SKAP2, a Candidate Gene for Type 1 Diabetes, Regulates β-Cell Apoptosis and Glycemic Control in Newly Diagnosed Patients

Tina Fløyel,[1] Kira Meyerovich,[2] Michala C. Prause,[1,3] Simranjeet Kaur,[1] Caroline Frørup,[1] Henrik B. Mortensen,[4,5] Lotte B. Nielsen,[4] Flemming Pociot,[1,5] Alessandra K. Cardozo,[2] and Joachim Størling[1,3]

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The single nucleotide polymorphism rs7804356 located in the Src kinase-associated phosphoprotein 2 (*SKAP2*) gene is associated with type 1 diabetes (T1D), suggesting *SKAP2* as a causal candidate gene. The objective of the study was to investigate if SKAP2 has a functional role in the β-cells in relation to T1D. In a cohort of children with newly diagnosed T1D, rs7804356 predicted glycemic control and residual β-cell function during the 1st year after diagnosis. In INS-1E cells and rat and human islets, proinflammatory cytokines reduced the content of SKAP2. Functional studies revealed that knockdown of SKAP2 aggravated cytokine-induced apoptosis in INS-1E cells and primary rat β-cells, suggesting an antiapoptotic function of SKAP2. In support of this, overexpression of SKAP2 afforded protection against cytokine-induced apoptosis, which correlated with reduced nuclear content of S536-phosphorylated nuclear factor-κB (NF-κB) subunit p65, lower nitric oxide production, and diminished CHOP expression indicative of decreased endoplasmic reticulum stress. Knockdown of CHOP partially counteracted the increase in cytokine-induced apoptosis caused by SKAP2 knockdown. In conclusion, our results suggest that SKAP2 controls β-cell sensitivity to cytokines possibly by affecting the NF-κB–inducible nitric oxide synthase–endoplasmic reticulum stress pathway.

Type 1 diabetes (T1D) is a chronic immune-mediated disease in which the pancreatic β-cells are destroyed. In this process, proinflammatory cytokines such as interleukin-1β (IL-1β) and interferon-γ (IFN-γ) contribute to T1D by causing β-cell apoptosis (1,2). A strong genetic component is present in T1D, with >60 risk loci identified (3–6). These loci contain numerous disease candidate genes, some of which have been shown to have functional roles in pancreatic β-cells, such as regulation of apoptotic cell death in response to cytokines (7–14).

Src kinase-associated phosphoprotein 2 (*SKAP2*) is located on chromosome 7p15.2 and harbors a single nucleotide polymorphism (SNP), rs7804356, in the third intron of *SKAP2*, which associates with T1D (5,15). Recently, a study found that the risk allele of this SNP correlates with lower *SKAP2* expression in Epstein-Barr virus–transformed B-lymphocyte lines as well as in $CD4^+$ and $CD8^+$ T cells (16), indicating that rs7804356 may affect T1D risk by regulating the *SKAP2* expression level.

SKAP2, which is also known as SKAP-HOM, is ubiquitously expressed and was initially identified in lymphocytes as a cytosolic adaptor protein and an Src kinase substrate (17,18). Loss-of-function studies revealed that SKAP2 is dispensable for T-lymphocyte activation in response to various stimuli but required for B-lymphocyte proliferative responses and adhesion induced by anti-IgM or lipopolysaccharide (19). In macrophage precursor cells, SKAP2 associates with actin and is involved in proliferation, migration, and chemotaxis (20,21). The function of SKAP2 in β-cells is currently unknown, but we recently

[1]Translational Type 1 Diabetes Research, Steno Diabetes Center Copenhagen, Gentofte, Denmark
[2]ULB Center for Diabetes Research, Université Libre de Bruxelles, Brussels, Belgium
[3]Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark
[4]Department of Pediatrics E, Herlev and Gentofte Hospital, Herlev, Denmark
[5]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark

Corresponding author: Joachim Størling, joachim.stoerling@regionh.dk

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reported that cytokines decrease the mRNA expression of *SKAP2* in human pancreatic islets (22), suggesting that *SKAP2* could play a functional role in β-cells for cytokine-mediated impairment. In this study, we show that rs7804356 in *SKAP2* can predict residual β-cell function and glycemic control in the 1st year after diagnosis in a cohort of children with newly diagnosed T1D. Further, we provide evidence that SKAP2 plays an important role in β-cells by regulating the sensitivity of the β-cells to cytokine-induced apoptosis.

RESEARCH DESIGN AND METHODS

Study Populations From the Hvidoere Study Group on Childhood Diabetes

The study population is described in Mortensen et al. (23). The cohort included 275 children (144 females), 84% White Caucasian, and age at clinical diagnosis was 9.1 years (range 0.2–16.8 years). The study was performed according to the criteria of the Helsinki II Declaration and was approved by the local ethics committee (permit number KA 04010gm) in each center as described in Mortensen et al. (23). HbA_{1c} analysis was performed centrally by automatic high-pressure liquid chromatography at onset and 1, 3, 6, 9, and 12 months after diagnosis. A combined expression of insulin dose-adjusted HbA_{1c} ($IDAA_{1c}$) was used to define the partial remission period in children and adolescents with T1D (24). Genotyping of rs7804356 was done using the KASPar system (KBioscience), and typing of the HLA class II DRB1 locus was performed as described (23,25). The T1D-associated SNP rs7804356 located in the *SKAP2* gene was tested for association with HbA_{1c} and $IDAA_{1c}$ levels at 1, 3, 6, 9, and 12 months after diagnosis in linear regression models, assuming additive allelic effects for the risk allele (T). The regression models were adjusted for the covariates sex, age, and HLA risk group.

Human Pancreatic Islet Expression Quantitative Trait Loci Data

The human islet RNA-sequencing data set consisting of 118 individual islet preparations was downloaded from the European Genome-phenome Archive with accession number EGAS00001001265 (26). Bam files were analyzed for quality control using SAMtools (27). Gene, exon, CDS, and other genomic features were defined based on GRCh37 annotation (Ensembl v75). The fragments per kilobase of transcript per million mapped reads (FPKM) values were computed using Cufflinks v.2.2.1 (28). Islet-expressed genes were identified using a criterion of FPKM >1 in at least 10% of the samples.

Cell and Islet Culture

The INS-1E rat insulinoma cell line (29), provided by Claes Wollheim and Pierre Maechler (University of Geneva, Geneva, Switzerland), was maintained in RPMI medium with GlutaMAX supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L β-mercaptoethanol (all from Life Technologies). The human fusion 1.1B4 cell line was obtained from Sigma-Aldrich and is a hybrid of primary human β-cells and the pancreatic ductal cell line PANC-1 (30). The cells were maintained as INS-1E cells but without β-mercaptoethanol. Isolation and culture of neonatal rat islets from Wistar rats (Taconic Biosciences, Ejby, Denmark) were performed as previously described (31). Primary rat β-cells were FACS-purified (FACSAria; BD Biosciences) from isolated islets from male Wistar rats (Charles River Laboratories, Brussels, Belgium) as described (12). Isolated human islets from five organ donors without diabetes were purchased from Prodo Laboratories, Inc. via tebu-bio. Donor information is provided in the Supplementary Material. Human islets were maintained in F10 nutrient mix medium plus GlutaMAX and 10% FBS and penicillin/streptomycin. For experimentation, the same medium but with 2% human serum (Sigma-Aldrich) instead of FBS was used. B lymphoblastoid cell lines (BLCLs) from 55 HapMap Centre d'Etude du Polymorphism Humain/Utah founders were obtained from the Coriell Institute for Medical Research (https://catalog.coriell.org) and cultured as previously described (9). The following cell lines were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research: [GM06985, GM06993, GM06994, GM07000, GM07022, GM07055, GM07056, GM07345, GM07357, GM11829, GM11830, GM11831, GM11832, GM11839, GM11840, GM11881, GM11882, GM11992, GM11993, GM11994, GM11995, GM12003, GM12005, GM12006, GM12043, GM12044, GM12056, GM12057, GM12144, GM12145, GM12146, GM12154, GM12155, GM12156, GM12234, GM12239, GM12248, GM12249, GM12264, GM12750, GM12751, GM12760, GM12761, GM12762, GM12763, GM12812, GM12813, GM12814, GM12815, GM12872, GM12873, GM12874, GM12875, GM12891, GM12892]. All cells and islets were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded in duplicate or triplicate in appropriate dishes 2–3 days prior to experimentation. Recombinant mouse and human IL-1β were obtained from BD Pharmingen and R&D Systems, respectively. Recombinant rat and human IFN-γ were from R&D Systems and PeproTech, respectively. Recombinant human tumor necrosis factor-α (TNF-α) was from R&D Systems. The concentrations and activities (in units per milliliter) of the cytokines and incubation times used for the cellular experiments depended on the exact cytokine batch and model system used and were chosen based on previous experience and optimizations for each of the models (9,10,14,31,32).

siRNA Transfection and Generation of Stable SKAP2-Overexpressing Clones

siGENOME SMARTpool siRNAs (Thermo Fisher Scientific) against rat and human *SKAP2* were used. The final concentration of siRNA was 30 nmol/L. A pool of nontargeting siRNA (Thermo Fisher Scientific) or the AllStars Negative Control siRNA (Qiagen) was used as negative control. Cells were transfected using Lipofectamine RNAiMAX (Thermo

Fisher Scientific) as previously described (31,33). Clones of INS-1E cells overexpressing SKAP2 were generated using a mammalian expression plasmid (pcDNA3.1) encoding hemagglutinin (HA)–tagged mouse SKAP2 (HA-mSKAP2) provided by Dr. Landian Hu (34) using DharmaFECT 1 (Thermo Fisher Scientific) as transfection reagent. Stable SKAP2-overexpressing clones were established by adding G418 to the culture medium at 200 μg/mL for selection and 50 μg/mL for continuous culture of cells. Two clones of INS-1E cells that expressed HA-mSKAP2 (#A and #B) were selected for experimentation. A pool of INS-1E cells stably transfected with an empty plasmid (pcDNA3.1) only containing the neomycin selection gene was used as control cells. Transient transfection of INS-1E cells with the HA-mSKAP2 plasmid or the empty pcDNA3.1 control plasmid was done using the SF Cell Line 4D-Nucleofector X Kit S and a 4D-Nucleofector (both from Lonza Group Ltd.). After trypsinization, 2×10^6 cells were transferred to four Eppendorf tubes, which were then centrifuged at 200*g* for 5 min. The media was discarded, and 100 μL Nucleofector solution and 4 μg plasmid were added to each tube. The solution was then transferred to a Nucleocuvette, and cells were transfected using the Nucleofector program FF-150, according to the manufacturer's recommendations. After transfection, the cells were seeded in appropriate cell culture dishes in RPMI medium supplemented with 10% FBS and 50 μmol/L β-mercaptoethanol (all from Life Technologies). The next day, the media was replaced with RPMI medium supplemented with 10% FBS, 50 μmol/L β-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Life Technologies). The cells were incubated for another day before experimentation.

Gene Expression

RNA extraction, cDNA synthesis, and gene expression were performed either as previously described (9,33) or by using the miRNeasy Mini Kit spin columns (Qiagen) for RNA extraction, the iScript cDNA Synthesis Kit (Bio-Rad Laboratories), and TaqMan assays (Applied Biosystems) for real-time PCR analyses on a CFX384 C1000 Thermal Cycler (Bio-Rad Laboratories). Gene expression was normalized to *Gapdh*, *Ppia*, *Hprt1*, or the geometric mean of *ACTB*, *GAPDH*, and *UBC*. The relative expression levels were calculated from a standard curve or by the ΔΔ threshold cycle method and presented as fold change (mean ± SEM).

Apoptosis and Caspase 3/7 Activity

Apoptotic cell death was evaluated using either propidium iodide (PI) and Hoechst 33342 staining, as described previously (14), or by the detection of DNA–histone complexes (nucleosomes) present in the cytoplasmic fraction of cells using the Cell Death Detection ELISA PLUS assay (Roche), also as described previously (35). For measurement of caspase 3/7 activity, the Caspase-Glo 3/7 Assay (Promega) was used according to the manufacturer's protocol. After measuring the caspase 3/7 activity, the CytoTox-Fluor assay measuring "cellular" protease activity was used to normalize the caspase 3/7 data.

Immunoblotting

Cells or islets were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) or in Laemmli buffer. Detergent-insoluble material was pelleted by centrifugation at 15,000*g* for 10 min at 4°C. The supernatants were stored at −80°C until further use. Protein concentration in cell lysate was determined by the Bradford method (Bio-Rad Laboratories). SDS-PAGE, preparation of nitrocellulose/polyvinylidene difluoride membranes, and immunoblotting were done as described previously (2,3,31). Primary antibodies used were: SKAP2 (#12926-1-AP; Proteintech Group), inducible nitric oxide synthase (iNOS) (#610332; BD Biosciences), cleaved caspase 3 (#9661; Cell Signaling Technology), CHOP (#sc-7351; Santa Cruz Biotechnology), phospho–nuclear factor-κB (NF-κB) p65 (#3033; Cell Signaling Technology), phospho-STAT1 (#7649; Cell Signaling Technology), β-actin (#ab6276; Abcam), α-tubulin (#T8203; Sigma-Aldrich), GAPDH (#ABS16; Millipore), Lamin A/C (#4C11; Cell Signaling Technology), and HA (#sc-805; Santa Cruz Biotechnology). Secondary horseradish peroxidase–conjugated antibodies were anti-rabbit IgG (#7074; Cell Signaling Technology), anti-mouse IgG (#7076; Cell Signaling Technology), and anti-goat IgG (#sc-2354; Santa Cruz Biotechnology). Immune complexes were visualized by chemiluminescence using LumiGLO (Cell Signaling Technology). Images were captured digitally on a Fujifilm LAS 4000, and quantitation was done by ImageQuant TL (GE Healthcare Life Sciences) software.

Statistics

Data are means ± SEM unless otherwise stated. For cellular and islet experiments, data were analyzed with ANOVA (GraphPad Prism) or Student *t* test (Excel) assuming equal variance, unless otherwise stated. Statistical analyses of clinical data were performed in SAS version 9.2 or R version 3.6. The expression quantitative trait loci (eQTL) effects were analyzed by ANOVA (GraphPad Prism). A P value ≤ 0.05 was considered statistically significant.

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

Rs7804356 in SKAP2 Predicts Glycemic Control in Patients With Newly Diagnosed T1D

To take previous genetic association studies a step further, we investigated if rs7804356 is associated with glycemic control the 1st year after diagnosis in a cohort consisting of 275 children with new-onset T1D (23). We found that

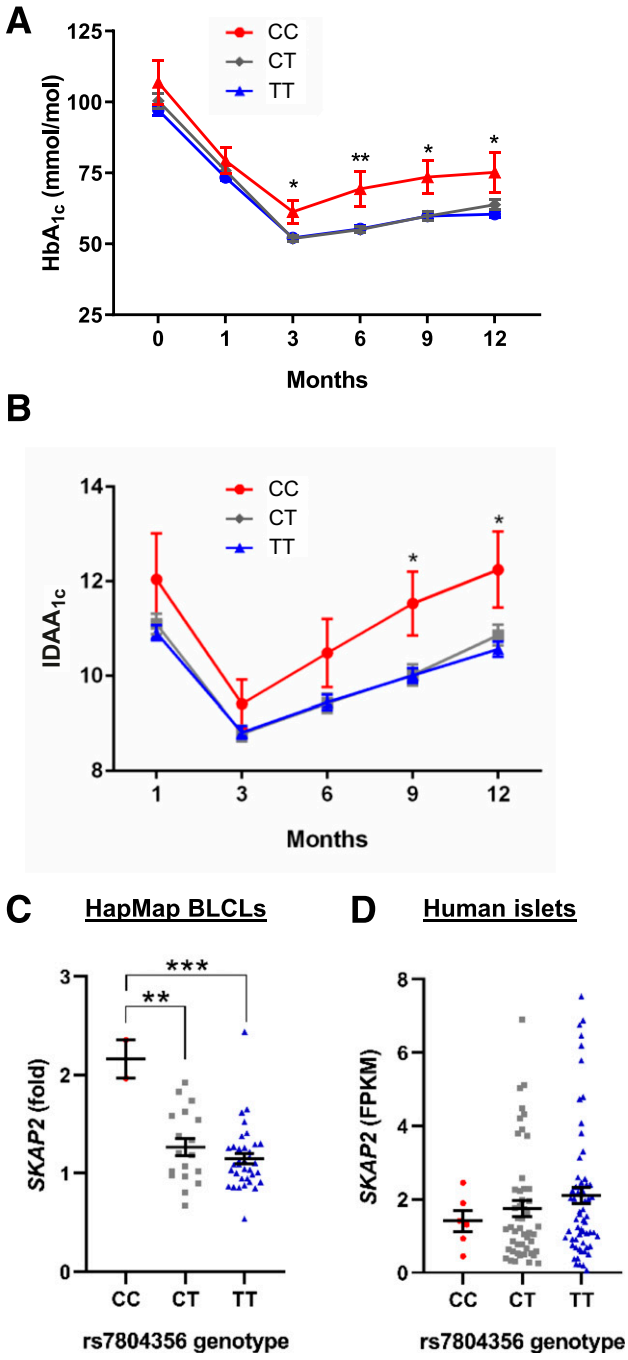

Figure 1—The T1D-associated SNP rs7804356 predicts residual β-cell function in children with newly diagnosed T1D. HbA_{1c} (mmol/mol) (*A*) and $IDAA_{1c}$ (*B*) in the Hvidoere cohort of newly diagnosed children with T1D according to rs7804356 genotype (red circles); CT (gray squares); and TT (blue triangles). Data are means ± SEM. The number of data points in each time point for each genotype was 230–245 for HbA_{1c} and 214–244 for $IDAA_{1c}$. *C*: *SKAP2* expression (fold change) in HapMap BLCLs according to rs7804356 genotype. The genotype distribution was CC, 2 (red); CT, 18 (gray); and TT, 35 (blue). *SKAP2* expression was determined by real-time quantitative PCR and normalized to the geometric mean of *ACTB*, *GAPDH*, and *UBC*. Data are means ± SEM. *D*: *SKAP2* expression (FPKM) in isolated human islets according to rs7804356 genotype. The genotype distribution was CC, 6 (red); CT, 50 (gray); and TT, 62 (blue). Data are means ± SEM. In *A* and *B*, $^*P < 0.05$, $^{**}P < 0.01$ indicate significance levels between CC and TT. In *C*, $^{**}P < 0.01$, $^{***}P < 0.001$. $IDAA_{1c}$, insulin dose-adjusted HbA_{1c}.

the C allele of rs7804356 was significantly associated with higher HbA_{1c} (millimoles per mole and percentage) levels at 3, 6, 9, and 12 months after diagnosis, indicating poorer glycemic control (Fig. 1*A* and Supplementary Fig. 1). After removing the 16% non-Caucasians from the data, we still found a significant association between the C allele and higher HbA_{1c} at 6 ($P = 0.047$) and 12 ($P = 0.012$) months and strong trends at 3 ($P = 0.057$) and 9 ($P = 0.062$) months. $IDAA_{1c}$, which is an estimate of residual β-cell function (24), was also significantly increased in carriers of the C allele at 9 and 12 months after disease onset (Fig. 1*B*). These findings show that rs7804356 genotypes can predict glycemic control and residual β-cell function in the 1st year after T1D diagnosis and strengthen a possible causal role of SKAP2 in T1D.

Rs7804356 Genotypes Affect SKAP2 Expression in Different Tissues

A recent study showed that rs7804356 genotypes correlate with the *SKAP2* expression level in immune cells (16). We confirmed an eQTL effect of rs7804356 on *SKAP2* expression in Epstein-Barr virus–transformed BLCLs from the HapMap Consortium with lower *SKAP2* expression in carriers of the T allele ($P = 0.0006$) (Fig. 1*C*). We then examined whether rs7804356 genotypes affect the expression level of *SKAP2* in human pancreatic islets using publicly available data performed on 118 individual islet preparations (26). There were no significant differences in *SKAP2* expression in human islets from donors carrying the different rs7804356 genotypes ($P = 0.3988$) (Fig. 1*D*). We next looked into possible eQTL effects of rs7804356 in other tissues as well using the public resource GTex Portal (https://gtexportal.org). We found an eQTL effect of the SNP on *SKAP2* in whole blood, pancreas, lymphocytes, and fibroblasts (Supplementary Table 1). Interestingly, as seen in Supplementary Table 1, rs7804356 also had an eQTL effect on various nearby HOXA genes in blood and fibroblasts. Collectively, these results suggest that rs7804356 genotypes are associated with altered *SKAP2* expression putatively in a tissue-specific manner.

Proinflammatory Cytokines Modulate SKAP2 Expression

We recently reported that exposure of human islets to proinflammatory cytokines for 48 h downregulates *SKAP2* expression at the mRNA level (22). We further characterized SKAP2 expression and regulation by cytokine rat insulin-secreting INS-1E cells and isolated rat and human islets. In INS-1E cells, IL-1β plus IFN-γ suppressed SKAP2 both at the mRNA and protein levels after treatment for 24 h (Fig. 2*A* and *B*). In rat islets, IL-1β plus IFN-γ did not

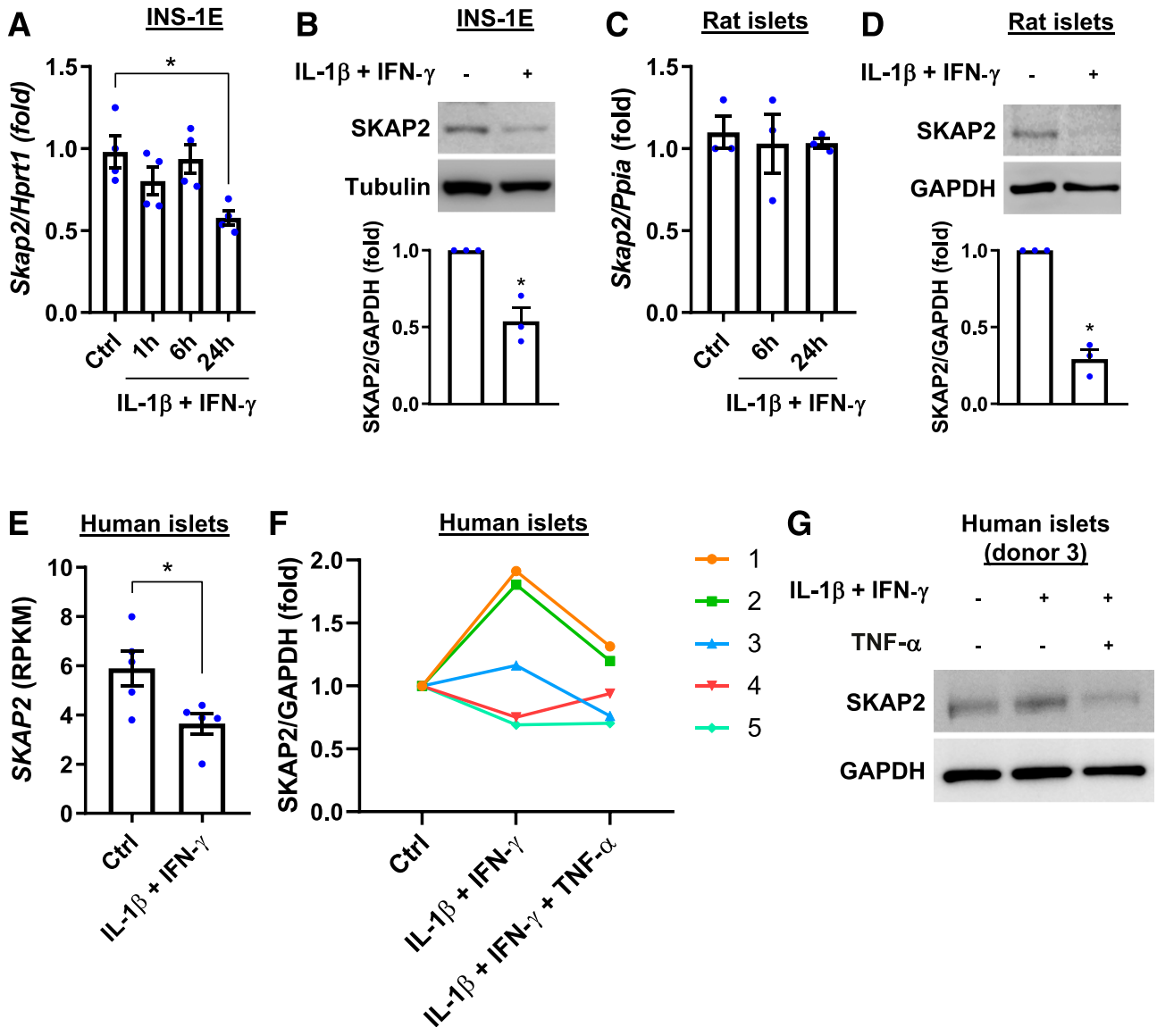

Figure 2—Effects of cytokines on SKAP2 expression. *A*: INS-1E cells were left untreated or exposed to cytokines (150 pg/mL mouse IL-1β plus 5 ng/mL rat IFN-γ) for 1, 6, or 24 h. *Skap2* mRNA expression was determined by real-time quantitative PCR and normalized to the housekeeping gene *Hprt1*. Data are means ± SEM of $n = 4$. *B*: Cell lysates from INS-1E cells left untreated or exposed to cytokines for 24 h were subjected to immunoblotting of SKAP2 and tubulin. Data are means ± SEM of $n = 3$. Representative blots are shown. *C*: Isolated rat islets were left untreated or exposed to cytokines (150 pg/mL mouse IL-1β plus 5 ng/mL rat IFN-γ) for 6 or 24 h. *Skap2* mRNA expression was determined by real-time quantitative PCR and normalized to *Ppia*. Data are means ± SEM of $n = 3$. *D*: Lysates from rat islets left untreated or exposed to cytokines for 24 h were subjected to immunoblotting of SKAP2 and GAPDH. Data are means ± SEM of $n = 3$. Representative blots are shown. *E*: Human islet *SKAP2* expression data extracted from a published RNA-sequencing study (36). Data are means ± SEM of $n = 5$. *F*: Lysates from five individual human islet preparations left untreated or exposed to cytokines (50 units/mL human IL-1β plus 1,000 units/mL human IFN-γ with or without 1,000 units/mL TNF-α) for 24 h were subjected to immunoblotting of SKAP2 and GAPDH. SKAP2 protein was normalized to GAPDH from each of the five human islet preparations (1–5). *G*: Representative blots from *F* are shown (donor 3). *$P < 0.05$ vs. control (Ctrl). RPKM, reads per kilobase of transcript per million mapped reads.

affect the mRNA expression but caused a marked reduction in SKAP2 at the protein level after cytokine exposure (Fig. 2*C* and *D*). Extraction of RNA-sequencing data from a published study (36) confirmed that a 24-h exposure to cytokines suppressed *SKAP2* mRNA expression in human islets (Fig. 2*E*). At the protein level, cytokines downregulated SKAP2 in three out of five individual islet preparations following exposure to cytokines for 24 h (Fig. 2*F* and *G*).

SKAP2 Has Antiapoptotic Activity in β-Cells

We investigated if SKAP2 regulates β-cell apoptosis in response to cytokines by using an siRNA knockdown (KD) approach. Transfection of INS-1E cells with a pool of siRNAs targeting *SKAP2* (siSKAP2) decreased *SKAP2* expression by ∼65% (Fig. 3*A*) and augmented both basal and cytokine-induced apoptosis (Fig. 3*B*), as compared with INS-1E cells transfected with a nontargeting negative

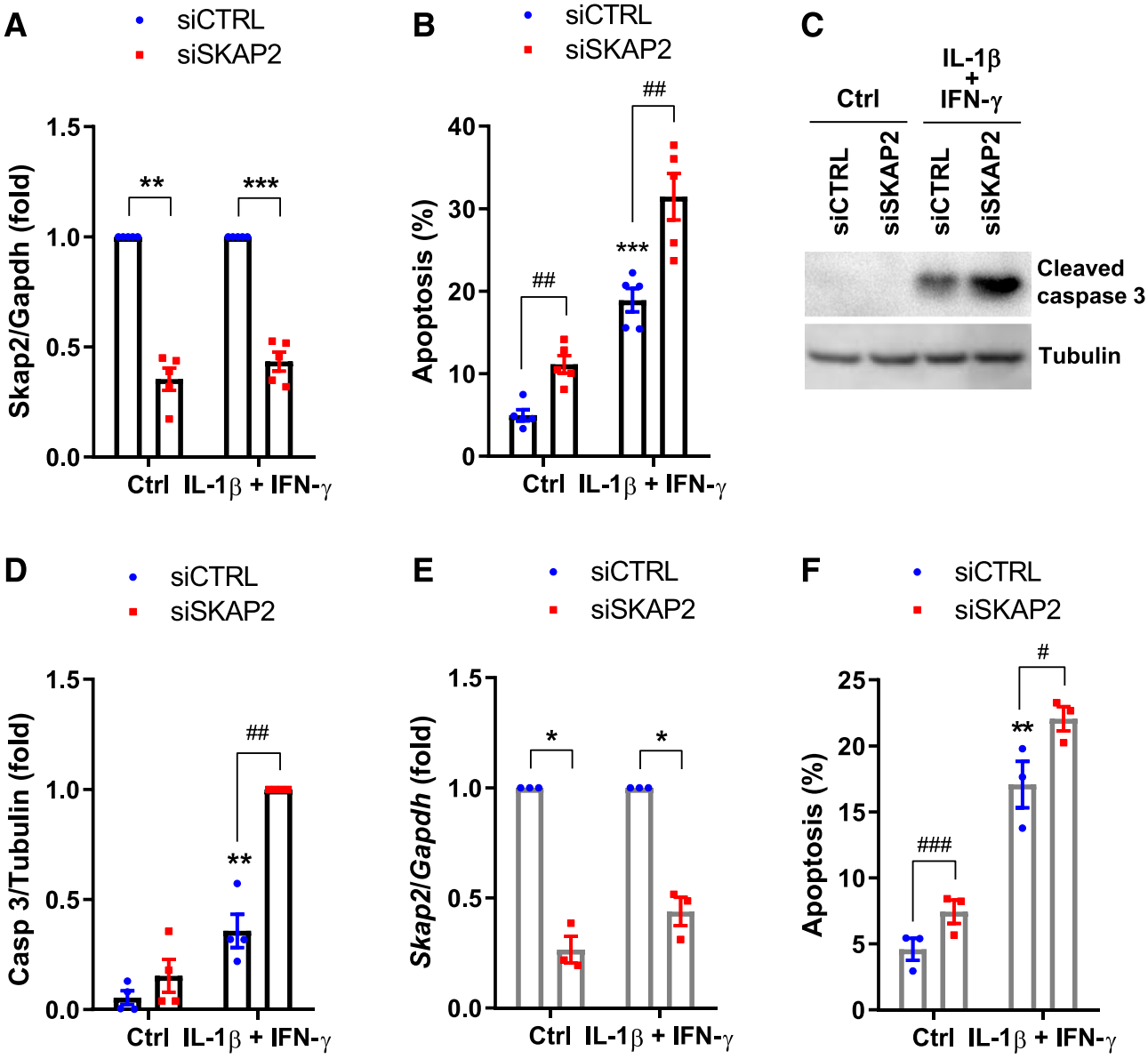

Figure 3—Effects of SKAP2 KD on cytokine-induced apoptosis. *A*: INS-1E cells were transfected with siCTRL or siRNA against SKAP2 (siSKAP2). Cells were left untreated or exposed to cytokines (500 pg/mL human IL-1β plus 36 ng/mL rat IFN-γ) for 16 h. *Skap2* mRNA expression was determined by real-time quantitative PCR and normalized to *Gapdh*. Data are presented as means ± SEM of $n = 5$. INS-1E cells transfected and treated as in *A* were subjected to measurement of apoptosis by Hoechst/PI staining (*B*) or immunoblotting of cleaved caspase (Casp) 3 (*C* and *D*). Data are means ± SEM of $n = 4$–5. Representative blots are shown. Purified primary rat β-cells were transfected as in *A* and left untreated or exposed to cytokines (2.5 ng/mL human IL-1β plus 72 ng/mL rat IFN-γ) for 24 h, after which *Skap2* mRNA expression (*E*) and apoptosis (*F*) were determined by real-time quantitative PCR and Hoechst/PI staining, respectively. *Skap2* expression was normalized to *Gapdh*. Data are means ± SEM of $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. siCTRL control (Ctrl); #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$.

control siRNA (siCTRL). These results were verified by a significant increase in cytokine-induced caspase 3 cleavage in *SKAP2*-silenced INS-1E cells (Fig. 3*C* and *D*). In FACS-purified primary rat β-cells, KD of SKAP2 also caused an increase in the apoptosis rate, further demonstrating an antiapoptotic function of SKAP2 (Fig. 3*E* and *F*). Finally, experiments performed using the recently established human hybrid cell line 1.1B4 (30) also revealed increased cytokine-induced apoptosis following SKAP2 KD despite that cytokines modestly upregulated SKAP2 expression in these cells (Supplementary Fig. 1).

To further establish an antiapoptotic function of SKAP2 in β-cells, we generated two clones (referred to as #A and #B) of INS-1E cells with stable overexpression of HA-tagged SKAP2 (Fig. 4*A*). Cytokine-induced apoptosis was reduced in both SKAP2-overexpressing clones compared with INS-1E cells transfected with an empty vector (pcDNA) (Fig. 4*B*). These results were substantiated by the

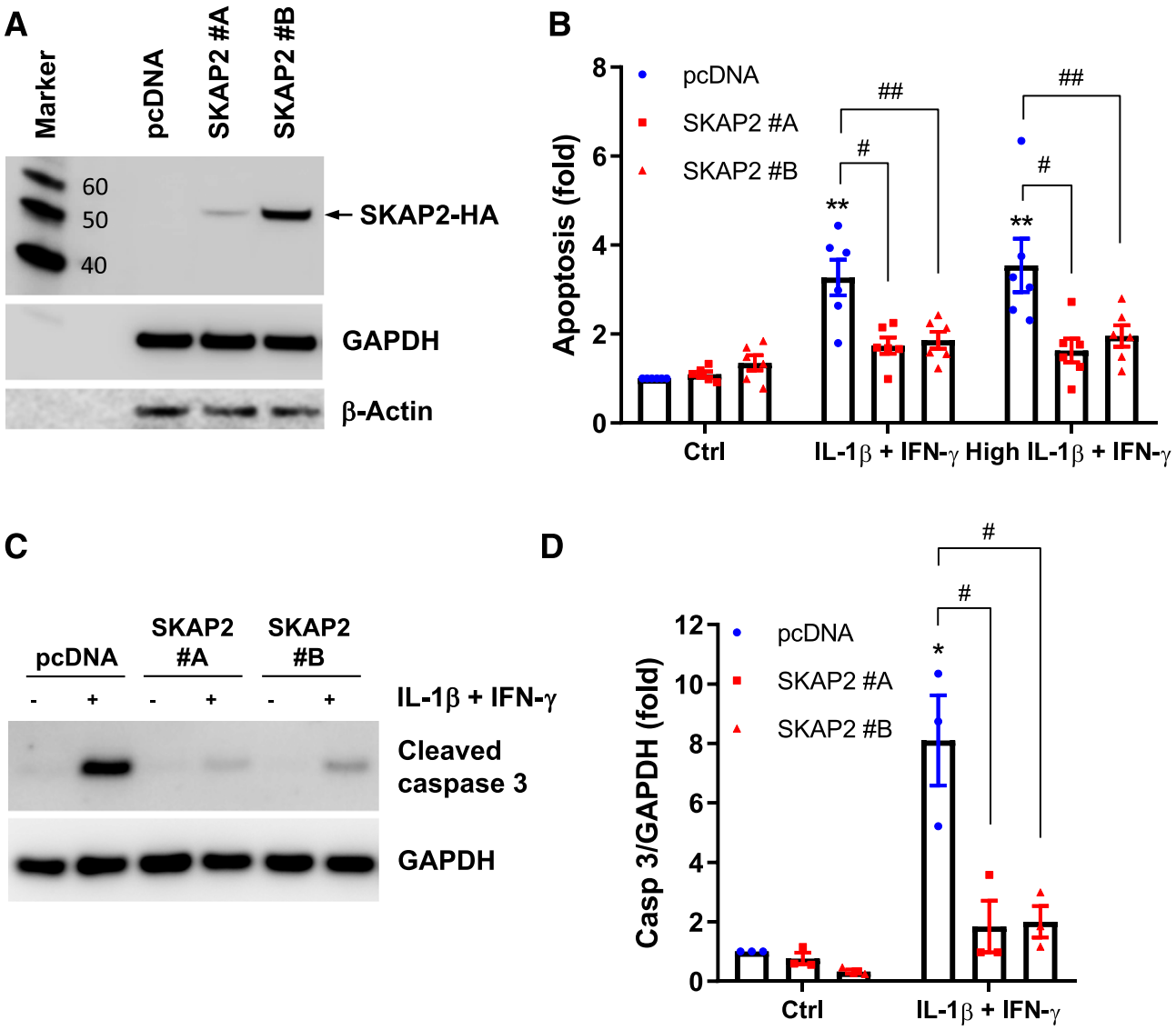

Figure 4—Effects of SKAP2 overexpression on cytokine-induced apoptosis. *A*: Immunoblot analysis using an anti-HA antibody of lysates from a pool of INS-1E cells stably transfected with an empty vector (pcDNA) or from individual clones (#A and #B) of INS-1E cells stably transfected with HA-tagged SKAP2. *B*: INS-1E pcDNA control cells and SKAP2-overexpressing clones (#A and #B) were left untreated or exposed to two different doses of cytokines (IL-1β plus IFN-γ: 150 pg/mL mouse IL-1β [mIL-1β] plus 5 ng/mL rat IFN-γ, and high IL-1β plus IFN-γ: 500 pg/mL IL-1β plus 36 ng/mL IFN-γ) for 16 h followed by measurement of apoptotic cell death by ELISA-based detection of cytoplasmic nucleosomes. Data are means ± SEM of $n = 6$. *C* and *D*: INS-1E pcDNA cells and SKAP2-overexpressing clones (#A and #B) were left untreated or exposed to 150 pg/mL mouse IL-1β plus 5 ng/mL rat IFN-γ for 16 h. Cell lysates were subjected to immunoblotting of cleaved caspase (Casp) 3 and GAPDH. Data are means ± SEM of $n = 3$. Representative blots are shown. $^*P < 0.05$, $^{**}P < 0.01$ vs. siCTRL control (Ctrl); $\#P < 0.05$; $\#\#P < 0.01$.

observation that SKAP2 overexpression suppressed cytokine-induced caspase 3 cleavage (Fig. 4*C* and *D*). Interestingly, although clone #A and #B expressed different levels of HA-tagged SKAP2 (Fig. 4*A*), both clones were protected to a similar degree against the detrimental effects of the cytokines.

SKAP2 Reduces iNOS Expression and Nuclear Phosphorylation of NF-κB p65

An important event in cytokine signaling is the induction of iNOS, leading to generation of nitric oxide (NO), which contributes to β-cell apoptosis via endoplasmic reticulum (ER) stress and potentiation of cytokine signaling (37–39). We therefore examined if overexpression of SKAP2 affected cytokine-induced iNOS expression and NO production in INS-1E cells. Cytokine-induced NO production was decreased in the SKAP2-overexpressing clones (#A and #B) compared with the pcDNA control cells (Fig. 5*A*), which correlated with lower iNOS expression (Fig. 5*B–D*).

As NF-κB and STAT1 are the primary activators of iNOS expression in β-cells exposed to IL-1β and IFN-γ (40,41), we next investigated if SKAP2 modulates cytokine-induced

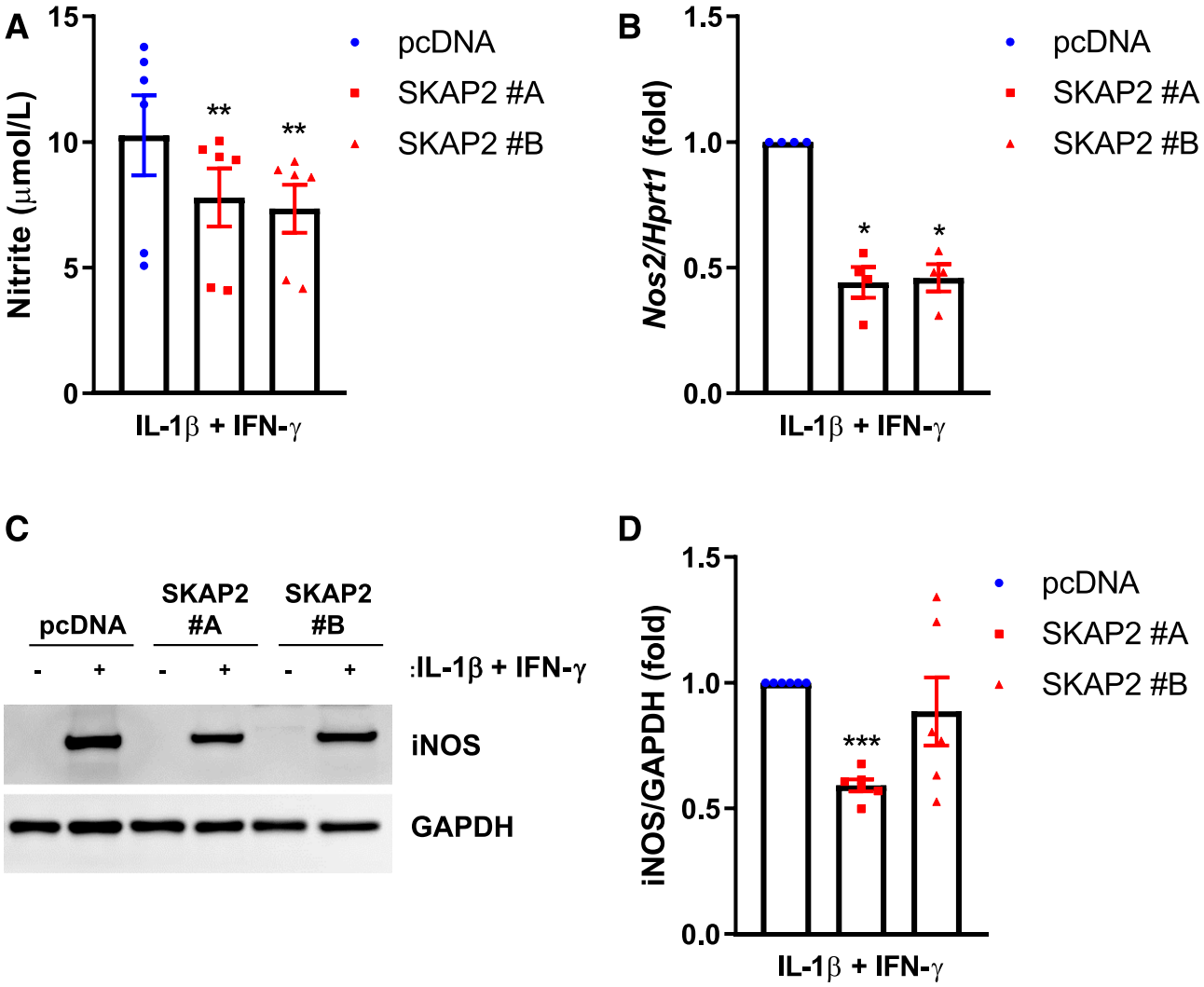

Figure 5—Effects of SKAP2 overexpression on cytokine-induced iNOS. *A*: INS-1E pcDNA control cells and SKAP2-overexpressing clones (#A and #B) were exposed to 150 pg/mL mouse IL-1β plus 5 ng/mL rat IFN-γ for 16 h. Accumulated nitrite (μmol/L) in the culture medium was measured by Griess assay. Data are means ± SEM of $n = 6$. *B*: INS-1E pcDNA cells and SKAP2-overexpressing clones (#A and #B) treated as in *A* for 4 h were analyzed for iNOS mRNA (*Nos2*) expression by real-time quantitative PCR and normalized to *Hprt1*. Data are means ± SEM of $n = 4$. *C* and *D*: Lysates from INS-1E pcDNA cells and SKAP2-overexpressing clones (#A and #B) treated as in *A* for 6 h were subject to immunoblotting of iNOS and GAPDH. Data are means ± SEM of $n = 6$. Representative blots are shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. pcDNA.

Ser536 phosphorylation of NF-κB p65 (P-p65) and Tyr701 phosphorylation of STAT1 (P-STAT1) in INS-1E cells. For this purpose, we performed experiments on INS-1E cells transiently transfected to overexpress SKAP2 or an empty pcDNA vector to substantiate the results obtained using the individual clones. Efficient transient transfection was demonstrated by transfecting with a plasmid encoding GFP, which resulted in a transfection rate of ∼40% (Fig. 6*A*). As observed for the stable SKAP2-overexpressing clones, transient overexpression of SKAP2 decreased cytokine-induced apoptosis, as measured by reduced cytokine-mediated caspase 3 cleavage (Fig. 6*B* and *C*). Nuclear, but not cytosolic extracts from SKAP2-transfected INS-1E cells showed a marked reduction (49%) in P-p65 and a minor (17%) decrease in P-STAT1 compared with pcDNA-transfected control cells (Fig. 6*D* and *E*). These results suggest that SKAP2 dampens proximal signaling via NF-κB.

SKAP2 Decreases Cytokine-Induced CHOP Expression

Because NO contributes to cytokine-induced apoptosis by inducing ER stress (42), we next examined if SKAP2 modulates the expression of CHOP, a classical marker of ER stress (43), as would be anticipated based on the inhibitory effect on iNOS expression and NO formation. Comparison of the expression of CHOP between the SKAP2-overexpressing INS-1E cells and the pcDNA control cells in response to cytokine exposure revealed diminished IL-1β plus IFN-γ–induced CHOP protein expression in the SKAP2-overexpressing cells (Fig. 7*A* and *B*). Notably, as

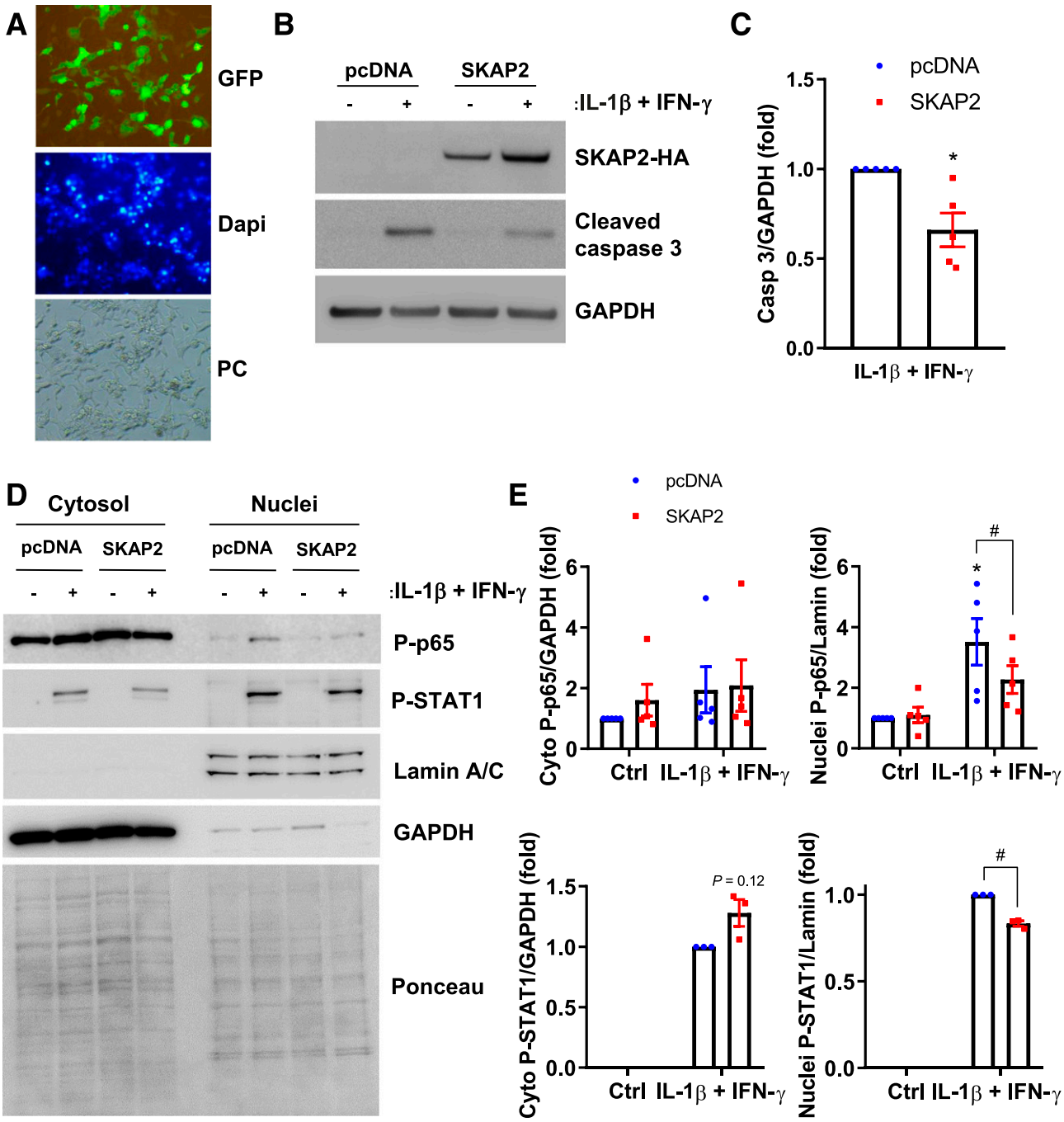

Figure 6—Effects of SKAP2 overexpression on phosphorylated p65–NF-κB and STAT1. *A*: INS-1E cells were transiently transfected with an expression plasmid encoding GFP to verify efficient transfection by microscopy. Nuclei staining was done by DAPI. *B* and *C*: INS-1E cells were transiently transfected with an empty vector (pcDNA) or a plasmid encoding HA-tagged SKAP2 before being left untreated or exposed to 150 pg/mL mouse IL-1β plus 5 ng/mL rat IFN-γ for 18 h. Lysates were subject to immunoblotting of HA-tagged SKAP2, cleaved caspase (Casp) 3, and GAPDH (*C*). Data are means ± SEM of $n = 5$. *D* and *E*: Cytosolic and nuclear extracts from INS-1E cells transfected and treated as in *B* for 60 min were prepared and subjected to immunoblotting of P-p65 and P-STAT1. Immunoblotting of Lamin A/C and GAPDH was used as cytosolic and nuclear markers, respectively. Total protein was stained by Ponceau. Representative blots are shown. Data are means ± SEM of $n = 3$–5. *$P < 0.05$ vs. pcDNA control (Ctrl); #$P < 0.05$.

opposed to overexpression, KD of SKAP2 did not modulate cytokine-induced CHOP expression (Fig. 7*C*). To determine the contribution of ER stress signaling via CHOP to SKAP2-dependent apoptosis regulation, we performed double-KD experiments in INS-1E cells in which SKAP2 and CHOP were silenced by siRNAs. Using this approach, it was observed that KD of CHOP abrogated the increase in cytokine-mediated apoptosis caused by KD of SKAP2 alone

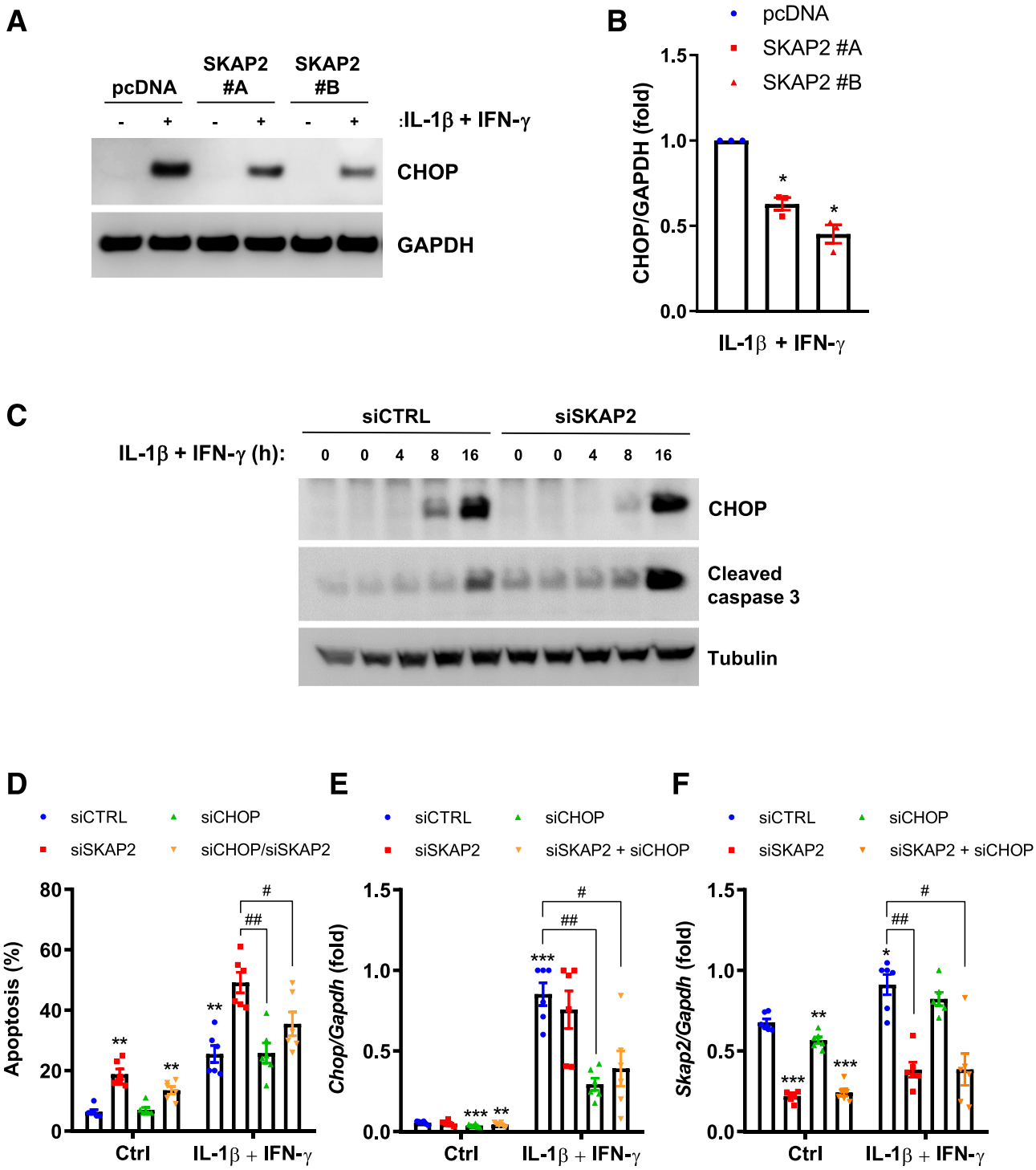

Figure 7—Association between SKAP2 and cytokine-induced CHOP. *A* and *B*: INS-1E pcDNA cells and SKAP2-overexpressing clones (#A and #B) were exposed to 150 pg/mL mouse IL-1β plus 5 ng/mL rat IFN-γ for 16 h. Lysates were subjected to immunoblotting of CHOP and GAPDH. Representative blots are shown. Data are means ± SEM of $n = 3$. *C*: INS-1E cells were transfected with siCTRL or siRNA against SKAP2 (siSKAP2). Cells were left untreated or exposed to cytokines (500 pg/mL human IL-1β plus 36 ng/mL rat IFN-γ) for the indicated time periods, after which CHOP and cleaved caspase 3 were analyzed by immunoblotting. Tubulin was used as loading control. Representative blots of three experiments are shown. INS-1E cells transfected as in *C* and exposed to cytokines for 16 h were analyzed for apoptosis (*D*), *Chop* mRNA expression (*E*), and *Skap2* mRNA expression (*F*). Data are means ± SEM of $n = 5$–6. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. pcDNA control (Ctrl)/cytokines; #$P < 0.05$; ##$P < 0.01$.

(Fig. 7*D*–*F*). Collectively, these results suggest that SKAP2 via regulatory effects on NF-κB and iNOS regulates cytokine-induced apoptosis in a CHOP-dependent manner. Interestingly, we also observed that apoptosis induced by thapsigargin, an inhibitor of the sarco- and ER calcium ATPase leading to ER calcium depletion and ER stress (44),

was significantly lower in SKAP2-overexpressing cells as compared with control cells (Supplementary Fig. 2).

DISCUSSION

In this study, we examined if the T1D candidate gene *SKAP2* has any functional roles in pancreatic β-cells relevant for the immune-mediated β-cell destruction observed in T1D. We show that the T1D-associated SNP rs7804356 in *SKAP2* is associated with glycemic control and residual β-cell function in the 1st year after diagnosis in children with new-onset T1D.

rs7804356 is located in the third intron of *SKAP2* and therefore does not affect the coding part of the gene. A recent study, however, demonstrated decreased *SKAP2* expression in B and T cells from patients with T1D carrying the T allele of rs7804356 (16), which we confirmed in HapMap BLCLs. The publicly available data on human islet eQTLs, which we used in this study, suggest that rs7804356 genotypes do not significantly modulate *SKAP2* expression in human pancreatic islets; however, if anything, the effect appears to be in the opposite direction (i.e., lowest *SKAP2* expression in carriers of the C allele of rs7804356). Considering that T is the risk allele, our finding is somewhat surprising and suggests complexity of how variation at the *SKAP2* locus affects disease risk in different tissues. Potentially, rs7804356 or other variants in the *SKAP2* locus may significantly affect *SKAP2* expression in islets under inflammatory conditions or in islets under direct immune attack preceding clinical T1D. Noteworthy, SKAP2 could have a dual role by acting both in the target tissue (the β-cells) and the effector tissue (the immune cells) in T1D. These effects could be counterregulatory (i.e., whereas higher expression of *SKAP2* has a protective effect in β-cells, higher *SKAP2* expression in immune cells could cause a more aggressive immune attack against the β-cells). If true, this makes the involvement of SKAP2 in T1D more complicated, but this may be what is reflected at the HbA_{1c}/$IDAA_{1c}$ data for the rs7804356 genotypes. That lower *SKAP2* expression is associated with more fragile β-cells is supported by the observation that cytokines suppressed SKAP2 at the protein level in INS-1E cells and rat islets and in most human islet preparations examined. The inconsistency observed in human islets, in which cytokines suppressed SKAP2 at the protein level in three out of five islet preparations underlines the natural heterogeneity of human islets as compared with rodents. Unfortunately, we do not have genotype information on the human islets used in this study, but potentially this may have impacted the effects of cytokines on SKAP2 protein expression.

Interestingly, the clinical data are supported by our in vitro data, which demonstrate an important apoptosis-regulatory role of SKAP2 in β-cells. KD of SKAP2 caused increased caspase activation and apoptosis in response to cytokines, whereas overexpression of SKAP2 protected against the deleterious effects of cytokines in β-cells. The potentiating effect of silencing SKAP2 on cytokine-induced apoptosis in INS-1E cells and primary rat β-cells together with the observed protective effect of overexpression of SKAP2 on cytokine-induced apoptosis in INS-1E cells are, to the best of our knowledge, the first evidence that SKAP2 possesses apoptosis-regulatory properties. Only limited information exists on the biological role of SKAP2. However, as mentioned, SKAP2 interacts with actin and seems important for actin organization (20,21,34,45). In this regard, it is interesting to note that reorganization of actin filaments is correlated to apoptotic cell death (46). Thus, it should be investigated if the antiapoptotic activity of SKAP2 is associated with an actin-regulatory effect in β-cells.

We found that the antiapoptotic effect of SKAP2 was accompanied by lower levels of phosphorylated NF-κB p65 and STAT1 in the nucleus, possibly accounting for the diminished iNOS expression and NO production. This suggests that SKAP2 regulates proximal cytokine signaling upstream of NF-κB and possibly also STAT1. This is conceivable with SKAP2 being an Src kinase substrate thereby involved in proximal signal transduction (17). Src kinases or Src-related kinases, including YES and FRK, have previously been reported to be expressed and functionally important in β-cells and islets (47,48).

Our data also show that SKAP2 overexpression correlates with lower CHOP induction following exposure to cytokines, suggesting reduced ER stress. Interestingly, our double-KD experiments revealed that the increased apoptosis level caused by KD of SKAP2 alone was partly reversed by simultaneous KD of CHOP, indicating that ER stress signaling via CHOP is involved in SKAP2-regulated apoptosis. Whereas overexpression of SKAP2 decreased cytokine-induced CHOP expression, KD of SKAP2 failed to increase the cytokine-induced CHOP level in INS-1E cells. This could be due to the finding that cytokines downregulate SKAP2 expression in the cells and further KD of SKAP2 by siRNA is insufficient to further modulate cytokine-induced CHOP expression. The fact that we observed that SKAP2 overexpression protected from both cytokine-induced and thapsigargin-induced apoptosis suggests that the antiapoptotic effect of SKAP2 is not limited to controlling proximal cytokine signaling events, but also regulates apoptosis-promoting mechanisms operating at or above the ER stress level.

Cytokines suppressed the mRNA level of *SKAP2* in INS-1E cells and human islets, but not in rat islets. In contrast, cytokines downregulated SKAP2 at the protein level in both INS-1E cells and rat islets, but not in human islets. These deviating observations could partly be due to species differences in sensitivity to cytokines and/or due to differences in the specific culturing and cytokine conditions and concentrations. However, it could also indicate that the regulation of SKAP2 in β-cells/islets is highly complex and involves regulatory mechanisms acting posttranscriptionally yet to be defined. Such mechanisms could include noncoding RNAs, including micro RNAs. Indeed, cytokines modulate miRNA expression in β-cells (49), some of which could target SKAP2 mRNA for translational inhibition.

However, further experiments are needed to clarify if miRNAs target SKAP2 in β-cells.

In conclusion, our study describes SKAP2 as a novel regulator of β-cell apoptosis. Prediction of the course of the residual β-cell function and glycemic control during disease progression in patients with T1D is important. The observation that rs7804356 in *SKAP2* is associated with glycemic control and residual β-cell function in children with new-onset T1D suggests that this SNP can serve as a predictive marker of β-cell function in at-risk and recent-onset individuals.

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