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BACH2, a Candidate Risk Gene for Type 1 Diabetes, Regulates Apoptosis in Pancreatic β-Cells via JNK1 Modulation and Crosstalk With the Candidate Gene *PTPN2*

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Type 1 diabetes is a chronic autoimmune disease characterized by specific destruction of pancreatic β-cells by the immune system. Linkage and genome-wide association studies have identified more than 50 loci across the human genome associated with risk of type 1 diabetes. Recently, basic leucine zipper transcription factor 2 (*BACH2*) has been associated with genetic risk to develop type 1 diabetes, in an effect ascribed to the immune system. We evaluated whether *BACH2* may also play a role in immune-mediated pancreatic β-cell apoptosis. BACH2 inhibition exacerbated cytokine-induced β-cell apoptosis in human and rodent β-cells by the mitochondrial pathway of cell death, whereas BACH2 overexpression had protective effects. BACH2 silencing and exposure to proinflammatory cytokines increased phosphorylation of the proapoptotic protein JNK1 by upregulation of mitogen-activated protein kinase kinase 7 (MKK7) and downregulation of PTPN2. JNK1 increased phosphorylation of the proapoptotic protein BIM, and both JNK1 and BIM knockdown protected β-cells against cytokine-induced apoptosis in BACH2-silenced cells. The present findings suggest that the type 1 diabetes candidate gene *BACH2* regulates proinflammatory cytokine–induced apoptotic pathways in pancreatic β-cells by crosstalk with another candidate gene, *PTPN2*, and activation of JNK1 and BIM. This clarifies an unexpected and relevant mechanism by which *BACH2* may contribute to diabetes.

Type 1 diabetes is a chronic autoimmune disease characterized by specific destruction of pancreatic β-cells by the immune system (1,2). There is an important inflammatory component in the initial phases of the disease (insulitis), which is triggered in the context of a "dialog" between invading immune cells and the target pancreatic β-cells (1). Polymorphisms in type 1 diabetes candidate genes may have a major effect in the triggering and evolution of insulitis in type 1 diabetes (2).

In recent years, linkage and genome-wide association studies have identified more than 50 loci across the human genome associated with risk of type 1 diabetes (3). Potential candidate genes have been described for >60% of these regions, explaining nearly 80% of heritability, but evidence for direct functional effect of these candidate genes is still limited (4,5). Until recently, nearly all candidate genes for type 1 diabetes were assumed to act at the immune system level (3,6), but recent findings by our group (7–11) and others (5) indicate that >60% of type 1

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diabetes candidate genes are expressed in pancreatic β-cells. We previously observed that some type 1 diabetes candidate genes, such as *PTPN2* and *IFIH1*, play a role in the modulation of innate immunity, antiviral responses, and activation of apoptosis in pancreatic β-cells (9,11,12). Other candidate genes, such as *GLIS3*, are related to β-cell phenotype (e.g., insulin expression and secretion) and susceptibility to proapoptotic stimuli (e.g., proinflammatory cytokines) (10,12).

Recent studies have associated the BTB and CNC homology 1, basic leucine zipper transcription factor 2 (*BACH2*) with genetic risk to develop type 1 diabetes (13,14) and other autoimmune diseases such as Crohn disease (15), celiac disease (16), and vitiligo (17). *BACH2* is a transcription factor that exerts its function at the immune system level and is implicated in the regulation of B cells toward plasma cell differentiation (18,19), immunoglobulin class switching, somatic hypermutation (20), as well as in the formation of regulatory T cells (21) and maintenance of the naive T-cell state (22). *BACH2* knockout (KO) mice have limited viability due to the development of severe inflammation in the spleen, lungs, and other tissues. Interestingly, sera from *BACH2* KO mice contain elevated levels of antinuclear and antidouble-stranded DNA autoantibodies, which are present in autoimmune diseases such as lupus erythematosus and rheumatoid arthritis (21).

BACH2 acts as a transcriptional repressor that generally binds to the Maf recognition element (MARE) and the antioxidant-responsive element (ARE) (23). It is implicated in mild oxidative stress–induced apoptosis in Raji cells (20) and in virus-mediated cell death in HeLa cells (24).

We presently report that the type 1 diabetes candidate gene *BACH2* is expressed and modulated by proinflammatory cytokines in rodent and human pancreatic islets. *BACH2* inhibition exacerbates cytokine-induced β-cell apoptosis via activation of the JNK1/BIM pathway, whereas opposite effects are observed after *BACH2* overexpression. These findings indicate that *BACH2* is a crucial factor in the regulation of cytokine-induced β-cell death, besides its role in the regulation of inflammation at the immune system level.

RESEARCH DESIGN AND METHODS

Culture of Human Islets, EndoC-βH1 Human β-Cell Line, Rat β-Cells, and INS-1E Cells

Male Wistar rats (Charles River Laboratories, L'Arbresle Cedex, France) were housed and used according to the guidelines of the Belgian Regulations for Animal Care. All experiments were approved by the local ethical committee. Islets were isolated by collagenase digestion and handpicked under a stereomicroscope. β-Cells were purified by autofluorescence-activated cell sorting (FACSAria; BD Bioscience, San Jose, CA) (25). Preparations contained 90 ± 1% β-cells (n = 11). β-Cells were cultured for 2 days in Ham's F-10 medium supplemented up to 10 mmol/L glucose as described (25). During cytokine exposure, cells were cultured in the same medium but without FBS.

Human islets were isolated from 17 nondiabetic organ donors (9 men and 8 women; age 53.3 ± 4.9 years; BMI 25.3 ± 0.7 kg/m^2) in Pisa, Italy, with the approval of the local ethics committee. Islets were isolated by enzymatic digestion and density-gradient purification and cultured in M199 medium containing 5.5 mmol/L glucose (26). The human islets were shipped to Brussels within 1–5 days of isolation. In Brussels, they were dispersed and cultured in Ham's F-10 medium containing 6.1 mmol/L glucose, 10% FBS, 2 mmol/L GlutaMAX, 50 μmol/L 3-isobutyl-1-methylxanthine, 1% BSA, 50 units/mL penicillin, and 50 μg/mL streptomycin. Treatment of the dispersed human islets with cytokines (see below) was done in the same medium but without FBS. The percentage of β-cells in the human islet preparations was 53 ± 5% (n = 17), as determined by staining with anti-insulin antibody (1:1,000; Sigma-Aldrich, Bornem, Belgium) and donkey anti-mouse IgG rhodamine (1:200; Lucron Bioproducts, De Pinte, Belgium).

The EndoC-βH1 human β-cell line (provided by Dr. R. Scharfmann, Centre de Recherche de l'Institut du Cerveau et de la Moelle épinière, Paris, France) was cultured in Matrigel-fibronectin–coated plates in low-glucose Dulbecco's modified Eagle's medium, as previously described (27).

Rat insulin-producing INS-1E cells (provided by Dr. C. Wollheim, Centre Médical Universitaire, Geneva, Switzerland) were cultured as previously described (28).

RNA Interference

The small interfering RNAs (siRNAs) used in this study are listed in Supplementary Table 1. Cells were cultured in antibiotic-free medium for at least 24 h before transfection. We have previously established the optimal siRNA concentration for β-cell transfection (30 nmol/L) (29). Cells were transfected using the Lipofectamine RNAiMAX lipid reagent (Invitrogen, Carlsbad, CA) (29,30). Allstars Negative Control siRNA (Qiagen, Venlo, the Netherlands) was used as a negative control (siCTRL); this control siRNA does not affect β-cell gene expression, function, or viability (29,30). Afterward, cells were cultured for a 48-h recovery period and subsequently exposed to cytokines.

Generation of Recombinant Adenovirus and Cell Infection

To overexpress BACH2 in human β-cells, we used a recombinant adenovirus containing the human BACH2 mRNA (GenBank: NM_021813; SIRION Biotech, Munich, Germany). The human BACH2 coding region was amplified by PCR from cDNA clone BC166613 purchased from Source BioScience (Berlin, Germany). The BACH2 coding region was then transferred by recombination into plasmid pADCMV-DEST containing the genome of a replication deficient Ad5-based vector deleted in E1/E3 genes. Presence and correctness of the BACH2-ORF in the resulting BAC-vector BA5-CMV-BACH2 was confirmed by DNA sequencing.

After 48-h preculture, EndoC-βH1 cells or dispersed human islets were infected with the recombinant adenovirus

encoding human BACH2 or with a control adenovirus encoding *Renilla* luciferase (31). Cells were infected for 3 h at 37°C in the absence of FBS at a multiplicity of infection of 5. The medium was then changed, and the cells were left to recover for 24 h before cytokine treatment.

Cytokine Treatment and Nitric Oxide Measurement

On the basis of previous dose-response experiments done by our group in rodent and human islet cells, the following cytokine concentrations were used: recombinant human interleukin (IL)-1β (R&D Systems, Abingdon, U.K.) at 10 or 50 units/mL as indicated; recombinant rat interferon (IFN)-γ (R&D Systems) at 100 and 500 units/mL for INS-1E cells and rat β-cells, respectively; and human IFN-γ (PeproTech, Rocky Hill, NJ) at 1,000 units/mL for the EndoC-βH1 human β-cell line and dispersed human islets (28,32,33). Culture supernatants were collected for nitrite determination (nitrite is a stable product of nitric oxide [NO] oxidation) at $OD^{540\ nm}$ using the Griess method (9).

Assessment of Cell Viability

The percentage of viable, apoptotic, and necrotic cells was determined after a 15-min incubation with the DNA-binding dyes propidium iodide (5 μg/mL; Sigma-Aldrich) and Hoechst 33342 (5 μg/mL; Sigma-Aldrich) (25,32). A minimum of 600 cells was counted in each experimental condition. Viability was evaluated by two independent observers, one of them unaware of sample identity. The agreement between findings obtained by the two observers was >90%. Results are expressed as percentage of apoptosis, calculated as number of apoptotic cells/total number of cells × 100. In some experiments, apoptosis was confirmed by Western blot analysis of cleaved caspases 9 and 3 (see below).

mRNA Extraction and Real-Time PCR

Poly(A)$^+$ mRNA was isolated from INS-1E cells, primary rat β-cells, EndoC-βH1 cells, and human islets using the Dynabeads mRNA DIRECT kit (Invitrogen) and reverse-transcribed as previously described (25,32,34). cDNAs from human leukocytes, thymus, and spleen, were obtained from Clontech Laboratories (Saint-Germain-en-Laye, France). The real-time PCR amplification reaction was done as described (25), using SYBR Green and compared with a standard curve (35). Expression values were corrected for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for rat β-cells or β-actin for human cells. Expression of these housekeeping genes is not modified under the present experimental conditions (9,36). We confirmed in the present data set that cytokine treatment and/or BACH2 knockdown (KD) do not affect GAPDH or α-tubulin expression, at respectively, mRNA and protein levels, in INS-1E cells when correcting for cell loss (data not shown). In the case of comparison among different tissues, samples were normalized by the average of three different housekeeping genes (*GAPDH*, β-actin, and ornithine decarboxylase antizyme 1 [*OAZ1*]). The primers used in this study are detailed in Supplementary Table 1.

Western Blot Analysis

Islets or cells were washed with cold PBS and lysed in Laemmli buffer. Immunoblot analysis was performed with the antibodies listed in Supplementary Table 1. Membranes were exposed to secondary peroxidase-conjugated antibody for 1 h at room temperature. Immunoreactive bands were revealed using the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) and detected using ChemiDoc XRS+ (Bio-Rad Laboratories). The densitometry of the bands was evaluated using Image Lab software (Bio-Rad Laboratories).

Statistical Analysis

Data are presented as means ± SEM. Comparisons were performed by two-tailed paired Student *t* test or by ANOVA, followed by Student *t* test with Bonferroni correction, as indicated. A *P* value of <0.05 was considered as statistically significant.

RESULTS

BACH2 Expression in Human and Rodent β-Cells Is Modified by Proinflammatory Cytokines

We first evaluated the expression of BACH2 mRNA in dispersed human islets, EndoC-βH1 human β-cell line, primary rat β-cells, and rat insulin-producing INS-1E cells compared with human cells and tissues involved in the immune response (leukocytes, thymus, and spleen; Fig. 1*A*) and protein level in INS-1E cells, EndoC-βH1 cells, human islets, and human lymphoblasts (Fig. 1*B*). BACH2 mRNA expression in dispersed human islets, which contained an average of 50% β-cells, was similar to the evaluated immune cells, whereas BACH2 expression in fluorescence-activated cell sorting–purified primary rat β-cells (>90% pure) was twofold higher, suggesting a high expression of this mRNA in primary β-cells (Fig. 1*A*). The cytokines IL-1β and IFN-γ induced a transitory increase in BACH2 mRNA expression in rat INS-1E and primary β-cells (Figs. 1*C* and *D*) but with lower values at 48 h in primary rat β-cells. This transitory increase in BACH2 expression was confirmed at the protein level in INS-1E cells (Fig. 1*F*). Lower BACH2 expression (nearly 50% reduction) was confirmed in dispersed human islets (mRNA level) and EndoC-βH1 cells (protein level) exposed to cytokines for 48 h (Figs. 1*E* and *G*). We also checked BACH2 expression in four independent preparations of dispersed human islets after 24 h of cytokine exposition and observed a nonsignificant trend for BACH2 inhibition (data not shown).

To investigate the potential role of BACH2 in global β-cell gene regulation under inflammatory conditions, we performed a Database for Annotation, Visualization and Integrated Discovery (DAVID) enrichment analysis for transcription factor binding sites in cytokine-modified human islet mRNAs, identified by RNA sequencing analysis (7), and ~40% (591 of 1,369) of cytokine-regulated genes showed

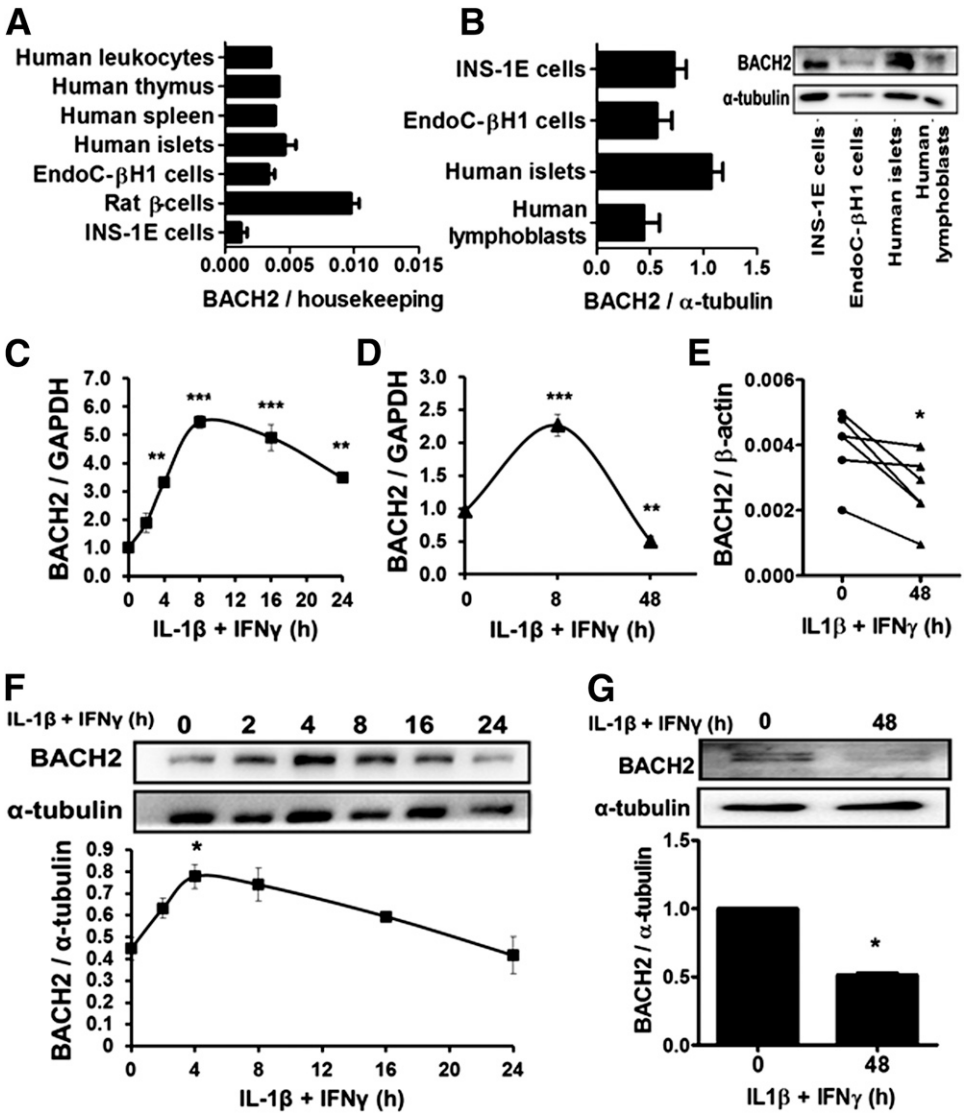

Figure 1—BACH2 is expressed in rodent and human β-cells, and its expression is modified by proinflammatory cytokines. *A*: BACH2 mRNA expression was evaluated in human islets, EndoC-βH1 cells, fluorescence-activated cell sorting–purified primary rat β-cells, and INS-1E cells. For comparison, BACH2 expression was also analyzed in human immune-related cells/tissues (leukocytes, thymus, and spleen). BACH2 mRNA expression was assayed by RT-PCR and normalized by the average of three different housekeeping genes (β-actin, *GAPDH*, and *OAZ1*). *B*: BACH2 protein expression was evaluated in INS-1E cells, EndoC-βH1 cells, human islets (200 islets/preparation), and human lymphoblasts (~10^4 cells/preparation). BACH2 protein expression was assayed by Western blot and normalized by α-tubulin. Results are means ± SEM of three independent preparations (n = 2 in the case of human islets). *C*: INS-1E cells were left untreated or treated with IL-1β + IFN-γ (10 and 100 units/mL, respectively) for 2, 4, 8, 16, and 24 h. BACH2 expression was assayed by RT-PCR. Primary rat β-cells were left untreated or treated with IL-1β + IFN-γ (50 and 500 units/mL, respectively) for 8 and 48 h (*D*), and dispersed human islets were left untreated or treated with IL-1β + IFN-γ (50 and 1,000 units/mL, respectively) for 48 h (*E*). BACH2 mRNA expression was assayed by RT-PCR and normalized by the housekeeping gene *GAPDH* (*C* and *D*) or β-actin (*E*). Results are means ± SEM of three to five independent experiments; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. untreated (i.e., not treated with cytokines). *F*: INS-1E cells were left untreated or treated as in *C*. *G*: EndoC-βH1 cells were left untreated or treated with IL-1β + IFN-γ (50 and 1,000 units/mL, respectively) for 48 h. BACH2 expression was assayed by Western blot. Representative Western blot (*top panel*) and densitometry are shown after normalization (*bottom panel*) for the protein content using α-tubulin. Results are means ± SEM of four independent experiments. *$P <$ 0.05 vs. untreated (i.e., not treated with cytokines); two-tailed paired Student t test.

transcription factor binding sites for BACH2. Importantly, BACH2 binding sites were ranked among the top five most enriched ones (Supplementary Fig. 1). This, as well as the high expression of *BACH2* in β-cells, suggests a putative functional role for this type 1 diabetes candidate gene not only in immune cells (19–22) but also in pancreatic β-cells.

BACH2 Inhibition Exacerbates Cytokine-Induced β-Cell Apoptosis

To examine the role of *BACH2* in β-cell death, specific siRNAs were designed to inhibit (KD) BACH2 expression in human (EndoC-βH1 cells and dispersed human islets) and rat cells. The different siRNAs that were used inhibited

BACH2 mRNA and/or protein expression by at least 50% under the different experimental conditions tested (Figs. 2*A*, *C*, and *G* and Supplementary Fig. 2*A* and *C–E*). The decrease in BACH2 expression led to a trend toward higher basal apoptosis in human (Fig. 2*I*) and rat (Supplementary Fig. 2*B* and *F*) β-cells, and a clear increase in cytokine-induced apoptosis in all four models as evaluated by nuclear dyes (Figs. 2*E* and *I* and Supplementary Fig. 2*B* and *F*). This increase in apoptosis after BACH2 KD was confirmed by the presence of higher expression of cleaved caspases 9 and 3 in cytokine-exposed INS-1E cells after 16 or 24 h exposure (Supplementary Fig. 2*G–I*). The observed

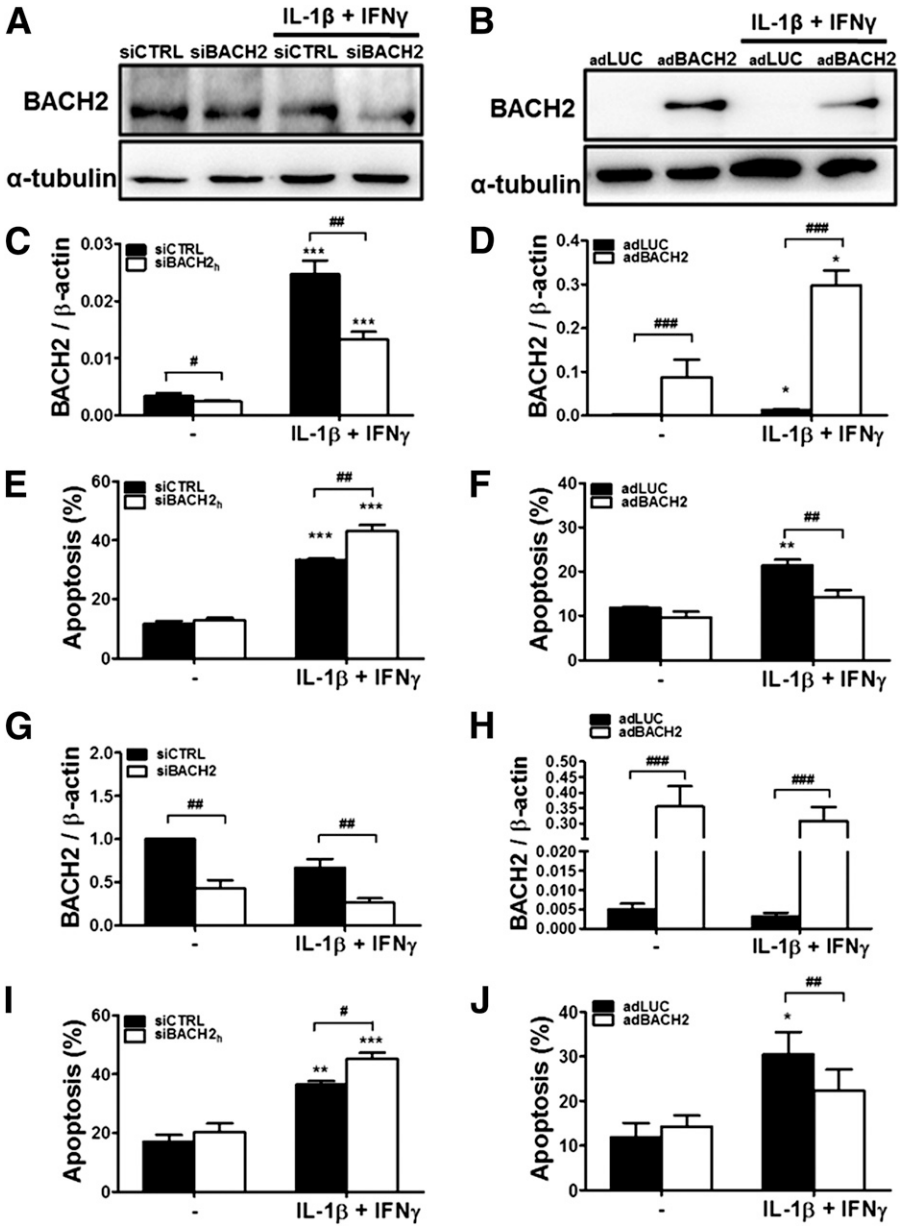

Figure 2—BACH2 inhibition exacerbates cytokine-induced apoptosis, whereas BACH2 overexpression prevents cytokine-induced β-cell death in human cells. EndoC-βH1 cells (*A–F*) or dispersed human islets (*G–J*) were transfected with siCTRL or with siRNA targeting human BACH2 (*A*, *C*, *E*, *G*, and *I*) or overexpressed BACH2 based on the use of an adenoviral (ad) vector encoding human BACH2 (*B*, *D*, *F*, *H*, and *J*). EndoC-βH1 cells or dispersed human islets were left to recover during 48 or 24 h, respectively. After this recovery period, cells were left untreated or treated with IL-1β + IFN-γ (50 and 1,000 units/mL, respectively) for 48 h. Western blot representative of BACH2 KD (*A*) or overexpression (*B*) in EndoC-βH1 cells. BACH2 mRNA expression was assayed by RT-PCR and normalized by the housekeeping gene β-actin (*C*, *D*, *G*, and *H*). Apoptosis after BACH2 KD (*E* and *I*) or BACH2 overexpression (*F* and *J*) was evaluated using Hoechst and propidium iodide staining. Results are means ± SEM of three to six independent experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs. untreated (i.e., not treated with cytokines) and transfected with the same siRNA; #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 as indicated by bars; ANOVA. adLUC, adenovirus encoding *Renilla* luciferase.

increase in caspase 9 suggests that BACH2 inhibition exacerbates IL-1β– and IFN-γ–induced apoptosis through the intrinsic mitochondrial pathway. In a mirror image of these experiments, BACH2 overexpression using an adenoviral vector (Figs. 2*B*, *D*, and *H*) decreased cytokine-induced apoptosis in EndoC-βH1 cells and dispersed human islets (Figs. 2*F* and *J*).

BACH2 Inhibition Downregulates the Antiapoptotic Proteins MCL-1 and B-Cell Lymphoma 2A1 and Increases Phosphorylation of the Proapoptotic Protein BIM

BACH2 inhibition did not increase cytokine-induced NO production by INS-1E cells (77.9 ± 4.9 to 48.7 ± 4.3 pmol/L/10^6 cells × 24 h, siCRTL and siBACH2, respectively) or primary rat β-cells (174.2 ± 3.3 to 109.7 ± 16.4 pmol/L/10^6 cells × 48 h, siCRTL and siBACH2, respectively), as evaluated by medium nitrite accumulation (there is actually less nitrite production in cells transfected with siBACH2), excluding a role for this radical in the observed increase in apoptosis after BACH2 inhibition.

The triggering of the intrinsic pathway of apoptosis in β-cells depends on the balance between anti- and pro-apoptotic B cell lymphoma 2 (BCL-2) proteins (37). BACH2 KD did not affect expression of the antiapoptotic proteins BCL-2 and BCL-XL but did cause a basal decrease in MCL-1 and BCL-2A1 (A1) expression, which was maintained for 8 h and 4 h after cytokine exposure for MCL-1 and A1, respectively (Fig. 3).

The BH3-only proteins death protein 5 (DP5), BCL-2 binding component 3 (p53 upregulated modulator of apoptosis [PUMA]), and BIM have been recently identified as mediators of cytokine-induced β-cell apoptosis (38–40). BACH2 KD, however, did not increase BIM, DP5, or PUMA expression (Supplementary Fig. 3). The pro-apoptotic activity of BIM is also regulated by phosphorylation at multiple serine and threonine residues (11,41). In line with this, BACH2 KD increased BIM phosphorylation at serine 65 (ser65) after 8 and 16 h of IL-1β and IFN-γ exposure (Figs. 4*A*–*C*), and similar results were observed when phospho-BIM was corrected for α-tubulin (data not shown). To evaluate whether BIM indeed contributes to cytokine-induced β-cell death in BACH2-inhibited cells, we used specific siRNAs to KD both BACH2 and BIM, achieving a >50% inhibition ($P < 0.05$) in the expression of both mRNAs (Supplementary Fig. 4 and data not shown). As observed previously (Fig. 2 and Supplementary Fig. 2), BACH2 KD exacerbated apoptosis after IL-1β plus IFN-γ treatment in INS-1E cells. BIM KD prevented this effect, which protected INS-1E cells from basal or cytokine-induced apoptosis in BACH2-silenced cells (Fig. 4*F*). The protective effect of BIM KD was confirmed by decreased expression of cleaved caspases 9 and 3 in INS-1E cells (Fig. 4*G*). There was a decrease of >70% ($P < 0.01$) for both cleaved caspases 9 and 3 when comparing cytokine-treated cells exposed to siBACH2 alone versus siBACH2 plus siBIM ($n = 4$; densitometry of Fig. 4*G* and other similar experiments; data not shown). Similar results were observed in primary rat β-cells (Fig. 4*H*), EndoC-βH1 cells (Fig. 4*I*), and dispersed human islets (Fig. 4*J*), in which BIM inhibition prevented BACH2 KD–induced apoptosis. The findings in human islets were confirmed with a second and independent siRNA targeting human BIM (Fig. 4*K*).

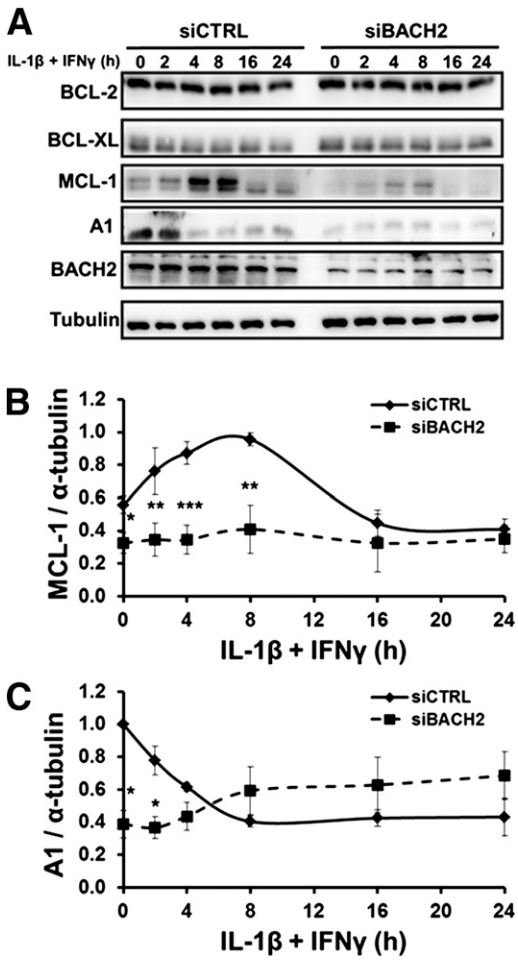

Figure 3—BACH2 inhibition downregulates basal expression of the anti-death proteins MCL-1 and A1. INS-1E cells were transfected with siCTRL or siBACH2. After 48 h of recovery, they were left untreated or treated with IL-1β + IFN-γ (10 and 100 units/mL, respectively) for 2–24 h. Expression of BCL-2, BCL-XL, MCL-1, A1, BACH2 (for KD confirmation), and α-tubulin (used as loading control) were measured by Western blot. *A*: Figure is representative of five to seven independent experiments. Densitometry results of MCL-1 (*B*) and A1 (*C*) are represented as means ± SEM of five to seven independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. siCTRL; ANOVA.

JNK1 Mediates Increase in BIM Phosphorylation in BACH2-Silenced Cells

It has been described under different experimental conditions that JNK1 may regulate BIM phosphorylation and consequent β-cell apoptosis (11). To determine whether JNK plays a role in the cytokine-induced increase of BIM phosphorylation in BACH2-inhibited cells, we measured phospho-JNK after IL-1β plus IFN-γ treatment in INS-1E

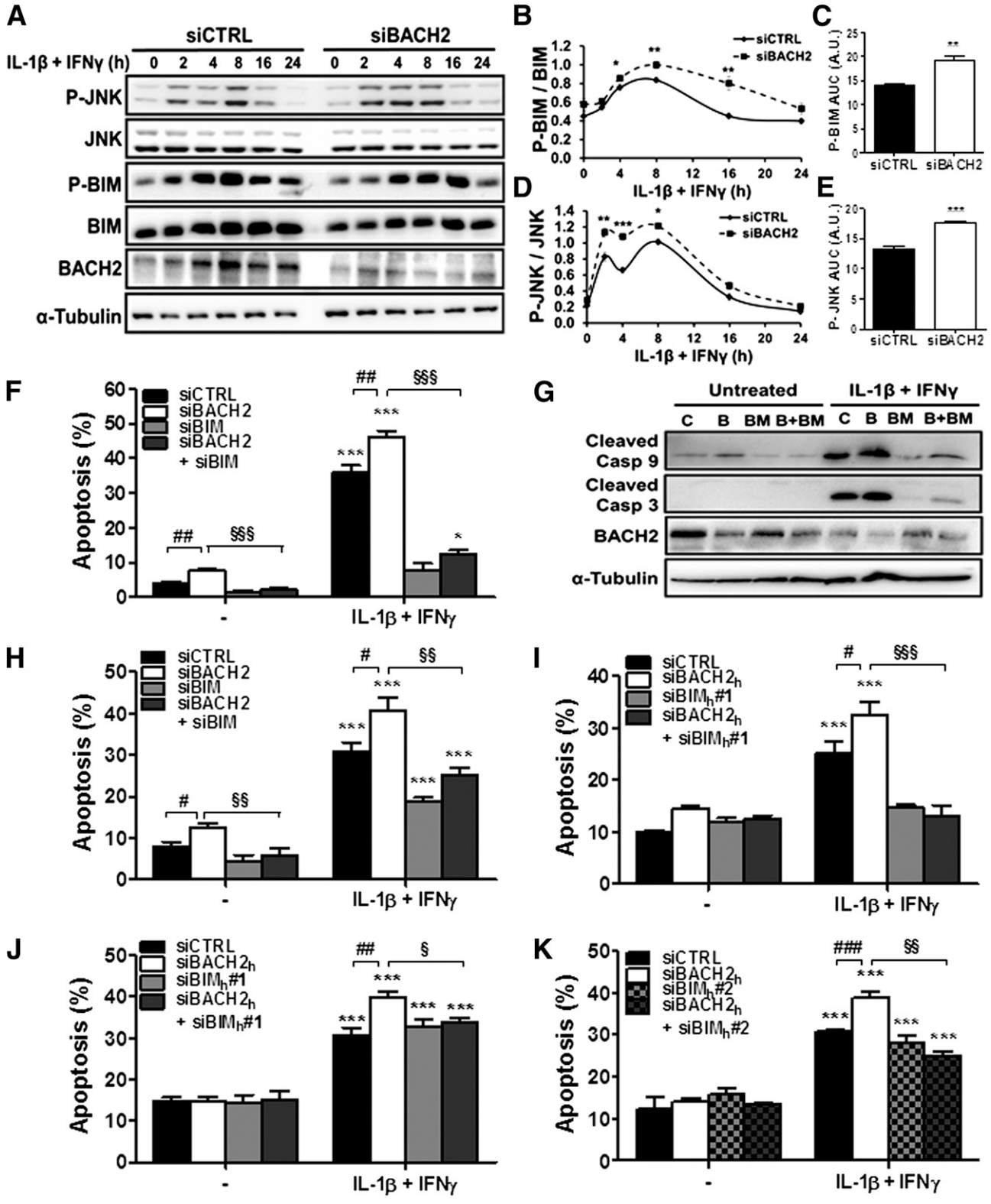

Figure 4—Inhibition of BACH2 increases phosphorylation of BIM (P-BIM) and JNK (P-JNK), and double-KD of BACH2 and BIM protects β-cells against cytokine-induced apoptosis. *A–E*: INS-1E cells were transfected with siCTRL or siBACH2. After 48 h of recovery, cells were exposed to IL-1β + IFN-γ (10 and 100 units/mL, respectively) for 2–24 h. Expression of P-BIM (Ser65), total BIM, P-JNK, JNK, BACH2 (for KD confirmation), and α-tubulin (used as loading control) were measured by Western blot. *A*: Figure is representative of five to six experiments. Densitometry results of P-BIM (Ser65) (*B*) and P-JNK (*D*). Quantification of the area under curve (AUC) (*C* and *E*) is shown as means ± SEM of five to six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. siCTRL. ANOVA (*B* and *D*) and *t* test (*C* and *E*). INS-1E cells (*F* and *G*), primary rat β-cells (*H*), EndoC-βH1 cells (*I*), and dispersed human islets (*J* and *K*) were transfected with siCTRL, siBACH2, siBIM, and the combination of siBACH2 and siBIM (*F* and *H*) or siBACH2 and two independent siRNAs against human

cells transfected with siCTRL or siBACH2 (Figs. 4*A*, *D*, and *E*). Phosphorylation of JNK was increased at early time points (2–8 h) of cytokine treatment (Figs. 4A and *D*), and calculation of the area under the curve for all evaluated points confirmed the significant increase in phospho-JNK in BACH2-silenced cells (Fig. 4*E*), with similar results observed when phospho-JNK was corrected for α-tubulin (data not shown). To evaluate whether JNK1 activation contributes to β-cell death in BACH2-inhibited cells, we performed a BACH2/JNK1 double-KD approach in INS-1E cells (Fig. 5*A*), achieving clear inhibition of the targeted mRNAs or proteins, especially after cytokine treatment (Supplementary Fig. 5*A* and *B*). Inhibition of BACH2 exacerbated apoptosis under the basal condition and after IL-1β plus IFN-γ treatment, and JNK1 KD prevented this increase (Fig. 5*A*). These results indicate that BACH2 inhibition leads to increased JNK1 activation, which contributes to the hyperphosphorylation of BIM at Ser65 and increased β-cell apoptosis.

BACH2 Silencing Induces JNK Phosphorylation by MKK7 Activation

Mitogen-activated protein kinase kinase 7 (MKK7) is the main kinase implicated in JNK activation during inflammation (42). To determine whether this MAPK is responsible for the observed increase in phospho-JNK in BACH2-inhibited cells, we used a BACH2/MKK7 double-KD approach (the KD confirmation is shown in Supplementary Fig. 5*C*–*E*). BACH2/MKK7 double KD partially prevented cytokine-induced JNK and BIM phosphorylation at 8 and 16 h (Fig. 5*B*), and siRNAs against BACH2 and MKK7 induced a >40% decrease ($P < 0.05$) in the respective RNAs at both 8 and 16 h (data not shown). Importantly, they also reduced cytokine-induced β-cell apoptosis in BACH2 KD cells to the same level observed in siCTRL-transfected cells (Fig. 5*C*). These results suggest that MKK7 is partly responsible for the increased JNK phosphorylation observed in BACH2-silenced cells.

Gadd45β is a key regulator of MKK7 activity by binding to its catalytic site and inhibiting its function (43,44). A recent study showed that Gadd45β is a BACH2 target gene in B cells (19). In line with these observations, BACH2 inhibition decreased cytokine-induced Gadd45β expression in INS-1E cells, primary rat β-cells, EndoC-βH1 cells, and in dispersed human islets (Supplementary Fig. 6). We also evaluated the expression of other MKK7 upstream proteins, namely, TRAF-6, TAK-1, and ASK1, but no significant changes were observed (data not shown). The role for Gadd45β in mediating BACH2 effects must be confirmed by additional coimmunoprecipitation experiments.

Expression of the Type 1 Diabetes Candidate Gene *PTPN2* Is Modulated by BACH2 in β-Cells

The type 1 diabetes candidate gene *PTPN2* modulates JNK1 activation in β-cells, and decreased PTPN2 expression augments cytokine-induced β-cell apoptosis by BIM phosphorylation (9,11). We thus evaluated whether BACH2 regulates PTPN2 in these cells. BACH2 inhibition significantly diminished cytokine-induced PTPN2 expression, mainly after 8 h of treatment (44% and 49% decrease in INS-1E cells and primary rat β-cells, respectively; Figs. 6*A* and *C*). Furthermore, a 51% and 32% decrease in PTPN2 expression was observed after 48 h treatment in EndoC-βH1 cells and dispersed human islets, respectively (Figs. 6*B* and *D*). This reduction in cytokine-induced PTPN2 expression was also evident at the protein level in INS-1E cells (Fig. 6*E*) and EndoC-βH1 cells (Fig. 6*F*). These results suggest that PTPN2 may be implicated in the regulation of cytokine-mediated β-cell apoptosis in BACH2-silenced cells and indicate, for the first time, a crosstalk between these two candidate genes for type 1 diabetes in pancreatic β-cells.

DISCUSSION

BACH2 is a transcription factor that has been genetically associated with risk of type 1 diabetes in several genome-wide association studies (13,14). This gene is located on chromosome 6q15 in a strong linkage disequilibrium block of 365 kb, in which *BACH2* is the only protein-coding locus (13,14). Different single nucleotide polymorphisms (SNPs) in *BACH2* have been associated with type 1 diabetes risk. The assumption up to now has been that particular SNPs in *BACH2* contribute to type 1 diabetes (13,14) and other autoimmune diseases (15,16) by regulating T- and B-cell differentiation, thus modulating autoimmune diseases by controlling the balance between tolerance and immunity (21,22). Our present findings revealed a similar *BACH2* expression in islet cells and in cells from the immune system. This, and the high prevalence of transcription factor binding sites for BACH2 in cytokine-treated human islet mRNAs, suggests that BACH2 may also play a relevant and direct role in β-cells exposed to an autoimmune assault.

BIM (siBIM$_h$#1 and siBIM$_h$#2) (*I*–*K*). After 48 h of recovery, they were left untreated or treated with IL-1β + IFN-γ for 24 h (*F* and *G*) or 48 h (*H*–*K*). Apoptosis was evaluated using Hoechst and propidium iodide staining (*F*, *H*–*K*). Results are means ± SEM of five to seven independent experiments. *$P < 0.05$ and ***$P < 0.001$ vs. untreated (i.e., not treated with cytokines) and transfected with the same siRNA; #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$; §$P < 0.05$, §§$P < 0.01$, and §§§$P < 0.001$ as indicated by the bars; ANOVA. *G*: INS-1E cells were transfected with siCTRL (C), siBACH2 (B), siBIM (BM), and the combination of siBACH2 and siBIM (B+BM). After 48 h of recovery, cells were exposed to IL-1β + IFN-γ (10 and 100 units/mL, respectively). Protein expression of cleaved caspase 9, cleaved caspase 3, BACH2 (for KD confirmation), and α-tubulin (used as loading control) were measured by Western blot. The figure is representative of three independent experiments.

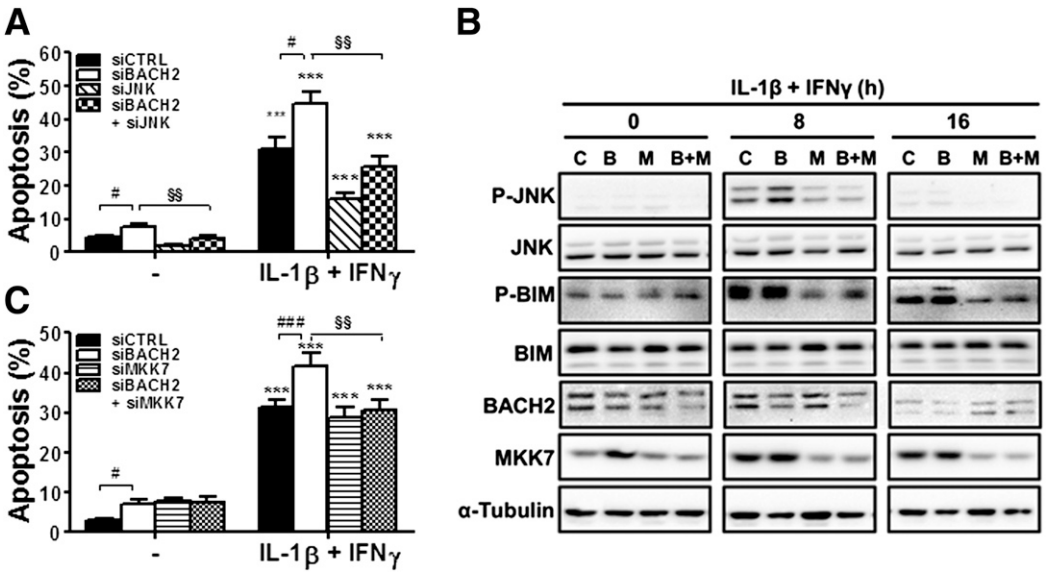

Figure 5—BACH2 silencing induces phosphorylation (P) of JNK (P-JNK) via MKK7 activation. *A*: INS-1E cells transfected with siCTRL, siBACH2, siJNK1, or the combination of siBACH2 and siJNK1 were exposed to IL-1β + IFN-γ (10 and 100 units/mL, respectively) for 24 h. Apoptosis was evaluated using Hoechst and propidium iodide staining. Results are means ± SEM of five independent experiments. ***$P < 0.001$ vs. untreated (i.e., not treated with cytokines) and transfected with the same siRNA; #$P < 0.05$; §§$P < 0.01$ as indicated by bars; ANOVA. *B*: INS-1E cells were transfected with siCTRL (C), siBACH2 (B), siMKK7 (M), and the combination of siBACH2 and siMKK7 (B+M). After 48 h of recovery, cells were exposed to IL-1β + IFN-γ (10 and 100 units/mL, respectively) for 8 or 16 h. Expression of P-JNK, JNK, P-BIM, BIM, MKK7, BACH2, and α-tubulin (used as loading control) were measured by Western blot. The figure is representative of three independent experiments. *C*: INS-1E cells were transfected with siCTRL, siBACH2, siMKK7, and the combination of siBACH2 and siMKK7. After 48 h of recovery, cells were exposed to IL-1β + IFN-γ (10 and 100 units/mL, respectively) for 24 h. Apoptosis was evaluated using Hoechst and propidium iodide staining. Results are means ± SEM of seven independent experiments. ***$P < 0.001$ vs. untreated (i.e., not treated with cytokines) and transfected with the same siRNA; #$P < 0.05$ and ###$P < 0.001$; §§$P < 0.01$ as indicated by the bars; ANOVA.

In agreement with this hypothesis, we observed that BACH2 is regulated by proinflammatory cytokines and that inhibition of BACH2 by specific siRNAs sensitizes human and rat pancreatic β-cells to cytokine-induced apoptosis. In a mirror image of these findings, BACH2 overexpression using an adenoviral vector decreases cytokine-induced apoptosis in human β-cells. Thus, BACH2 seems to have an antiapoptotic role in pancreatic β-cells. In contrast, BACH2 silencing protects against polyinosinic:polycytidylic acid–induced apoptosis in HeLa cells (24), and overexpression of BACH2 in Raji B cells exacerbates cell death upon exposure to a mild oxidative stress stimulus (45,46). These observations suggest that BACH2 may promote or inhibit apoptosis in a cell- and stimuli-specific manner. In line with this, other transcription factors involved in inflammation and apoptosis, such as nuclear factor-κB, have proapoptotic roles in pancreatic β-cells, but are antiapoptotic in other cell types, such as fibroblasts (47).

The presently observed increase in apoptosis in BACH2-deficient cells is secondary to the activation of the intrinsic apoptotic pathway of cell death, as indicated by a higher activation of caspases 9 and 3 and dependency on specific BH3-only proteins. β-Cell survival depends on the balance between anti- and proapoptotic BCL-2 proteins (37). BACH2 inhibition results in lower levels of the antiapoptotic BCL-2 proteins MCL-1 and A1 under basal condition and, at least for MCL-1, after exposure to cytokines. In line with these findings, BACH2 upregulation in patients with B-cell lymphoma correlates with lower levels of BCL-2 protein (48), whereas inhibition of nuclear translocation of BACH2 leads to an increase in BCL-XL, BCL-2, and MCL-1 expression levels in lymphoma cells (45). Furthermore, reduced survival of BACH2-deficient regulatory T cells has been associated with decreased expression of BCL-2 and MCL-1 (49). BACH2 is thus implicated in the regulation of the expression of BCL-2 antiapoptotic proteins in different cell models, which may partly explain its role in target cells of an autoimmune response or in cancer cells.

A systematic analysis of the other side of the coin, namely, the proapoptotic proteins potentially implicated in the intrinsic pathway of cell death in BACH2-silenced cells, revealed an increase in BIM phosphorylation, which was preceded by an augmented activation of JNK1. Importantly, the combined KD of BACH2 and JNK1 or BIM prevented the deleterious effect of BACH2 inhibition in rodent and human β-cells, confirming that JNK1 and BIM both contribute to cell death. The BH3-only protein BIM has a primary role in the control of β-cell death by regulating the activation of the proapoptotic members of the BCL-2 protein family, BAX and BAK (50). Activation of BIM is driven by different pre- and posttranscriptional events (41). Thus, alternative splicing or hyperphosphorylation of BIM has been previously shown to enhance its

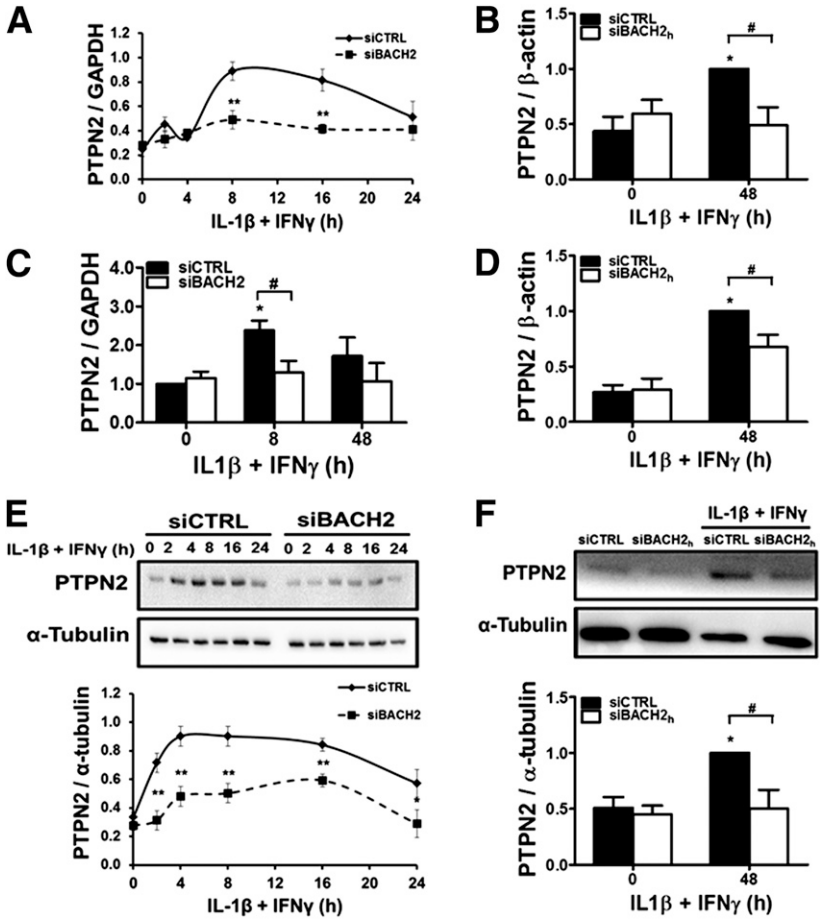

Figure 6—The expression of the type 1 diabetes candidate gene *PTPN2* is modulated by *BACH2* in rodent and human β-cells. *A–F*: Cells were transfected with siCTRL or siBACH2. After 48 h of recovery, they were left untreated or treated for 2–24 h with IL-1β + IFN-γ in INS-1E cells (*A* and *E*), for 8 and 48 h in primary rat β-cells (*C*), or for 48 h in EndoC-βH1 cells (*B* and *F*) and dispersed human islets (*D*). Expression of PTPN2 was analyzed by RT-PCR and normalized by the housekeeping gene *GAPDH* (*A* and *C*) or β-actin (*B* and *D*). Protein expression of PTPN2 was analyzed by Western blot and normalized by the housekeeping protein α-tubulin in INS-1E cells (*E*) and EndoC-βH1 cells (*F*). The figure shows a representative Western blot and quantification of four independent experiments (*E* and *F*). Results are means ± SEM of four to five independent experiments. **P* < 0.05 and ***P* < 0.01 vs. untreated (i.e., not treated with cytokines); #*P* < 0.05 as indicated by the bars; ANOVA.

proapoptotic activity in pancreatic β-cells (10,11). We presently show that BACH2 inhibition increases BIM phosphorylation by JNK1 activation, increasing β-cell apoptosis.

To clarify the mechanism by which BACH2 inhibition increases JNK1 phosphorylation and BIM phosphorylation, we focused on the regulation of JNK1 activation. We observed that the hyperphosphorylation of JNK1 in BACH2-silenced cells is mediated by MKK7. Indeed, inhibition of MKK7 partially prevents JNK1 phosphorylation and cytokine-induced apoptosis in BACH2-silenced β-cells. These results were supported by decreased expression of Gadd45β in BACH2-silenced cells. That reduced amounts of Gadd45β lead to an increase in MKK7 activity in fibroblast-like synoviocytes, granulocytes, and macrophages has been previously described (43,44). Interestingly, Gadd45β KO mice have augmented synovitis and joint destruction in a model of rheumatoid arthritis (43).

We previously showed that the phosphatase PTPN2 controls JNK1 phosphorylation in pancreatic β-cells, inhibiting IFN-induced JNK1 activation and, consequently, reducing the proapoptotic capacity of BIM (11). We presently show that inhibition of BACH2 decreases cytokine-induced PTPN2 expression and, in agreement with our previous results, exacerbates β-cell apoptosis (9,11). Of note, PTPN2 is also a candidate gene for type 1 diabetes and other autoimmune diseases, such as Crohn disease, arthritis, and celiac disease (2). This is the first indication of an interaction between two candidate genes for type 1 diabetes, namely *BACH2* and *PTPN2*, that crosstalk at the β-cell level and increase cytokine-induced activation of a proapoptotic pathway (JNK1/BIM phosphorylation). Taking into account that PTPN2 does not have binding sites for BACH2 in its promoter (data not shown), further studies are needed to elucidate the mechanisms by which BACH2 regulates PTPN2 expression.

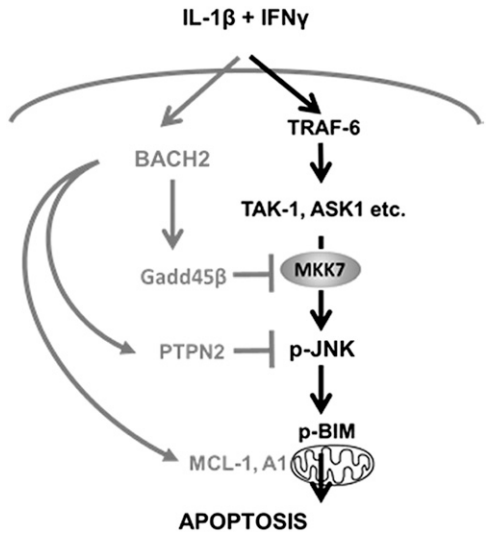

Figure 7—Proposed model for the role of the candidate gene *BACH2* in β-cell apoptosis. IL-1β + IFN-γ induces MKK7 activation, probably via TRAF-6, TAK-1, and ASK1 activation. This leads to phosphorylation of JNK (p-JNK) and BIM (p-BIM) and consequent increase in β-cell apoptosis by the intrinsic mitochondrial pathway. BACH2 functions as a modulator of this proapoptotic pathway by increasing: *1*) Gadd45β, which inhibits MKK7 activity; *2*) PTPN2, which inhibits p-JNK; and *3*) MCL-1 and A1, antiapoptotic members of the BCL-2 family, which inhibit the mitochondrial steps of apoptosis. In individuals with lower BACH2 expression due to particular genetic polymorphisms and/or the inhibitory effects of cytokines, the compensatory mechanisms described above are hampered, contributing to higher pancreatic β-cell apoptosis.

In conclusion, we demonstrate that the type 1 diabetes candidate gene BACH2 regulates proinflammatory cytokine-induced β-cell apoptosis by modulation of the JNK1/BIM pathway and antiapoptotic members of the BCL-2 family, MCL-1, and A1 (Fig. 7). These observations provide a mechanistic explanation for an unexpected effect of BACH2 at the β-cell level. Furthermore, they broaden our understanding of the dialog between candidate genes, pancreatic β-cells, and the immune system in the early stages of type 1 diabetes.

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Author Contributions. L.Marr. and I.S. contributed to the original idea and the design of the experiments; researched data; contributed to discussion; and wrote, revised, and edited the manuscript. R.S.D.S. researched data, contributed to discussion, and revised and edited the manuscript. L.Mars. and P.M. researched data and revised and edited the manuscript. D.L.E. contributed to the original idea and the design and interpretation of the experiments; contributed to discussion; and wrote, revised, and edited the manuscript. D.L.E. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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