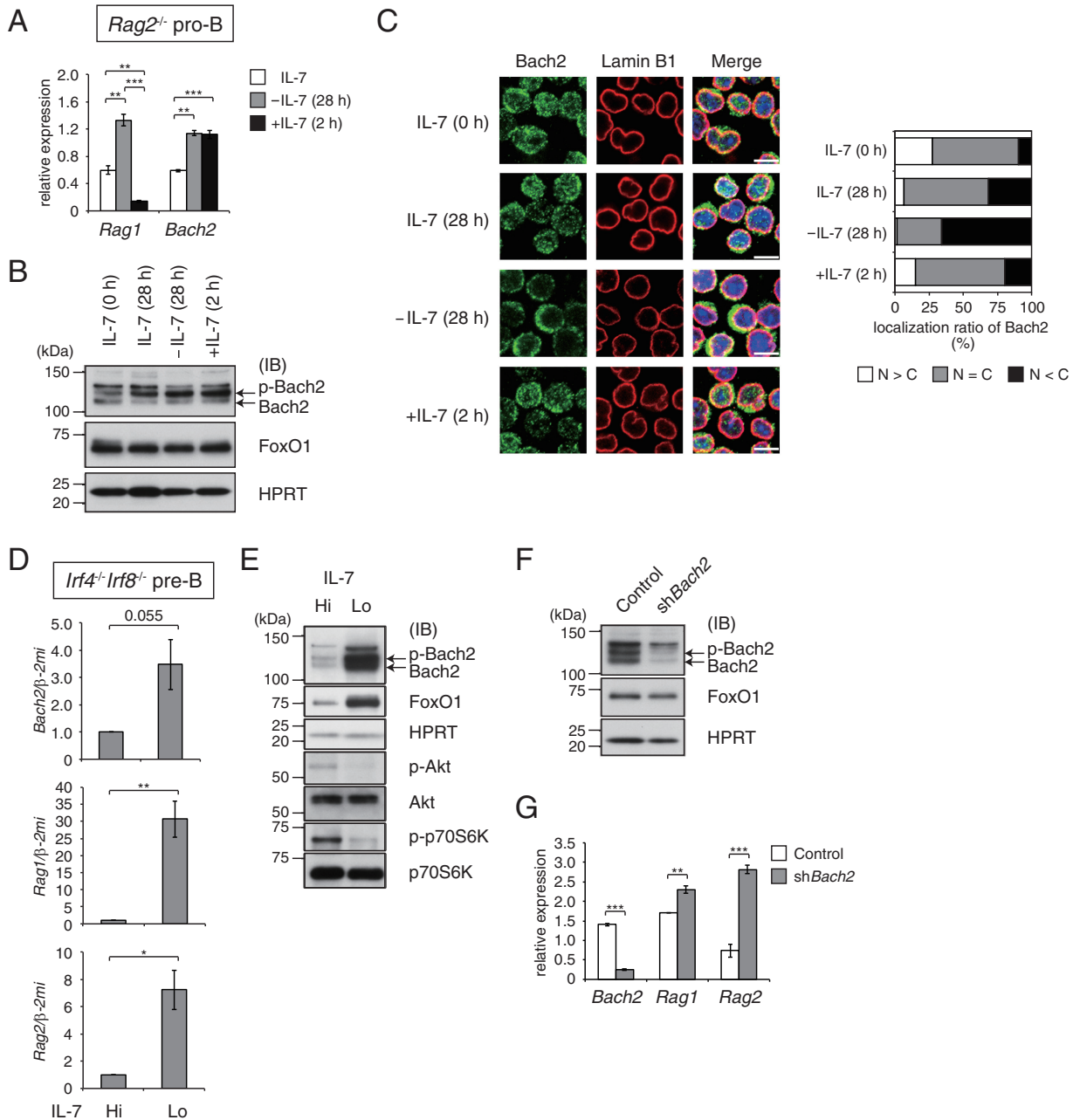


FIG 1 Bach2 negatively regulates expression of *Rag* genes in *Rag2*^{-/-} pro-B and *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells. (A to C) IL-7 was withdrawn for 28 h (-IL-7) from cultures of *Rag2*^{-/-} pro-B cells, and the cultures were restimulated for 2 h with IL-7 (+IL-7); shown are quantitative RT-PCR analysis of *Rag1* and *Bach2* expression (A), immunoblot analysis of Bach2 and FoxO1 (B), and immunohistochemistry for Bach2 protein (C). (C) Bach2 (green) and lamin B1 (red) distribution in cells (left); the subcellular localization of Bach2 was evaluated by classification of cells (*n* = 100) for each condition into three classes (right): cytoplasm dominant (*N* < *C*), nucleus and cytoplasm (*N* = *C*), and nucleus dominant (*N* > *C*). Bar, 10 μm. (D and E) Immunoblot analysis of Bach2, FoxO1, phosphorylated Akt (p-Akt), total Akt, p-p70S6K, and p70S6K (D) or quantitative RT-PCR analysis of *Bach2* and *Rag1/2* expression (E) in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells cultured with 5.0 ng/ml (Hi) or 0.1 ng/ml (Lo) of IL-7 for 48 h. (F and G) Immunoblot analysis of Bach2 and FoxO1 (F) or RT-PCR analysis of *Bach2* and *Rag1/2* expression (G) in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells transfected with a control vector (Control) or vector targeting Bach2 (*shBach2*). Then, cells were sorted on the basis of GFP expression and cultured under IL-7 Lo conditions for 48 h. For panels B, E, and F, HPRT served as an internal control. IB, immunoblot; p-Bach2, phosphorylated Bach2. For panels A, D, and G, relative expression was normalized by β₂-microglobulin (B2M) mRNA, and the mean expression and standard error of the mean (SEM) data were from three independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

withdrawal promoted the cytoplasmic accumulation of Bach2, restimulation of IL-7R clearly promoted the nuclear localization of Bach2 (Fig. 1C).

We next examined the effect of IL-7 withdrawal in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells. Compared with *Rag2*^{-/-} pro-B cells, the amounts of both mRNA and protein of Bach2

were markedly increased in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells by IL-7 withdrawal (Fig. 1D and E). Bach2 was present in phosphorylated and unphosphorylated forms. Nuclear accumulation of Bach2 was also promoted in these cells (see Fig. S1 in the supplemental material). The expressions of *Rag1/2* transcripts were also induced (Fig. 1D). We should note that while mRNA expression of *Bach2* was induced in pro-B and pre-B cells when IL-7 was low, the amount of protein was significantly increased only in pre-B cells. To examine the effect of Bach2 on *Rag1/2* expression, we performed knockdown of *Bach2* in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells (Fig. 1F). The expression of *Rag1/2* gene showed higher levels in cells with *Bach2* knockdown than in control cells (Fig. 1G). Bach2 might directly or indirectly repress *Rag1/2* expression in these cells.

mTOR complexes (mTORCs) regulate Bach2 expression and protein stability via phosphorylation at S535. In the above-described experiments, the expression of *Bach2* showed a pattern similar to that of *Rag* genes, which are direct targets of FoxO1. As expected, we identified FoxO1-binding peaks on the *Bach2* locus by examining previously reported chromatin immunoprecipitation sequencing (ChIP-seq) data (2, 18). Binding of FoxO1 to these regulatory regions was confirmed by ChIP-PCR (see Fig. S2A and B in the supplemental material), indicating *Bach2* as a direct target of FoxO1. Since IL-7R signaling is coupled to the PI3K-AKT-FoxO1 pathway, we examined the effect of the pharmacological inhibition of PI3K with LY294002 on Bach2 and FoxO1. In the presence of IL-7, the treatment with LY294002 of *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells resulted in only a slight accumulation of FoxO1 protein (Fig. 2A). As expected from this observation, the mRNA expression of FoxO1 targets *Bach2* and *Rag1/2* remained low in the presence of LY294002 compared with their expression under low IL-7 conditions (Fig. 2B) (2). Surprisingly, the amount of Bach2 protein substantially increased in response to LY294002 (Fig. 2A). Nuclear accumulation of Bach2 was enhanced under such conditions (see Fig. S3 in the supplemental material). We surmise that the PI3K pathway regulates protein stability and the subcellular localization of Bach2 in pre-B cells. mTORC1 may be involved in this regulation since it directly phosphorylates Bach2 (17).

To investigate this possibility, we used rapamycin, the specific inhibitor for mTORC1, or AZD8055, the inhibitor for both mTORC1 and mTORC2, in the presence of IL-7. Treatment of the cells with rapamycin resulted in Bach2 protein accumulation without affecting the FoxO1 protein amount or inducing *Bach2* mRNA (Fig. 2C and D). In contrast, AZD8055 treatment resulted in robust accumulation of Bach2 and FoxO1 proteins (Fig. 2C), with induction of both *Bach2* and *Rag1/2* mRNA (Fig. 2D). The treatments with these inhibitors enhanced nuclear accumulation of both Bach2 and FoxO1 (see Fig. S4A and B in the supplemental material). These findings were further verified by a biochemical fractionation of endogenous proteins in these cells, showing an increase of nuclear Bach2 in response to rapamycin (Fig. 2E). Based on these results, we suggest two distinct pathways regulating Bach2 under IL-7R. One is the direct, transcriptional regulation of *Bach2* gene by FoxO1 in pre-B cells, where FoxO1 activity is negatively regulated by mTORC2 in addition to PI3K-AKT. The other is the reduction of the amount and nuclear accumulation of Bach2 protein in pre-B cells mediated by the IL-7R-PI3K-mTORC1 cascade. The above-described observations also suggest an intimate association between cytoplasmic retention and protein stability of Bach2.

To examine whether the stability of Bach2 protein is regulated by the IL-7R-PI3K-mTORC1 cascade, the half-life of endogenous Bach2 protein in the pre-B cells in the absence of rapamycin was compared to that in the presence of rapamycin. The half-life of Bach2 was slightly increased in the presence of rapamycin, whereas that of FoxO1 was not affected (Fig. 3A). It should be noted that more of the faster-moving signal of Bach2 remained in the presence of rapamycin at 1 h after the chase than in the absence of rapamycin. To examine whether phosphorylation of Bach2 is involved in this regulation, we overexpressed wild-type Bach2 or a derivative of Bach2 with an S535A mutation, which we have reported recently as a critical phosphorylation site in mature B cells (17). Importantly, the slower-migrating band of phosphorylated Bach2 (p-Bach2) almost disappeared when Bach2S535A was expressed in these cells (Fig. 3B). Bach2S535A

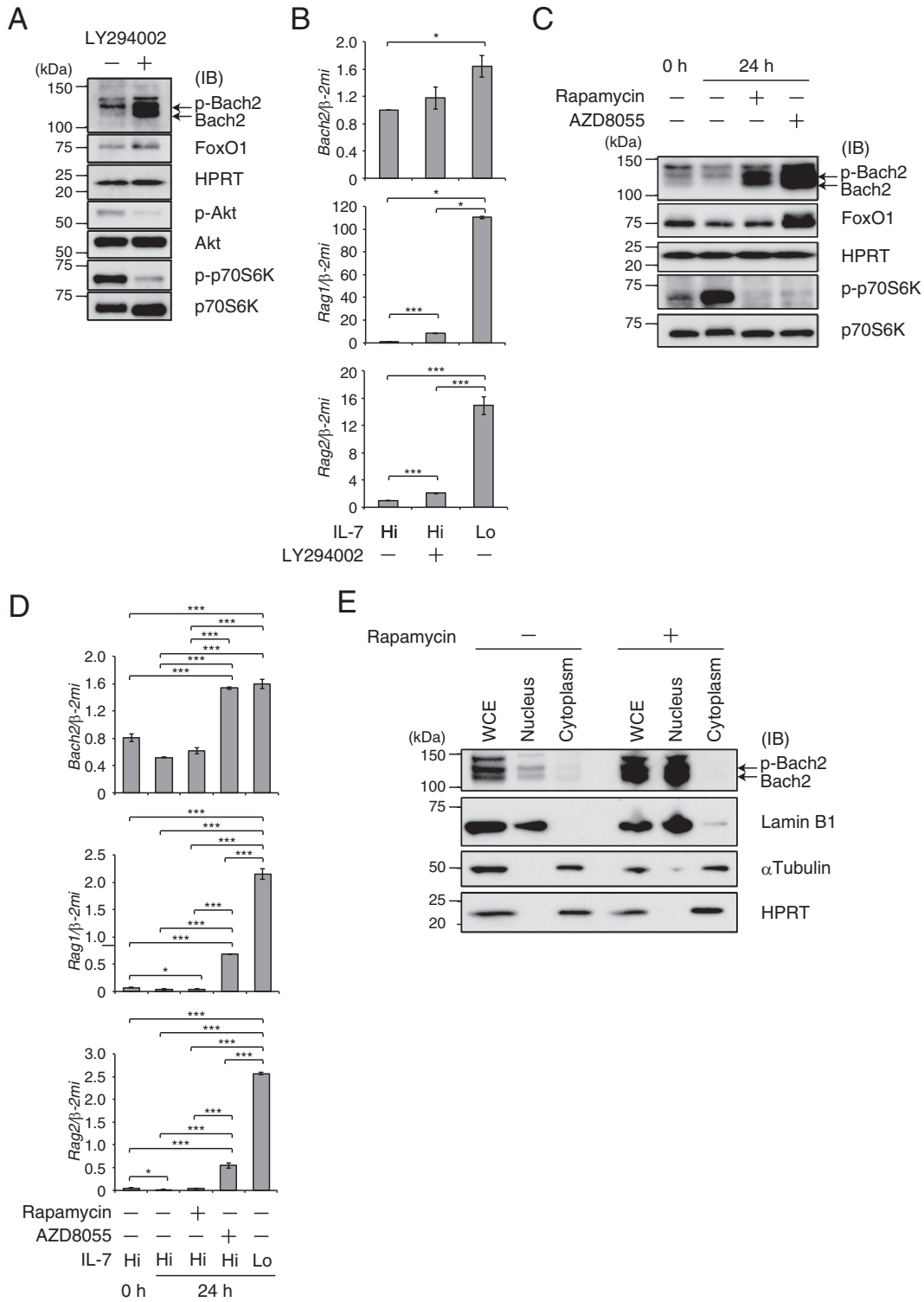


FIG 2 Presence of two distinct signaling pathways regulating Bach2 transcripts and protein. (A and B) Immunoblot analysis (A) or quantitative RT-PCR analysis (B) in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells cultured in the presence of LY294002 under IL-7 Hi conditions for 48 h. (C and D) Immunoblot analysis (C) or quantitative RT-PCR analysis (D) in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells cultured in the presence of rapamycin or AZD under IL-7 Hi conditions for 24 h. For panels A and C, HPRT served as an internal control. IB, immunoblot; p-Bach2, phosphorylated Bach2. For panels B and D, data under IL-7 Lo conditions for 24 h are shown as a control. Relative expression was normalized by B2M mRNA, and the expression and SEM data were from three independent experiments. *, *P* < 0.05; ***, *P* < 0.001. (E) Subcellular localization of Bach2 in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells cultured in the presence of rapamycin. Lamin B1 served as a control for the nuclear fraction, and α-tubulin and HPRT were used as controls for the cytoplasm fraction. WCE, whole-cell extract.

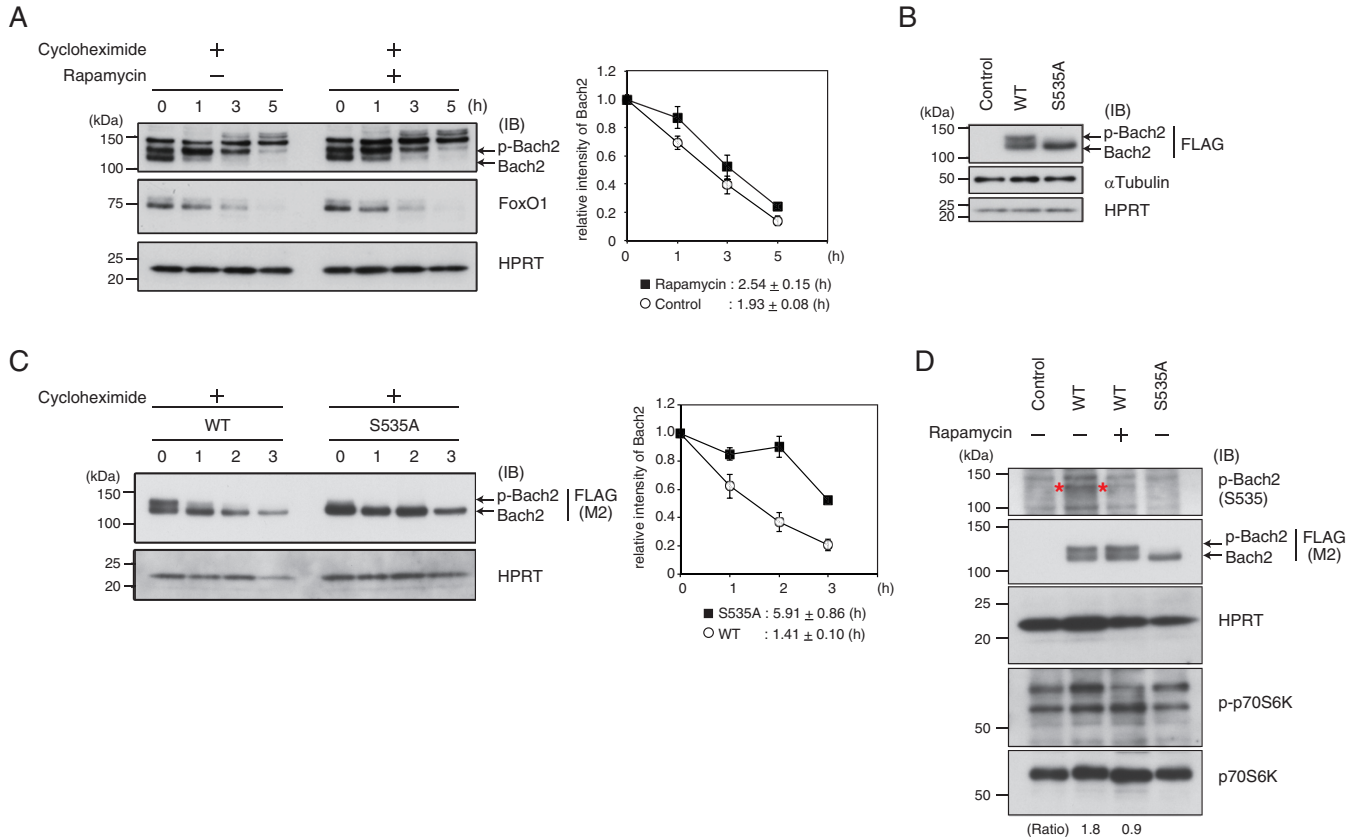


FIG 3 mTORC1-mediated Bach2 phosphorylation at S535 promotes cytoplasm localization and protein degradation. (A) Half-life of Bach2 protein in *lrf4*^{-/-} *lrf8*^{-/-} pre-B cells cultured with or without rapamycin in the presence of cycloheximide under IL-7 Hi conditions. (Left) Immunoblot analysis of Bach2 and FoxO1. (Right) Relative intensity of total Bach2 protein. The amounts of protein were normalized with HPRT. The means and SEM were from three independent experiments. (B) Immunoblot analysis in *lrf4*^{-/-} *lrf8*^{-/-} pre-B cells transduced with control vector or vectors expressing FLAG-tagged wild-type (WT) Bach2 or Bach2 S535A. (C) Half-life of WT Bach2 or Bach2 S535A. *lrf4*^{-/-} *lrf8*^{-/-} pre-B cells transduced with FLAG-tagged WT Bach2 or Bach2 S535A and cultured in the presence of cycloheximide under IL-7 Hi conditions. (Left) Immunoblot analysis of FLAG-Bach2. (Right) Relative intensity of FLAG-Bach2 protein, p-Bach2, and total Bach2. The amounts of proteins were normalized with HPRT. The means and SEM were from three independent experiments. (D) Immunoblot analysis in *lrf4*^{-/-} *lrf8*^{-/-} pre-B cells transduced as described for panel B and then cultured with or without rapamycin. Specific signals detected with anti-p-S535 antibody were marked with asterisks. The ratio was calculated as the division of p-Bach2 signal with anti-S535 antibody by signals detected with anti-FLAG of WT Bach2 with/without rapamycin. For panels A to D, HPRT or α -tubulin served as an internal control. Each immunoblot analysis was representative of three independent experiments. IB, immunoblot; p-Bach2, phosphorylated Bach2.

showed a significantly longer half-life than did wild-type Bach2 in the *lrf4*^{-/-} *lrf8*^{-/-} pre-B cells (Fig. 3C).

To further examine Bach2 phosphorylation, we generated a monoclonal antibody specifically recognizing Bach2 with phosphorylation of S535. Whole-cell extracts were prepared from Plat-E cells transfected with pcDNA wild-type Bach2 (WT) or Bach2 S535A, which showed two specific bands for WT Bach2 or one band for Bach2 S535A as previously reported (17). By using the monoclonal antibody, we detected two signals with WT Bach2-expressing cells (see Fig. S5 in the supplemental material). These signals were not detected with Bach2 S535A-expressing cells, suggesting that the monoclonal antibody detected Bach2 with phosphorylation of S535. We found that wild-type Bach2 overexpressed in the pre-B cells was phosphorylated at this residue, which was reduced upon the inhibition of mTORC1 (Fig. 3D). Combining these data with those of our previous report (17), we conclude that phosphorylation of Bach2 at S535 by mTORC1 promotes cytoplasmic retention of Bach2 as well as its degradation in pre-B cells.

The Bach2-Ccnd3 axis controls G₁ arrest during pre-B cell development. As the withdrawal of IL-7R signaling strictly regulated Bach2 expression in the pre-B cells, Bach2 might possess critical roles at this differentiation stage. To elucidate Bach2 function, we performed gene expression analysis by using *lrf4*^{-/-} *lrf8*^{-/-} pre-B cells transduced with control vector or Bach2 expression vector (Fig. 4A). Based on the

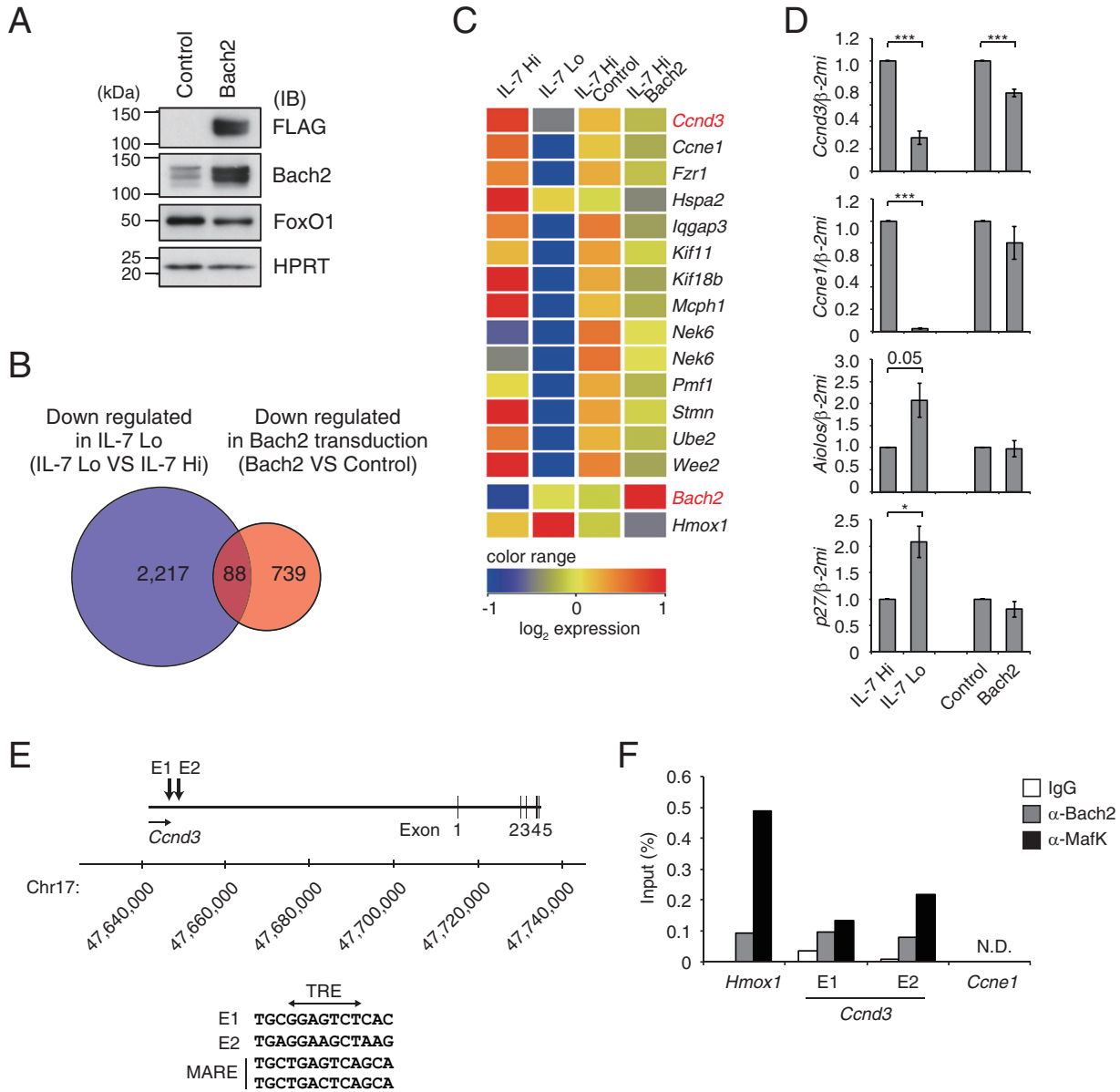


FIG 4 *Ccnd3* gene as a putative target of Bach2. (A) Immunoblot analysis of indicated proteins in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells transduced with control or Bach2 expression vector and then sorted based on GFP expression. HPRT served as an internal control. (B) Venn diagram of microarray analysis in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells comparing downregulated genes with IL-7 attenuation (blue) and Bach2 transduction with IL-7 Hi (red). Among 2,305 genes downregulated with IL-7 attenuation (21), 88 genes overlapped genes that were downregulated by Bach2 transduction. (C) Heatmap of cell cycle-related gene expression in a comparison of IL-7 Hi and IL-7 Lo conditions and of transduction of control vector versus Bach2. Transcripts of *Hmox1* were shown to be one of the direct targets of Bach2. (D) Quantitative RT-PCR analysis of *Ccnd3*, *Ccne1*, *Aiolos*, and *p27* in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells as described for panel C. Relative expression was normalized by β2M, and the means and SEM were from three independent experiments. *, *P* < 0.05; ***, *P* < 0.001. (E) MARE-like sequences E1 and E2 at *Ccnd3* locus. Bach2 binding sequences detected in these regions were shown along with MARE consensus. E, enhancer. (F) Binding of Bach2 and MafK to regulatory elements in the *Hmox1*, *Ccnd3* E1 and E2, and *Ccne1* loci in cells cultured with 0.1 ng/ml of IL-7 for 48 h, assessed by ChIP with control IgG, anti-Bach2, or anti-MafK. The data are the averages from three independent experiments. N.D., not detected.

expression of *Hmox1*, which is a well-known Bach2 target gene (22), we selected 827 genes downregulated more than *Hmox1* (i.e., 1.3-fold) in the Bach2-transduced cells compared to the control (Fig. 4B). These data were coanalyzed with previously published data sets of genes downregulated with the withdrawal of IL-7 (2), resulting in the identification of 88 common genes (Fig. 4B; see also Table S1 in the supplemental material). Among these genes, 13 genes were related to the cell cycle progression (Fig. 4C; see also Table S2 in the supplemental material). Consistent with this, the expres-

sions of *Ccnd3* and *Ccne1* were significantly repressed in *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells with IL-7 withdrawal (Fig. 4D), and *Bach2* overexpression in these cells resulted in reduced expression of *Ccnd3* but not *Ccne1* (Fig. 4D). The expression of *Aiolos*, a known repressor of *Ccnd3* (23), was not altered upon *Bach2* transduction. Importantly, we discovered several Maf recognition element (MARE)-like sequences, which could be bound by *Bach2* and small Maf heterodimer, near the *Ccnd3* locus (Fig. 4E). Bindings of both *Bach2* and its partner MafK to these regions were confirmed by using quantitative ChIP-PCR (Fig. 4F). Therefore, we suggest *Ccnd3* as a direct target gene of *Bach2*.

Cell cycle arrest is required for IgL rearrangement in pre-B cells (23, 24). Since an inhibition of cyclin D3 expression is expected to cause G₁ arrest (24), we examined the importance of the *Bach2*-*Ccnd3* axis for cell cycle arrest during pre-B cell differentiation. By using *Bach2*-RFP mice (where RFP is red fluorescent protein) (10), pre-B cells in the bone marrow were sorted into *Bach2*-RFP high (*Bach2*^{hi}) and low (*Bach2*^{lo}) fractions based on the expression of RFP (Fig. 5A). The *Bach2*^{hi} population was larger in small pre-B cells than in large pre-B cells (Fig. 5A, upper panels). Intracellular staining of FoxO1 protein showed that its amount was higher in the *Bach2*^{hi} population than in the *Bach2*^{lo} population (see Fig. S6 in the supplemental material), confirming the regulatory relationship between FoxO1 and *Bach2* gene expression. Small pre-B cells undergo cell cycle arrest (3). Surprisingly, an *in vivo* BrdU labeling experiment revealed that *Bach2*^{hi} small pre-B cells but not *Bach2*^{lo} cells underwent cell cycle arrest (Fig. 5A, lower panels). The expressions of genes in *Bach2*^{hi} and *Bach2*^{lo} small pre-B cells were compared. A large reduction of *Ccnd3* expression was observed in the *Bach2*^{hi} population compared with the *Bach2*^{lo} population (Fig. 5B). Importantly, the expression of the *Rag1* gene was induced in this population, whereas *Rag2* expression was similar in these cells (Fig. 5B). Thus, these results suggest that *Bach2*^{hi} small pre-B cells undergo cell cycle arrest and IgL rearrangement, due in part to reduced expression of *Ccnd3* and increased expression of *Rag1*.

To investigate the requirement of *Bach2* in cell cycle arrest of small pre-B cells, we performed cell cycle analysis in wild-type and *Bach2*-deficient B cells during B cell development. Compared with wild-type cells, *Bach2*-deficient small pre-B cells failed to arrest at G₁ phase, and more of the mutant cells than wild-type cells were in the S phase (Fig. 5C). *Bach2*-deficient pro-B cells also showed an increase in the ratio of cells in S phase (Fig. 5C). Interestingly, a larger fraction of *Bach2*-deficient immature and mature B cells entered S phase than wild-type cells. These results suggest that *Bach2* is an important regulator of cell cycle arrest in B cells.

The expressions of genes related to cell cycle and recombination were compared between wild-type and *Bach2*-deficient pre-B cells. In wild-type cells, the expression of *Ccnd3* was lower in small pre-B cells than large pre-B cells, which was again consistent with the cell cycle arrest of the small pre-B cells. *Ccnd3* mRNA was unexpectedly lower in *Bach2*-deficient large pre-B cells than in wild-type cells, and unlike what was seen in wild-type cells, it was not further reduced in small pre-B cells (Fig. 5D). These alterations in *Bach2*-deficient pre-B cells suggest that cell cycle regulators other than *Ccnd3* are also deregulated in the absence of *Bach2*. In contrast, the expression of *Rag1* was normally induced in *Bach2*-deficient small pre-B cells, whereas *Igk* transcript tended to be higher in *Bach2*-deficient small pre-B cells than in wild-type cells. These observations together suggest that a proper coordination of cell cycle arrest and immunoglobulin gene rearrangement in pre-B cells was lost in the absence of *Bach2*.

Bach2 controls G₁ arrest in mature B cells. Mature B cells remain dormant until they encounter a cognate antigen. To examine whether this dormancy is affected by *Bach2* deficiency, we compared gene expression in splenic naive B cells purified from wild-type and *Bach2*-deficient splenic B cells and found that the expression of *Prdm1* was derepressed in *Bach2*-deficient B cells in accord with our previous reports (Fig. 6A and B) (15, 16). Surprisingly, we found that the expression of *Rag1* and *Rag2* was also upregulated in mature B cells, in which these genes are not normally induced (25). *Bach2*-deficient B cells also showed a slight upregulation of *Ccnd3* as well as a striking

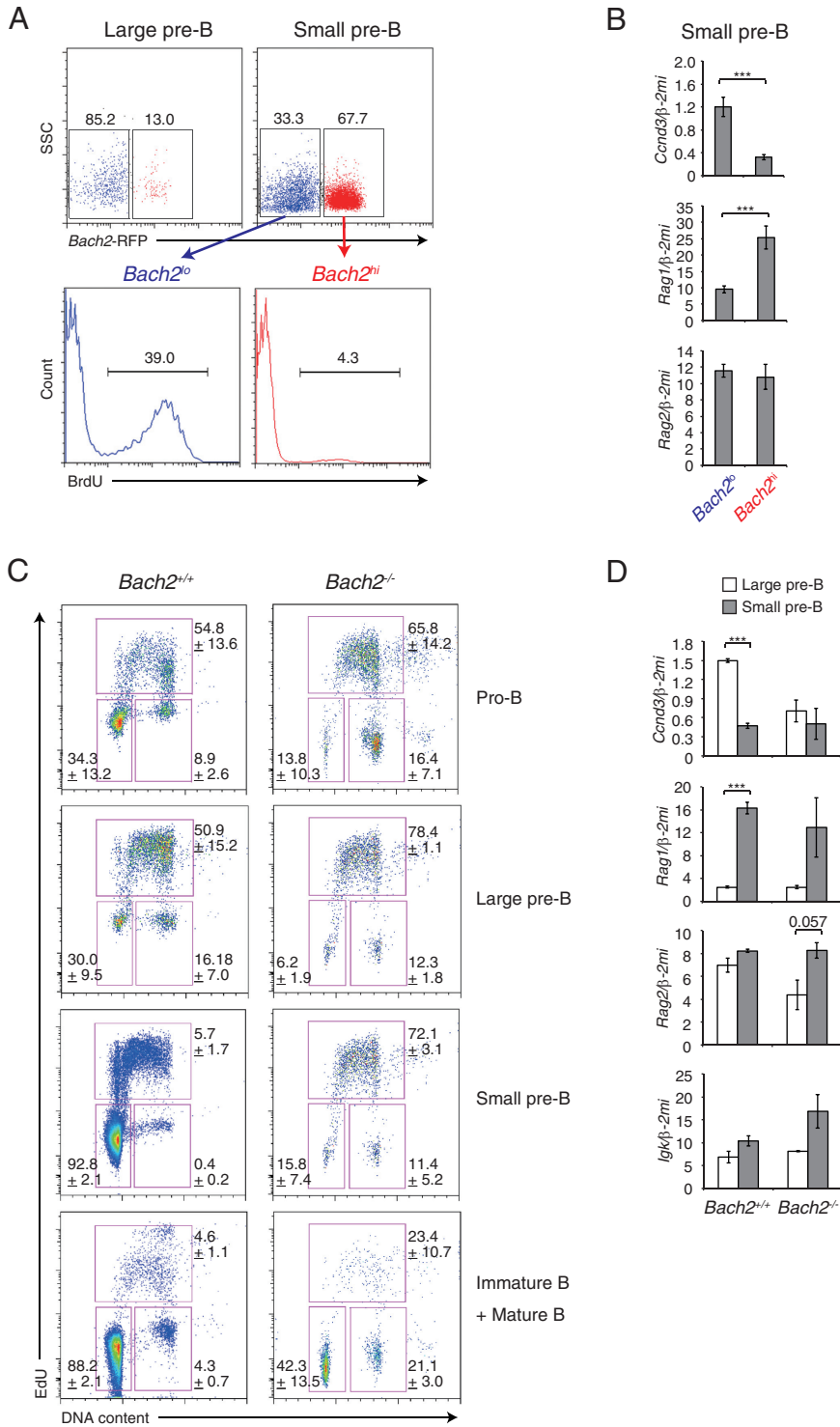


FIG 5 Bach2 is required for proper G₁ cell cycle arrest during B cell development. (A) Flow cytometry analysis of *Bach2* transcripts in bone marrow pre-B cells of *Bach2*-RFP mice. Data are representative of results for four mice in one experiment. (B) Quantitative RT-PCR analysis of *Ccnd3*, *Rag1*, and *Rag2* in small pre-B cell fractions sorted by RFP expression. *Bach2*^{hi} and *Bach2*^{lo}, *Bach2*-RFP high and low, respectively. (C) Cell cycle analysis by using flow cytometry in bone marrow-derived pro-B, large pre-B, small pre-B, and immature and mature B cells from wild-type (*Bach2*^{+/+}) and *Bach2*-deficient (*Bach2*^{-/-}) mice. Data are representative of results for three mice for each genotype in one experiment and are shown as means and SEM. (D) Quantitative RT-PCR analysis of *Ccnd3*, *Rag1*, *Rag2*, and *Igk* transcripts comparing results for large pre-B cells and small pre-B cells from wild-type or *Bach2*-deficient mice. One experiment was performed using three mice for each genotype. For panels B and D, relative expression was normalized by B2M, and the means and SEM were from four (B) or three (D) mouse samples. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

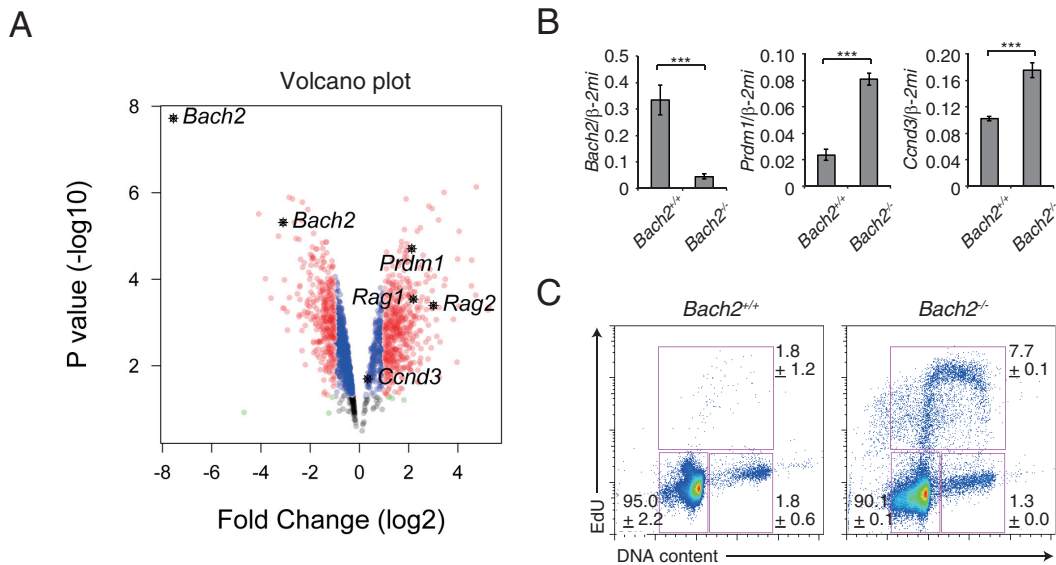


FIG 6 Abnormal regulation of cell cycle in *Bach2*-deficient splenic B cells. Comparison analysis of splenic B cells from wild-type or *Bach2*-deficient mice. (A) Volcano plot for microarray data. (B) Quantitative RT-PCR analysis of *Bach2*, *Ccnd3*, and *Prdm1* compared in splenic B cells from wild-type or *Bach2*-deficient mice. Relative expression values were normalized by B2M, and the means and SEM were from three mice for each genotype in one experiment. ***, $P < 0.001$. (C) Cell cycle analysis by using flow cytometry. Data are representative of two independent experiments using one or two mice, totalling three mice for each genotype, and shown as means and SEM.

increase in the ratio of cells in S phase (Fig. 6). Of note, while the number of mature B cell is reduced in *Bach2*-deficient mice (14), we found a larger number of apoptotic cells in *Bach2*-deficient mature B cells (see Fig. S7 in the supplemental material). Therefore, these results suggest that Bach2 functions in properly promoting G₁ arrest in not only pre-B cells but also mature B cells.

The mTOR-Bach2 axis controls CSR under the BCR signaling cascade. A portion of mature B cells undergo CSR along the way to PC differentiation in response to antigen stimulation, and the frequency of CSR is reduced by the PI3K and mTORC pathways (4, 26). To clarify the importance of Bach2 as a regulatory target of these pathways in the regulation of CSR and PC differentiation, we used splenic B cells isolated from BCR-transgenic mice (27), which can be induced to undergo PC differentiation accompanied with IgG1-type CSR *ex vivo*, with a higher efficiency than that of wild-type cells (K. Ochiai, submitted for publication). Upon BCR stimulation with NP-Ficoll *ex vivo*, mTOR was immediately activated, and FoxO1 was phosphorylated in these cells (Fig. 7A). Moreover, the two specific bands of Bach2 were shifted to the slower-migration form, indicating an increased phosphorylation of Bach2. At this time point, the inhibition of mTOR by using rapamycin or AZD8055 blocked the activation of mTOR as judged by the reduction of phosphorylation of p70 S6K. Under these conditions, phosphorylation of FoxO1 was effectively inhibited by AZD8055 but not rapamycin. These observations were similar to the results in pre-B cells, indicating that mTORC2 phosphorylated and inhibited FoxO1 in mature B cells. Two bands of Bach2 were detected with the anti-phosphorylated S535 antibody upon BCR stimulation, and these were reduced only with AZD8055 in a manner similar to that of FoxO1 (Fig. 7A). These observations suggest that both faster and slower bands include Bach2 with phosphorylation at S535. Next, Bach2 protein accumulation was examined by immunohistochemistry. Localization of Bach2 tended to shift from nucleus to cytoplasm by BCR stimulation (Fig. 7B). Treatment with rapamycin or AZD8055 resulted in enhanced nuclear localization of Bach2. Next, we examined the stability of Bach2 protein in these cells. The half-life of endogenous Bach2 protein in the absence of AZD8055 was compared to that in the presence of AZD8055. The faster-migrating Bach2 band disappeared within 1.5 h in the absence of AZD8055, whereas it remained in the

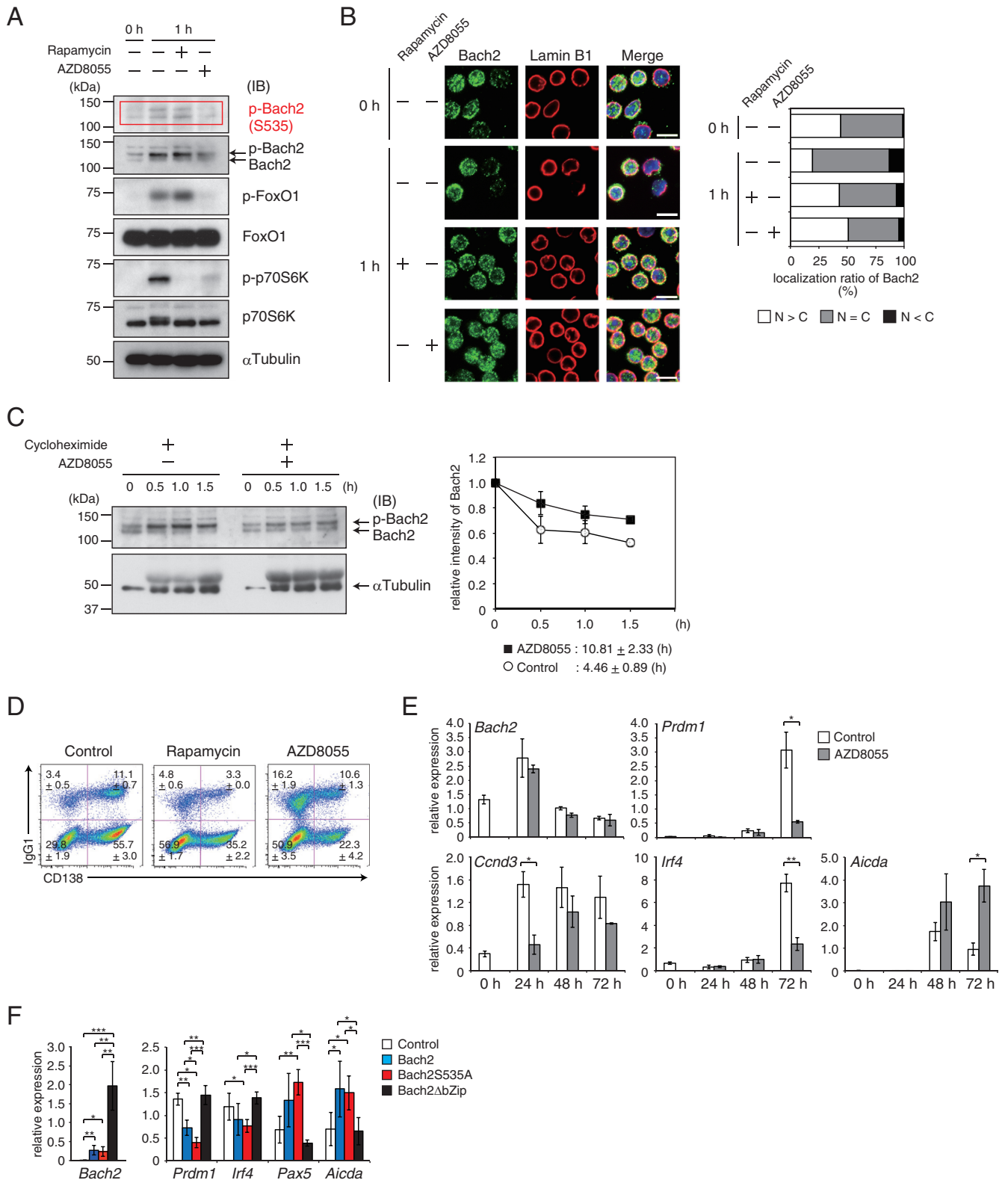


FIG 7 Decision of CSR and PC differentiation via the mTORC-Bach2 cascade. Splenic B cells were isolated from B1-8^{hi} mice and stimulated with IL-2, IL-4, IL-5, CD40L, and NP-Ficoll. Cells were cultured for 1 h in the presence of rapamycin or AZD8055 (A and B). (A) Immunoblot analysis for phosphorylated Bach2 at S535 (p-Bach2), total Bach2, phosphorylated FoxO1 (p-FoxO1), FoxO1, p-p70S6K, and p70S6K. α -Tubulin served as an internal control. (B) Immunohistochemistry for Bach2 protein. (Left) Bach2 (green) and lamin B1 (red) distribution in cells. (Right) The subcellular localization of Bach2 was evaluated by classification of 100 cells as described for Fig. 1C. Bar, 10 μ m. (C) Half-life of Bach2 protein. Cells were cultured with or without AZD8055 in the presence of cycloheximide under IL-7 Hi conditions. (Left) Immunoblot analysis of Bach2. (Right) Relative intensity of total Bach2 protein. The amounts of protein were normalized with α -tubulin. Data are from one experiment using three mice and are shown as means and SEM. (D) Flow-cytometric analysis of CD138 and IgG1. Data are representative

(Continued on next page)

presence of AZD8055 (Fig. 7C). Importantly, the half-life of Bach2 was prolonged in the presence of AZD8055 (Fig. 7C). These observations suggest that, similar to IL-7R signaling in pre-B cells, the stability of Bach2 protein is controlled by mTOR downstream of the BCR signal. BCR-mediated activation of mTOR promoted phosphorylation of Bach2 and its cytoplasmic retention and degradation, which are together expected to result in inactivation of Bach2 during PC differentiation.

The inhibition of only mTORC1 by rapamycin reduced the percentages of both IgG1-positive and CD138-positive cells (Fig. 7D), suggesting that mTORC1 activity is required for both CSR and PC differentiation. In contrast to rapamycin, the inhibition of mTORC1/2 by AZD8055 resulted in a higher frequency of IgG1-positive cells and a reduction of CD138-positive cells (Fig. 7D). To investigate whether Bach2 is involved in the effects of AZD8055, we compared wild-type and *Bach2*-deficient B1-8^{hi} splenic B cells by staining for IRF4, CD138, and IgG1. While AZD8055 decreased the percentages of IRF4- and CD138-positive plasma cells among wild-type cells, it did not substantially affect plasma cell differentiation of *Bach2*-deficient cells (see Fig. S8A in the supplemental material). FoxO1 protein was expressed at the same level in wild-type and *Bach2*-deficient cells (see Fig. S8B in the supplemental material). Therefore, these results indicated that Bach2 was required for the effects of AZD8055.

Upon examining mRNA expression, we found reduced expression of *Prdm1* and *Irf4* with AZD8055 (Fig. 7E), which was consistent with the inhibition of PC differentiation. While the expression of *Aicda* was reduced by 72 h after stimulation in control cells, it was sustained at a higher level at 72 h in the presence of AZD8055 (Fig. 7E). Importantly, the expression of *Ccnd3* was reduced transiently at 24 h with AZD8055. Since the expression of *Bach2* mRNA was not largely affected by the inhibition of mTORC1/2 (Fig. 7E), these effects appeared at least in part due to the nuclear accumulation of Bach2 protein (Fig. 7B). Consistent with this interpretation, transduction of these cells with retroviruses expressing wild-type Bach2 or Bach2 S535A resulted in expected alterations in gene expression. The expression of *Prdm1* was reduced by both wild-type Bach2 and Bach2 S535A, with the latter showing a stronger effect (Fig. 7F). Both wild-type Bach2 and Bach2 S535A-transduced cells also reduced the expression of *Irf4* and increased that of *Pax5* and *Aicda*. However, there was no significant difference in their effects. These effects were not observed with a transduction of Bach2ΔbZip, which cannot bind to DNA. These results strongly support the idea that the BCR-mTOR cascade controls CSR and PC differentiation by targeting Bach2 protein.

DISCUSSION

In this study, we explored Bach2 regulation under receptors that couple to mTOR signaling in B cells (Fig. 8). By using *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells, we found that mTORC1 inhibited Bach2 protein accumulation in the nucleus and promoted its degradation. As our previous report indicated, withdrawal of IL-7 signaling activated FoxO1 (2), which promoted the expression of *Bach2* mRNA (Fig. 1D). Inhibition of PI3K was insufficient to promote FoxO1 activity in the presence of IL-7, whereas inhibition of mTORC1 and mTORC2 resulted in FoxO1 activation (Fig. 2C and D). Considering that inhibition of mTORC1 did not promote FoxO1 activation (Fig. 2C and D), IL-7 signaling may associate with mTORC2-FoxO1 regulation in a PI3K-independent manner. Thus, the IL-7R-mTOR cascade regulates Bach2 at both transcriptional and posttranslational steps in B cells. Distinct from these responses in pre-B cells, Bach2 seems to be unresponsive to attenuation of IL-7R signaling in pro-B cells (Fig. 1). This difference between pro-B and pre-B cells may be due to the absence or presence of pre-BCR and other signaling

FIG 7 Legend (Continued)

of three mice analyzed in one experiment and are shown as the means and SEM. (E) Quantitative RT-PCR analysis of *Bach2*, *Prdm1*, *Ccnd3*, *Irf4*, and *Aicda* for the indicated time points in the presence of AZD. One experiment was performed using three mice. (F) Quantitative RT-PCR analysis of *Bach2*, *Prdm1*, *Irf4*, *Pax5*, and *Aicda* in cells transduced with control, WT Bach2, Bach2 S535A, or Bach2ΔbZip expression vectors and sorted based on GFP expression. One experiment was performed using four mice. For panels A and C, α -tubulin served as an internal control. IB, immunoblot. For panels D and F, relative expression was normalized by B2M, and the means and SEM were from three (E) or four (F) mouse samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

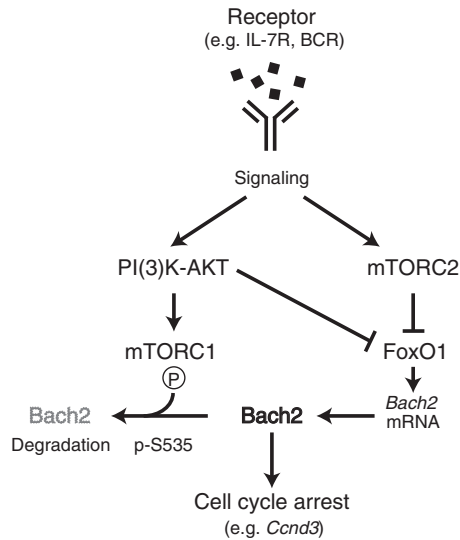


FIG 8 Schematic representation of mTOR-Bach2 cascade in pre-B and mature B cells. Signaling from receptor couples to mTORCs, and Bach2 is regulated under each mTORC1 and mTORC2 at the protein level and transcript level, respectively. mTORC1 mediates Bach2 phosphorylation of serine at 535, which promotes cytoplasmic retention and degradation of Bach2 protein. FoxO1, which is negatively regulated by mTORC2, directly activates *Bach2* expression. Therefore, attenuation of receptor signaling reduces mTOR activity, and it enables Bach2 to control the cell cycle by targeting specific genes, such as *Ccnd3*. Our data suggest that the mTOR-Bach2 cascade functions in pre-B cells with IL-7R and in mature B cells with BCR.

pathways, such as MEK/extracellular signal-regulated kinase (ERK), which are also regulated under pre-BCR. In our recent report, we detected multiple phosphorylation sites on Bach2 protein, which include candidate MEK/ERK consensus sites (observation from reference 17). It is possible that a combination of multiple phosphorylations of Bach2 protein regulates the activity of Bach2. Along B cell differentiation, Bach2 is essential for promoting CSR via inhibiting *Prdm1* expression (15). While the occurrence of CSR is controlled under the mTORC2-FoxO1 cascade (26), it is not clear whether FoxO1 directly regulates the expression of mRNA required for CSR. Importantly, we elucidated that the mTOR-Bach2 cascade controlled CSR. Transduction of Bach2 S535A resulted in a lower expression of *Prdm1* than that of wild-type Bach2 (Fig. 7F). Importantly, AZD8055 increased CSR in wild-type B cells but not in *Bach2*-deficient B cells, indicating that Bach2 is a critical effector of mTORC2 for the regulation of CSR. These observations together suggest that FoxO1 may regulate CSR in part by activating the expression of Bach2 in B cells. Thus, the mTORC-Bach2 cascade regulates both pre-B cell differentiation and mature B cell activation.

Prdm1 has been well established as a direct target gene of Bach2 in B cells (15–17). In this study, we newly identified *Ccnd3* as a direct target gene of Bach2 (Fig. 4), which is known to promote cell cycle transition from G₁ to S phase. We showed that *Bach2* expression was associated with the reduction of *Ccnd3* expression as well as cell cycle arrest in *Bach2*-RFP pre-B cells (Fig. 5A and B). Furthermore, *Bach2*-deficient B cells in the bone marrow and spleen showed entry to S phase at stages where normally cells are stalled at G₁ phase (Fig. 5C and 6C). *Ccnd3* expression was upregulated in *Bach2*-deficient splenic B cells. These observations indicate that the repression of *Ccnd3* by Bach2 contributes to G₁ arrest along the course of B cell development. We should note that *Ccnd3* expression was lower in *Bach2*-deficient large pre-B cells than in wild-type cells, and it showed no further reduction in small pre-B cells. Genetic deficiency of *Bach2* might have an effect on the basal level of *Ccnd3* expression in pre-B cells. Thus, we surmise that Bach2 controls proper cell cycle arrest along B cell development by regulating not only *Ccnd3* but also additional regulatory genes of the cell cycle.

During B cell differentiation, DNA rearrangement of immunoglobulin genes is

caused by Rag1 and Rag2 in pro-B and pre-B cells. Bach2 regulates the expression of these genes as well. A previous report showed an activation of *Rag1/2* gene by Bach2 in *BCR-ABL*-transformed pre-B cells (18). However, our data showed both a positive and a negative correlation between Bach2 and *Rag1/2* expression. As positive correlation, we observed higher expression of *Rag1* expression in the *Bach2*^{hi} pre-B fraction (Fig. 5B). As negative correlation, less nuclear Bach2 localization was accompanied with more *Rag1* expression in *Rag2*^{-/-} pro-B cells (Fig. 1A and C). In addition, *Rag1/2* expression was upregulated in *Bach2* knockdown *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells as well as *Bach2*-deficient mature B cells (Fig. 1G and 6A). Such contradicting observations might be caused by the modulation of Bach2 by different signaling pathways under receptors. In addition to mTOR, various signaling molecules may tune the interactions of Bach2 with mediators or cofactors, resulting in both activator and repressor functions. Therefore, Bach2 might control *Rag1/2* expression in both activation and repression depending on the situations.

We presented the contribution of the mTOR-Bach2 cascades along B cell differentiation. Since recent studies have revealed the importance of Bach2 not only in B cells but also in the differentiation of multipotent progenitors, common lymphoid progenitors, and T-lymphoid cells (10, 13, 28), this cascade may be used in a reiterative manner to control proliferation and differentiation of hematopoietic cells.

MATERIALS AND METHODS

Mice. Mice used in this study were described previously: *Bach2*-deficient mice (14), *Bach2*-RFP mice (10), and BCR transgenic B1-8^{hi} mice (27). All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Tohoku University Environmental & Safety Committee.

Cells and culture conditions. *Rag2*^{-/-} pro-B cells and *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells were cultured as described previously (2, 19). Pharmacological inhibitors were from Calbiochem: PI3K inhibitor LY294002, mTORC1 inhibitor rapamycin, and mTORC1/2 inhibitor AZD8055. For experiments using *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells, 5 μ M LY294002, 20 nM rapamycin, or 500 nM AZD8055 was supplemented in culture medium. For experiments using splenic B cells, cells were isolated as described previously (29). B1-8^{hi} splenic B cells were stimulated with IL-2 (100 U/ml), recombinant mouse IL-4 (5 ng/ml), recombinant mouse IL-5 (1.5 ng/ml), recombinant mouse CD40 ligand (0.2 ng/ml) (all R&D Systems), and NP(40)-Ficoll (0.01 ng/ml; Biosearch Technologies Inc.), and 0.5 nM rapamycin or 10 nM AZD8055 was supplemented in culture medium.

Retroviral vectors and transduction. Retroviral vectors encoding mouse WT Bach2 (10) or Bach2 S535A (17) with an amino-terminal FLAG tag and green fluorescent protein (GFP) have been reported previously. Retroviral vectors encoding mouse Bach2 Δ bZip (in this study) were constructed by subcloning of cDNA segments from pcDNA3 Bach2 Δ bZip (30). Plasmids encoding short hairpin RNA oligonucleotides were constructed as described previously (2). The oligonucleotide sequence for short hairpin RNA directed against *Bach2* mRNA was 5'-GGGTGCTAAACTTCTACCAAAC-3'. The generation of retroviral stocks was as described previously (2). Cells were suspended in retroviral supernatant supplemented with Polybrene (4 μ g/ml) and were centrifuged for 90 min at 2,500 rpm at 32°C. After centrifugation, cells were incubated for 3 h at 37°C and then were washed and cultured under the appropriate conditions. After 48 h, transduced cells were sorted on the basis of GFP expression.

RT-PCR. For reverse transcription (RT)-PCR, total RNA was prepared by using the RNeasy Plus minikit (Qiagen) or the RNeasy Plus microkit (Qiagen) according to the manufacturer's instructions, and cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen). The SYBR qPCR master mix (Clontech) and Applied Biosystems 7500 Fast real-time PCR system were used for quantitative PCR (for a list of primers, see Table S3 in the supplemental material).

Immunoblot analysis and immunohistochemistry. Protein extracts were prepared as described previously (2). Each sample was separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore) and examined by immunoblot analysis as described previously (17). The primary antibodies used were as follows: anti-Bach2 (N1; homemade) and anti-phosphorylated S535 of Bach2 (#17; homemade); anti-FoxO1 (2880), anti-Akt (9272), and anti-p70 S6 kinase (9202) (all from Cell Signaling); antibody to phosphorylated FoxO1 (9461), Akt (4058), and p70 S6 kinase (9205) (all from Cell Signaling); anti-lamin B1 (M-20) (sc-6217), anti-hypoxanthine phosphoribosyltransferase (anti-HPRT) (FL-218) (sc-20975), and anti- α -tubulin (B-7) (sc-5286) (all from Santa Cruz); and anti-FLAG M2-peroxidase (A8592; Sigma). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, HRP-conjugated anti-goat IgG, and HRP-conjugated anti-mouse IgG (all from GE Healthcare). Fractionated proteins were isolated by using a subcellular protein fractionation kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. For determination of the half-life of Bach2 protein, bands obtained from immunoblots were scanned and measured by using ImageJ software and normalized by that of HPRT. Immunohistochemistry was performed by using anti-Bach2 (N1) or anti-FoxO1 (9461; Cell Signaling) or anti-lamin B1 (sc-6217; Santa Cruz) as described previously (2).

Flow cytometry. Cells were washed in staining buffer and phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.05% sodium azide and were blocked with Fc receptor (2.4G2; Tombo Bioscience). Cells were stained with biotin- or fluorescence-coupled antibodies in staining buffer using standard procedures. Anti-mouse mouse antibodies specific for B220 (RA3-6B2), CD43 (S7), IgM (II/41), IgG1 (A85-1), and CD138 (281-2) were from BD Pharmingen. Each population of B cell differentiation stage is gated on the basis of surface marker as follows: pro-B (B220⁺ CD43^{hi} IgM⁻), pre-B (B220⁺ CD43^{lo} IgM⁻), immature and mature B in bone marrow (B220⁺ CD43^{lo} IgM⁺); and large and small pre-B cells were gated with side scatter (SSC) and forward scatter (FSC) for cell size. Mature B cells in spleen were gated with B220⁺. For intracellular staining, washed cells were then fixed by incubation for 10 min at 25°C with 1% paraformaldehyde and permeabilized in staining buffer containing 0.03% saponin (Fluka). Staining buffer containing 0.3% saponin was used to incubate the cells with normal rabbit IgG (sc-2027; Santa Cruz) or anti-FoxO1 antibody (sc-11350; Santa Cruz) or anti-IRF4 (sc-6069; Santa Cruz) in the presence of 5% donkey serum (Jackson ImmunoResearch). Labeling was detected using a Cy5-coupled donkey anti-goat antibody (Jackson ImmunoResearch). For cell cycle analysis, the Click-iT EdU Alexa Fluor 488 flow cytometry assay kit (C10425; Thermo Fisher) was used according to the manufacturer's instructions. Data were collected with the FACSCanto II system and analyzed with FlowJo software (TreeStar).

Microarray analysis. Total RNAs were prepared and labeled with Cy3 by using the low input quick amp labeling kit (Agilent). A SurePrint G3 mouse GE microarray slide was used according to the manufacturer's instructions (Agilent). The data were detected on an Agilent scanner. Data were analyzed with the GeneSpring software package (Agilent).

ChIP assay. ChIP was performed as previously described (2). Chromatin from *Irf4*^{-/-} *Irf8*^{-/-} pre-B cells was isolated and then sonicated to obtain DNA fragments ranging in size from 100 to 500 bp. Chromatin fragments were immunoprecipitated with normal rabbit IgG (sc-2027; Santa Cruz), anti-FoxO1 (sc-11350; Santa Cruz), anti-Bach2 antiserum (N1; homemade), or anti-MafK (sc-477; Santa Cruz). After reversal of formaldehyde cross-links, specific DNA sequences were assessed by quantitative real-time PCR. Primers used in ChIP PCR are shown in Table S3.

Statistical analysis. Statistical analysis was performed with the Welch two-sample *t* test using the open-source statistical programming environment R.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/MCB.00418-17>.

SUPPLEMENTAL FILE 1, PDF file, 7.1 MB.

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K.O. and K.I. designed and T.T. performed the majority of the experiments with assistance from K.O. K.O. performed the ChIP assay, the analysis of *Bach2*-RFP mice by using flow cytometry, the microarray of the comparison between wild-type versus *Bach2*-deficient splenic B cells, the immunoblot analysis of retrovirus transduction using B1-8^{hi} splenic B cells, and the experiments using *Bach2*-deficient splenic B cells with B1-8^{hi} background. A.M. supported the screening of *Bach2* binding sites in *Ccnd3* and *Ccne* locus. Y.K. assisted A.M. in designing and generating the specific antibodies against *Bach2* S535 phosphorylation. N.S. assisted A.M. in experiments. M.M. supported with data analysis the microarray that K.O. performed. T.K. contributed to analysis and evaluation of data. T.T., K.O., and K.I. wrote the manuscript.

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