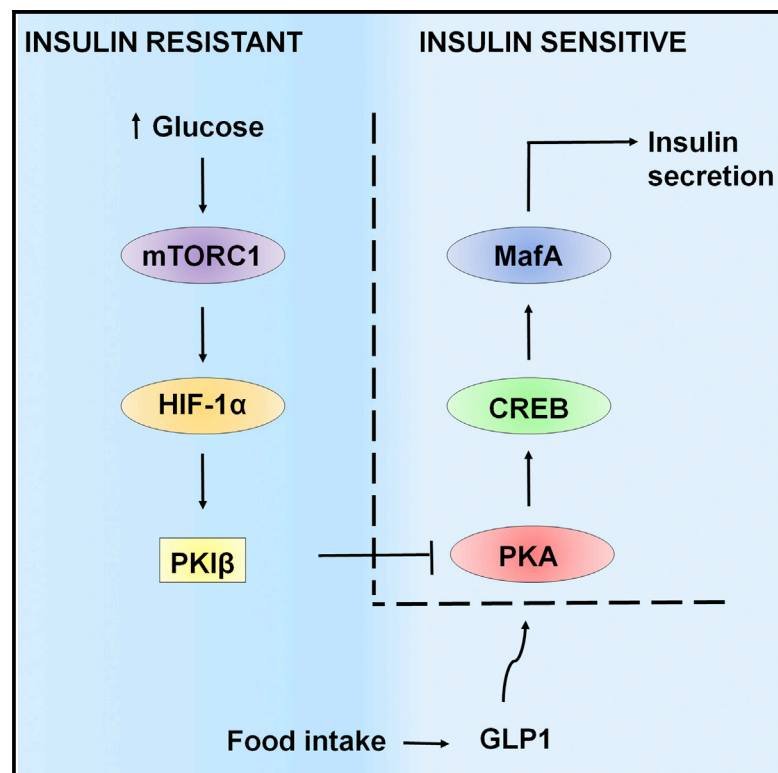


Feedback Inhibition of CREB Signaling Promotes Beta Cell Dysfunction in Insulin Resistance

Graphical Abstract



Authors

Emilie Blanchet, Sam Van de Velde, ..., Klaus Kaestner, Marc Montminy

Correspondence

montminy@salk.edu

In Brief

Blanchet et al. find that the CREB pathway promotes insulin secretion via induction of the transcription factor MafA. Beta cell CREB activity is disrupted in chronic hyperglycemia due to upregulation of the protein kinase A inhibitor beta and to consequent reductions in MafA expression.

Highlights

- CREB and CRTC2 promote insulin secretion by stimulating MafA expression
- Beta cell CREB activity is disrupted in insulin resistance
- Chronic hyperglycemia induces pancreatic islet PKIβ expression
- Disruption of the PKIβ gene improves islet function in insulin resistance

Accession Numbers

GSE60158



Feedback Inhibition of CREB Signaling Promotes Beta Cell Dysfunction in Insulin Resistance

Emilie Blanchet,¹ Sam Van de Velde,¹ Shigenobu Matsumura,¹ Ergeng Hao,¹ John LeLay,^{1,2} Klaus Kaestner,^{1,2} and Marc Montminy^{1,*}

¹Peptide Biology Laboratories, Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA

²Department of Genetics, Institute for Diabetes, Obesity and Metabolism, University of Pennsylvania School of Medicine, 3400 Civic Center Boulevard, Philadelphia, PA 19104-5156, USA

*Correspondence: montminy@salk.edu

<http://dx.doi.org/10.1016/j.celrep.2015.01.046>

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

SUMMARY

Although persistent elevations in circulating glucose concentrations promote compensatory increases in pancreatic islet mass, unremitting insulin resistance causes deterioration in beta cell function that leads to the progression to diabetes. Here, we show that mice with a knockout of the CREB coactivator CRTC2 in beta cells have impaired oral glucose tolerance due to decreases in circulating insulin concentrations. CRTC2 was found to promote beta cell function in part by stimulating the expression of the transcription factor MafA. Chronic hyperglycemia disrupted cAMP signaling in pancreatic islets by activating the hypoxia inducible factor (HIF1)-dependent induction of the protein kinase A inhibitor beta (PKIB), a potent inhibitor of PKA catalytic activity. Indeed, disruption of the PKIB gene improved islet function in the setting of obesity. These results demonstrate how crosstalk between nutrient and hormonal pathways contributes to loss of pancreatic islet function.

INTRODUCTION

The second messenger cAMP has been shown to mediate effects of incretin hormones, in part through induction of the Ser/Thr protein kinase PKA. Blocking cAMP signaling in beta cells through targeted disruption of the Gs α subunit of the heterotrimeric G protein leads to severe glucose intolerance and beta cell apoptosis (Xie et al., 2007). Conversely, mutations that increase PKA activity, either via disruption of the R1 α regulatory subunit (Song et al., 2011) or via a gain-of-function mutation in the PKA catalytic subunit (Kaiharu et al., 2013), enhances insulin secretion.

cAMP promotes cellular gene expression via the PKA-mediated phosphorylation of the CREB family of activators and via the de-phosphorylation of the cAMP-regulated transcriptional coactivators (CRTC2). Following its activation, CREB has been found to promote islet function in part by upregulating the insulin

receptor substrate 2 (IRS2) in beta cells (Jhala et al., 2003; Park et al., 2006). Although deletion of the single family member CREB1 alone has no effect on beta cell function under normal chow conditions (Shin et al., 2014), expression of a dominant-negative CREB polypeptide A-CREB, which blocks all three family members (CREB1, ATF1, and CREM), leads to severe hyperglycemia due in part to reductions in glucose-stimulated insulin secretion (Jhala et al., 2003).

IRS2-dependent increases in insulin signaling are thought to promote islet growth through the activation of mTORC1 complexes and subsequent induction of the hypoxia-inducible factor HIF1 (Van de Velde et al., 2011; Gunton et al., 2005). Although the mTORC1-HIF pathway appears to be critical for adaptive expansion of pancreatic islet mass, beta cell function deteriorates in the setting of chronic insulin resistance (Zhao et al., 1998). Based on the ability for GLP1 agonists to improve beta cell function in this setting, we examined the potential role of CREB and CRTC2 in mediating these effects.

RESULTS

Exposure of INS1 insulinoma cells to the cAMP agonist forskolin (FSK) promoted the phosphorylation of CREB at Ser133 and the de-phosphorylation of CRTC2 within 30 min (Figure 1A). By contrast, exposure to depolarizing concentrations of KCl (40 mM) stimulated CREB phosphorylation to a lesser extent. Under basal conditions, CRTC2 was sequestered in the cytoplasm of beta cells from cultured islets; exposure to FSK triggered CRTC2 nuclear translocation (Figure 1B).

GLP1 and other incretin hormones are released from intestinal cells only following nutrient ingestion (Holst et al., 2011), so we analyzed effects of oral glucose tolerance testing (OGTT) in mice with a beta-cell-specific knockout of CRTC2 (MIP-T2KO; Figure S1A). Although they were almost comparable to control littermates by intraperitoneal glucose tolerance test (IPGTT), MIP-T2KO mice showed impaired glucose tolerance by OGTT test (Figures 1C and S1B). Indeed, we observed similar differences between control and MIP-T2KO mice following intraperitoneal (i.p.) injection of exendin-4 in addition to glucose (Figure S1C). Arguing against an increase in insulin resistance, circulating glucose concentrations decreased identically in MIP-T2KO mice and control littermates following insulin

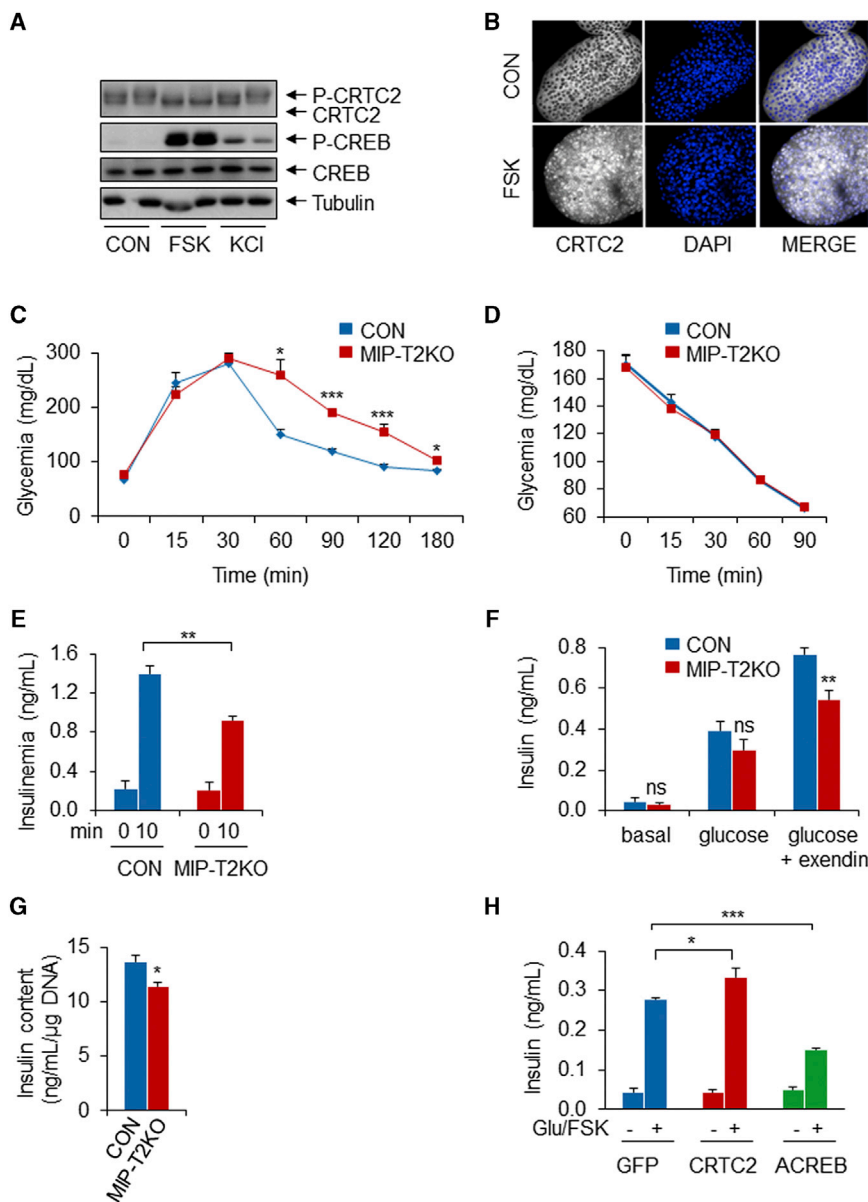


Figure 1. Impaired Glucose Tolerance in Mice with a Beta-Cell-Specific Knockout of CRTC2

(A and B) De-phosphorylation and nuclear translocation of CRTC2 in INS1 (A) and mouse pancreatic islets (B) following exposure to cAMP agonist forskolin (FSK) or KCl.

(C) Oral glucose tolerance testing (OGTT) of mice with a knockout of CRTC2 in beta cells (MIP-T2KO) relative to control littermates ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $n = 10$).

(D) Insulin tolerance testing of control and MIP-T2KO mice.

(E) Circulating insulin concentrations in MIP-T2KO and control littermates 10 min following administration of glucose ($**p < 0.01$; $n = 10$).

(F) Effect of glucose alone (20 mM) or plus exendin (10 nM) on insulin secretion from primary cultured islets of MIP-T2KO and control littermates ($**p < 0.01$; $n = 6$).

(G) Insulin content in pancreatic islets from CRTC2 mutant or control littermates ($*p < 0.05$; $n = 6$).

(H) Effect of CRTC2 or dominant-negative A-CREB overexpression on insulin secretion from INS1 cells ($*p < 0.05$; $**p < 0.001$; $n = 5$). Data are shown as mean \pm SEM.

administration (Figure 1D). Rather, MIP-T2KO mice secreted less insulin in response to oral glucose gavage, suggesting that beta cell function is disrupted in these animals (Figure 1E).

We evaluated effects of CRTC2 on insulin secretion from cultured islets in response to cAMP. Following 10 min exposure to exendin-4 plus high glucose (20 mM), insulin release from MIP-T2KO islets was reduced 30% (Figure 1F) relative to control islets. Exposure to glucose alone stimulated insulin secretion comparably in cultured islets from wild-type and MIP-T2KO mice (Figure 1F). Insulin (*Ins1* and *Ins2*) gene expression and protein content were also modestly decreased in MIP-T2KO islets (Figures 1G and S1D). Conversely, overexpression of CRTC2 in INS1 insulinoma cells enhanced insulin secretion in response to GLU/FSK. These effects were blocked by expression of a dominant-negative CREB polypeptide called A-CREB (Figure 1H).

(*Syt4*, *Syt5*, and *Syt7*), proteins that function as major calcium sensors for synaptic vesicles (Iezzi et al., 2004, 2005; Figure S1E).

Realizing the importance of MafA for insulin expression and secretion, we addressed the potential role of this transcription factor in mediating effects of CREB on beta cell function. Exposure of isolated mouse islets to FSK increased the expression of MafA (Figure S1F), which promotes beta cell maturation. Indeed, islet MafA expression was reduced by two-thirds in pancreatic islets from MIP-T2KO mice relative to control animals by immunohistochemical analysis (Figure 2B). As a result, beta cell maturity gene expression (*Glp1r*, *Pc*, and *Pdx1*) was also downregulated in CRTC2 mutant islets (Figure S1F). Conversely, CRTC2 overexpression in INS1 cells increased MafA expression (Figure 2C).

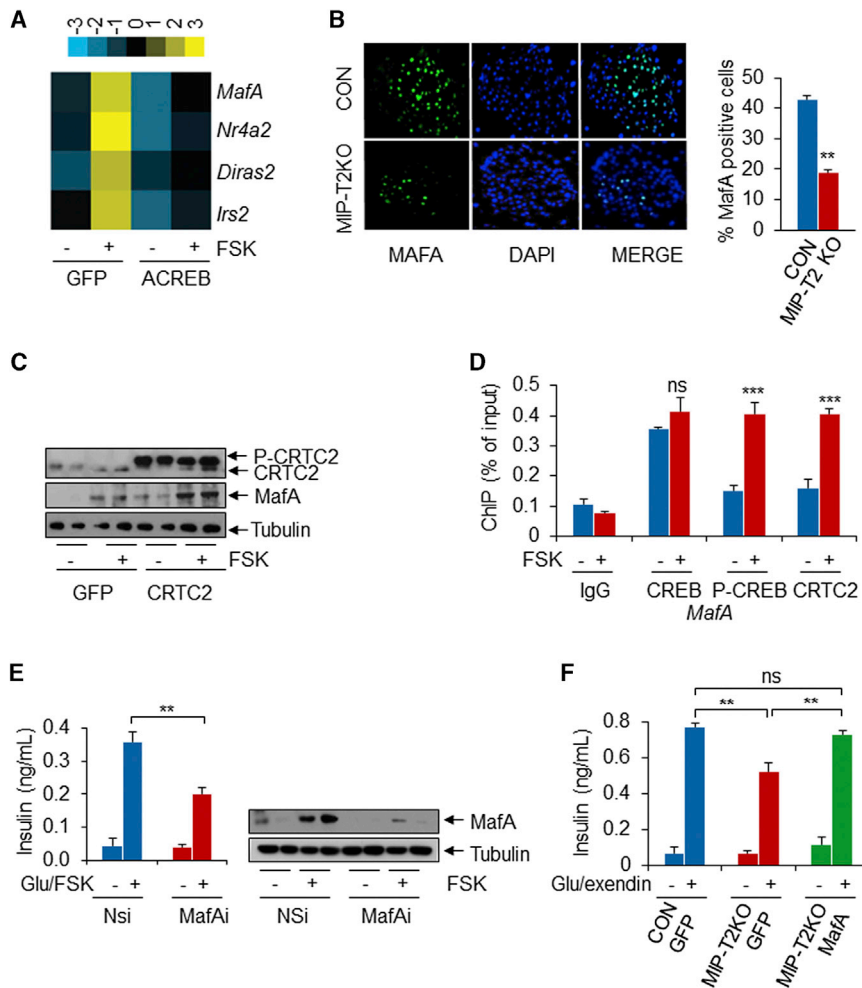


Figure 2. CRTC2 Stimulates MafA Expression

(A) RNA-seq analysis of INS1 cells exposed to FSK. Top genes upregulated 2-fold or better indicated. Effect of dominant-negative A-CREB expression shown. (B) Immunohistochemical assay of MafA staining in islets from pancreatic sections of control and MIP-T2KO mice. Right, relative number of MafA-positive cells in control and MIP-T2KO sections shown (** $p < 0.01$; $n = 5$). (C) Effect of CRTC2 overexpression on MafA protein amounts in INS1 cells. Exposure to FSK indicated. (D) Chromatin immunoprecipitation (ChIP) assay of INS1 cells showing amounts of CREB and CRTC2 recruited to the MafA promoter under basal conditions and following exposure to FSK (** $p < 0.001$; $n = 6$). (E) Effect of MafA depletion on insulin secretion from INS1 cells (* $p < 0.05$; ** $p < 0.01$; $n = 6$). (F) Effect of adenoviral GFP control or MafA overexpression on insulin secretion from MIP-T2KO mice islets compared to control (** $p < 0.01$; $n = 6$). Data are shown as mean \pm SEM.

We evaluated whether MafA is a direct target gene for CREB and CRTC2. Supporting this notion, the MafA promoter contains a consensus cAMP response element (CRE) that is constitutively occupied by CREB in INS1 cells (Figures 2D and S1G). In line with its cytoplasmic sequestration in unstimulated cells (Screaton et al., 2004), amounts of CRTC2 over the MafA promoter were low under basal conditions but increased following FSK treatment, when CRTC2 is translocated to the nucleus. Inhibiting CREB occupancy, through A-CREB overexpression, downregulated MafA gene in INS1 cells (Figure S1H).

We tested whether MafA activity contributes to cAMP-dependent increases in insulin secretion. In line with this notion, RNAi-mediated depletion of MafA decreased insulin content and secretion from INS1 cells, whereas MafA overexpression increased them (Figures 2E, S1I, and S1J). Indeed, adenoviral expression of MafA restored insulin secretion to wild-type levels in CRTC2 mutant pancreatic islets exposed acutely to glucose plus exendin (Figure 2F). Taken together, these results demonstrate that CRTC2 promotes beta cell function in part by upregulating MafA in response to cAMP.

Although initially compensated by an increase in pancreatic islet mass, prolonged insulin resistance causes impairment in

beta cell function that is thought to reflect the downregulation of certain beta cell factors including MafA. Having seen that CREB and CRTC2 promote MafA expression in response to cAMP signals, we tested effects of insulin resistance on this pathway in pancreatic islets. By contrast with the robust upregulation of MafA and other CREB target genes by FSK in pancreatic islets from lean mice, FSK had only modest effects on islets from high-fat diet (HFD) mice (Figures 3A, S2A, and S2B). Similarly, effects of FSK on insulin secretion were attenuated in HFD islets relative to control (Figure S2C). In principle, the loss of CREB activity in HFD-fed mice could reflect chronic increases in a number of nutrients including circulating glucose or free fatty acids. To test the role of hyperglycemia in this process, we performed high-carbohydrate diet (HCD) feeding studies. Although it had no effect on body weight, HCD feeding impaired glucose tolerance and insulin secretion after only 8 weeks (Figures S2D–S2G). Similar to islets from HFD mice, pancreatic islets from HCD animals showed only modest increases in insulin secretion and CREB target gene expression following exposure to FSK (Figures S2H and S2I).

We wondered whether exposure of cultured pancreatic islets to high concentrations of glucose mimics effects of hyperglycemia on the CREB pathway. Supporting this idea, prolonged exposure of either INS1 cells or cultured pancreatic islets to high glucose (20 mM; 72 hr) inhibited the expression of MafA and other CREB target genes in cells exposed to FSK (Figures 3B, S3A, and S3B). Exposure to 10-mM glucose was not sufficient for PKIB induction in INS1 cells. Indeed, FSK-dependent increases in insulin secretion were substantially downregulated in

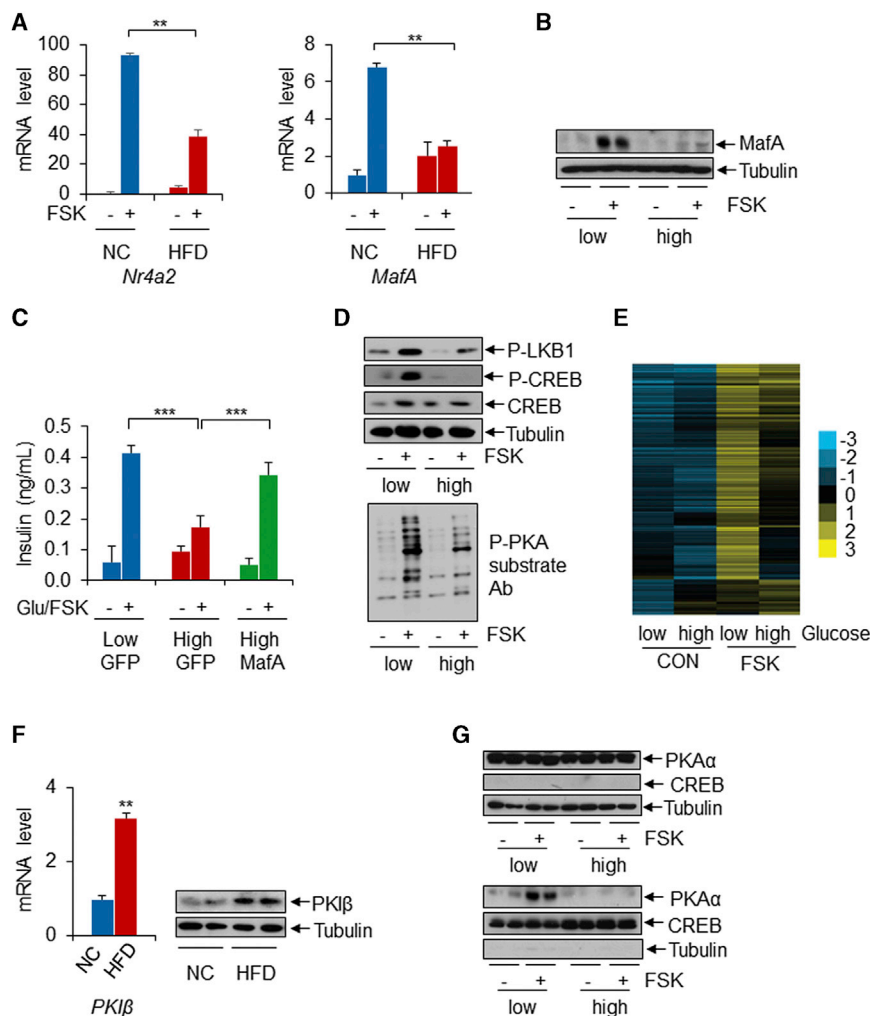


Figure 3. Disruption of the Beta Cell CREB Pathway in Chronic Hyperglycemia

(A) Relative induction of CREB target genes by FSK in cultured pancreatic islets from normal chow (NC)- or high-fat diet (HFD)-fed mice. mRNA amounts for NR4A2 and MafA shown (** $p < 0.01$; $n = 6$).

(B) Effect of prolonged exposure to high glucose on FSK-induced increases in MafA protein amounts.

(C) Effect of chronic high-glucose and adenoviral MafA expression on insulin secretion from INS1 cells (** $p < 0.001$; $n = 6$).

(D) Immunoblots showing effect of prolonged high-glucose exposure on CREB phosphorylation (left) and on cellular PKA activity (right) in primary cultured mouse islets exposed to FSK. Effect of glucose on phosphorylation of LKB1 at a consensus PKA site (Ser431) also shown.

(E) RNA-seq analysis of INS1 cells showing effect of prolonged exposure to high glucose on down-regulation of cAMP-inducible genes. Effect of FSK shown.

(F) Effect of HFD feeding on PKIβ mRNA (left) and protein expression (right) in mouse islets (** $p < 0.01$; $n = 4$).

(G) Effect of chronic high-glucose treatment on nuclear shuttling of PKA catalytic subunit in INS1 cells exposed to FSK. Cytoplasmic (top) and nuclear (bottom) fractions shown. Data are shown as mean \pm SEM.

INS1 cells exposed to high glucose and FSK (Figure 3C). The impairment in insulin secretion under chronic high-glucose conditions was partially rescued by MafA overexpression in both INS1 cells and primary islets (Figures 3C and S3C).

We addressed the mechanism by which high glucose exposure attenuates CREB activity. Although total amounts of CREB were unchanged, high-glucose treatment disrupted the FSK-induced phosphorylation of CREB in both INS1 cells and pancreatic islets (Figures 3D and S3D). Moreover, phospho-CREB amounts were also downregulated in cultured islets from HFD and HCD mice (Figures S3E and S3F). Arguing against an effect on adenylyl cyclase or phosphodiesterase activities, exposure to FSK increased intracellular cAMP concentrations comparably in control and high-glucose-treated INS1 cells (Figure S3G). Rather, cellular PKA activity was substantially reduced in islets chronically exposed to glucose, as visualized in immunoblot assays with phospho-PKA substrate antiserum (Figure 3D). Consistent with its effects on CREB phosphorylation, prolonged exposure of INS1 cells to high glucose largely inhibited the induction of cAMP-responsive genes (Figure 3E; Table S2).

PKIs have been shown to bind with high specificity and affinity to PKA; they also contain a potent nuclear export signal that maintains PKA in the cytoplasm (Taylor et al., 2005; Fantozzi et al., 1994; Wen et al., 1994, 1995). PKIB was selectively upregulated in INS1 cells and cultured pancreatic islets following prolonged exposure to high glucose (Figures S4A and S4B). Indeed, PKIB mRNA and protein amounts were also increased in pancreatic islets, but not other metabolic tissues, from HFD- and HCD-fed mice relative to controls (Figures 3F, S4C, and S4D).

In immunohistochemical studies, we detected PKIB in beta cells of the pancreatic islets, but not in surrounding glucagon-producing alpha cells or in acinar cells of the exocrine pancreas (Figure S4E). Similar to its effects in mouse islets, chronic glucose exposure also promoted PKIB expression in human islets, thereby attenuating CREB phosphorylation and target gene expression (Figures S4F–S4J).

Based on its proposed role in nuclear export, we reasoned that PKIB may reduce CREB phosphorylation by blocking the nuclear accumulation of PKA. Supporting this idea, exposure to FSK promoted an increase in nuclear amounts of PKA catalytic

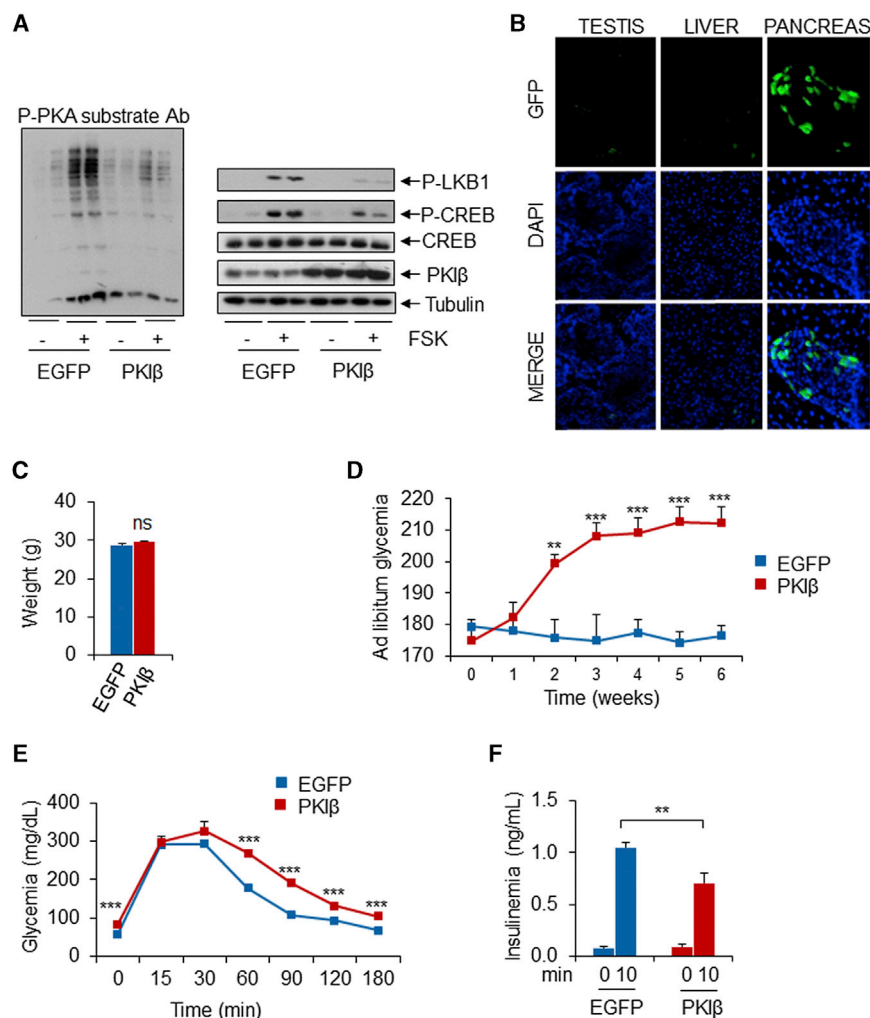


Figure 4. PKIB Disrupts Beta Cell Function

(A) Immunoblots showing effect of dsAAV-MIP-PKIB expression on PKA activity using phospho-PKA substrate antibody (left) or phospho-CREB and phosphor-LKB antiserum (right) in MIN6 cells (n = 4). (B) Immunohistochemical analysis of GFP expression from AAV-encoded EGFP virus in testis, liver, and pancreas 10 days after injection (n = 3). (C and D) Body weight (C) as well as circulating ad libitum glucose concentrations in mice infected with PKIB or control EGFP (D; **p < 0.01; ***p < 0.01; n = 5). (E and F) Circulating glucose (OGTT) and insulin concentrations in mice following oral glucose administration (F; **p < 0.01; ***p < 0.01; n = 5). Data are shown as mean ± SEM.

circulating concentrations of insulin in response to OGTT, indicating that PKIB expression is sufficient to disrupt beta cell function (Figures 4E and 4F).

We used mice with a knockout of PKIB to determine whether the upregulation of this inhibitor in response to HFD feeding contributes to the deterioration in pancreatic islet function. Although they were otherwise unremarkable (Belyamani et al., 2001), NC-fed PKIB KO mice were modestly more glucose tolerant relative to wild-type littermates by OGTT (Figure S5A). When placed on a HFD, however, PKIB mutants had lower fasting blood glucose concentrations and were substantially more glucose tolerant (Figures 5A and S5B–S5D). Moreover, circulating insulin concentrations rose 3-fold in PKIB knockout mice following glucose administration, but they remained unchanged in controls (Figure 5B). Similarly, MafA expression was strongly reduced in pancreatic sections from control mice following HFD feeding, but it remained high in PKIB KO mice (Figure 5C). Taken together, these results demonstrate that the upregulation of PKIB in insulin resistance disrupts beta cell CREB activity and insulin secretion.

In the setting of insulin resistance, chronic hyperglycemia is thought to promote beta cell hypertrophy in part through the induction of the energy-sensing kinase mTOR. Indeed, prolonged exposure of pancreatic islets to glucose or FSK triggers the mTORC1-dependent activation of HIF-1α (Van de Velde et al., 2011) in beta cells, prompting us to evaluate the role of this pathway in mediating the induction of PKIB. Exposure of INS1 cells to glucose or FSK increased HIF-1α protein amounts after 12–24 hr followed by increases in PKIB protein amounts (Figure 5D).

Consistent with a requirement for mTOR activity, exposure to the mTORC1 inhibitor rapamycin effectively blocked the upregulation of PKIB in cells exposed to FSK (Figure 5E). Moreover,

subunit in INS1 cells maintained under low-glucose conditions, but not in cells maintained on high glucose (Figure 3G).

Although PKI potentially inhibits PKA activity, the importance of this pathway for insulin secretion is unclear, as increases in circulating glucose modulate insulin secretion primarily through calcium signaling. In that event, the PKIB would cause only modest changes in circulating glucose and insulin concentrations. To evaluate the effects of this inhibitor on glucose homeostasis, we employed a double-stranded AAV8 vector expressing PKIB under control of the mouse insulin promoter, which targets transgene expression specifically to beta cells. Overexpression of AAV8-encoded PKIB in MIN6 insulinoma cells reduced PKA activity and correspondingly disrupted CREB phosphorylation in response to FSK treatment (Figure 4A). Following i.p. administration, a control AAV-MIP-EGFP virus was expressed in pancreatic islets, but not other tissues such as liver and testis (Figure 4B). Ad libitum circulating glucose concentrations increased progressively over a 6-week period in mice expressing AAV-encoded PKIB (Figures 4C and 4D). Although their body weights were identical to controls, PKIB-overexpressing animals became relatively glucose intolerant and they had lower

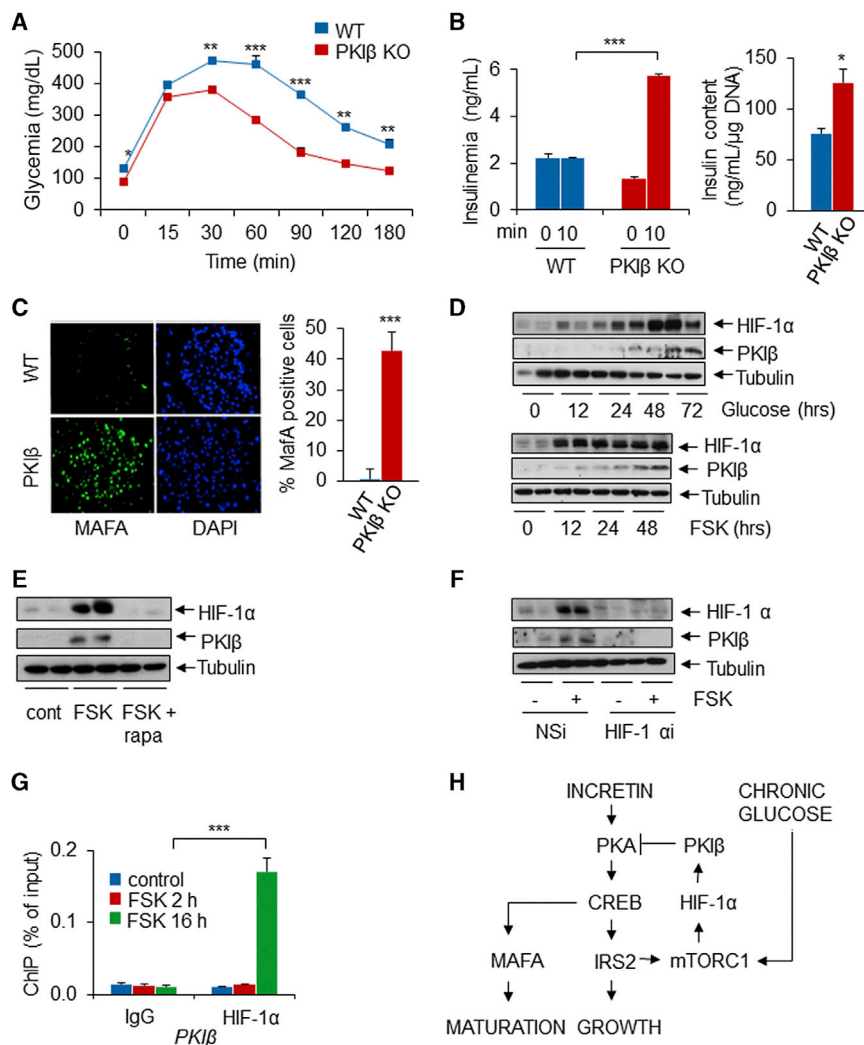


Figure 5. Knockout of PKIB Improves Pancreatic Islet Function in Insulin Resistance

(A) OGTT of PKIB knockout and control littermates maintained on a HFD for 12 weeks (** $p < 0.01$; *** $p < 0.01$; $n = 6$).

(B) Circulating insulin concentrations in control and PKIB mutant littermates under basal conditions and 10 min following oral glucose administration. Right, relative insulin content in isolated pancreatic islets (* $p < 0.05$; *** $p < 0.01$; $n = 6$).

(C) Immunohistochemical analysis showing MafA staining in pancreatic sections from HFD-fed control mice and PKIB^{-/-} littermates.

(D) Time course of PKIB mRNA accumulation in INS1 cells exposed to FSK ($n = 4$).

(E) Effect of mTORC1 inhibitor rapamycin on PKIB mRNA amounts in INS1 cells exposed to FSK for 14 hr ($n = 4$).

(F) Effect of HIF-1 α RNAi-mediated knockdown on PKIB mRNA levels in INS1 cells ($n = 4$).

(G) ChIP assays showing effect of short- (2 hr) or long-term (16 hr) exposure to FSK on HIF-1 α occupancy over the PKIB promoter (*** $p < 0.001$; $n = 6$).

(H) Feedback regulation of beta cell CREB activity in insulin resistance. Glucose- and incretin-dependent increases in CREB/CRTC2 activity during feeding promote the expression of IRS2, which in turn mediates induction of mTORC1 complexes. When hyperglycemia is prolonged, increases in HIF-1 protein trigger expression of PKIB, which downregulates the CREB/CRTC2 pathway by binding to and inhibiting PKA. Data are shown as mean \pm SEM.

RNAi-mediated depletion of HIF-1 α also disrupted PKIB induction, whereas HIF-1 α overexpression potentiated it (Figures 5F, S5E, and S5F). The effect of HIF on PKIB expression appears direct; HIF1 protein amounts over consensus binding sites on the PKIB promoter increased following prolonged, but not short-term, FSK treatment of INS1 cells by ChIP assay (Figure 5G). Indeed, selective induction of endogenous HIF1, by administration of the prolyl hydroxylase inhibitor DMOG, also up-regulated PKIB expression, demonstrating that HIF is sufficient for induction of PKIB in beta cells (Figures S5G and S5H).

DISCUSSION

Taken together, our results show how CRTC2 mediates effects of incretin hormones through upregulation of MafA and other CREB target genes that promote insulin gene expression and secretion (Figure 5H). In response to nutrient stress, GLP1 and glucose promote pancreatic islet function through increases in mTORC1 activity that culminate in the induction of the HIF pathway. When insulin resistance is prolonged, HIF1 inhibits

PKIB in cultured islets, chronic treatment of insulin-resistant mice with GLP-1R agonist improved glucose intolerance in vivo (Irwin et al., 2009). Incretin signaling accounts for a large fraction of post-prandial glucose disposal, and the attenuation of this pathway in type II diabetes is thought to contribute significantly to the increased glucose excursions that are characteristic of this disease (Holst et al., 2011). Indeed, a sizable percentage of type II diabetic patients appear to be unresponsive to GLP1 agonist therapy (Buysschaert et al., 2012; Hall et al., 2013; Preumont et al., 2012), potentially reflecting the upregulation of PKIs. Although the mechanism is unclear, PKIA also appears to be upregulated in pancreatic islets from type II diabetic individuals (Gunton et al., 2005). Reducing the expression of PKIs in beta cells may provide therapeutic benefit in this setting.

EXPERIMENTAL PROCEDURES

Animals

All studies except for HFD and HCD studies were performed using 10- to 12-week-old male mice in a C57Bl6 background. Animals were adapted to their

environment for 1 week before studies and were housed in colony cages with a 12 hr light/12 hr dark cycle in a temperature-controlled environment. C57BL6 were purchased from Jackson Laboratories. For HFD studies, C57BL6 mice were fed a HFD for 20 weeks beginning at 8 weeks of age (60% kcal fat; D12492; Research Diet). Age-matched C57BL6 on normal chow were used as controls. For HCD studies, 8-week-old male C57BL6 were maintained in diet with 73% carbohydrate during 10 weeks (AIN-93M; Research Diet). PKIB mice were purchased from MMRRC (B6.129P2-*Pkib*^{tm1^{ldz}/Mmmh}). MIP-CreERT mice were a gift from Dr. Phillipson of the University of Chicago. The *CRTC2*^{flox/flox} mice are homozygous for a “floxed” *CRTC2* allele, in which *CRTC2* exons 1 and 5 are flanked by *loxP* target sites for Cre recombinase. Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Salk Institute.

In Vivo Analysis

At 10 weeks of age, *CRTC2* flox/flox MIP-Cre/ERT mice (MIP-T2 KO mice) were fed with tamoxifen (Sigma; T5648) dissolved in corn oil at 100 mg/kg body weight (once per day for 5 days). Following tamoxifen administration, the mice were housed for 5–10 days before being used and analyzed for Cre-recombinase activity. MIP-T2 KO mice fed with corn oil were used as controls. Following an overnight fast, mice were administered glucose (1 g/kg) by oral gavage or by i.p., and glucose levels were measured every 30 min over a 3-hr period. Insulin tolerance was tested by i.p. injection of 6-hr fasted mice with 1 U/kg of insulin (Humulin R; Eli Lilly) followed by blood glucose measurements every 15 min until 1 hr. For insulin dosage, blood was taken from the tail vein after 10 min of glucose gavage. dsAAV infections were carried out at a dose of 4×10^{11} genomes (vg) per mouse. Viruses were administered i.p. in a total volume of 800 μ l of sterile saline solution containing 5% sorbitol.

Cell Culture

INS-1 insulinoma cells were cultured in RPMI (Corning cellgro) containing 10% fetal bovine serum (Gemini Bio Products), 100 μ g/ml penicillin-streptomycin, and 1 mM sodium pyruvate (Corning cellgro). MIN6 cells were grown in DMEM (Corning cellgro) with 10% FBS, 100 μ g/ml penicillin-streptomycin, and 70 μ M beta-mercaptoethanol. For chronic glucose experiments, cells were maintained under low (2.8 mM) or high (20 mM) glucose for 3 days. Exendin-4 (10 nM), FSK (10 μ M), KCl (40 mM), DMOG (1 mM), and rapamycin (50 nM) were added to cells as indicated.

cAMP Measurement

Cellular cAMP levels were measured using an ELISA kit (Cayman Chemical Company) according to manufacturer's instructions.

RNAi

The sequences of the oligonucleotides used for target rat *mafA* were as follows: 5'-GAGGAUCUGUAVUGGAUGA-3' and 5' UCAUCCAGUACAGAUC CUC-3'. As a negative control, RNA duplexes targeting GFP were used: 5'-GCAAGCUGACCCUGAAGUUC-3' and 5'-GAACUUCAGGGUCAGCUUGC-3'. After annealing, 100 pmol of synthetic duplex was transfected using Lipofectamine 2000 reagent (Invitrogen) per well (6-well plates) and cells were harvested 48 hr later.

Islets Isolation and Human Islets

Islets isolation was performed as described previously (Shapiro et al., 1996). Briefly, pancreata from 10- to 12-week-old mice were injected with Liberase (0.2 mg/ml; Roche Applied Science) and digested at 37°C for 15 min. Preparations were washed with Hank's buffered salt solution, and the dissociated islets were purified on Histopaque gradients (Sigma-Aldrich) and cultured in RPMI with 10% FBS for 3 days before testing. Human islets were supplied by the Integrated Islets Distribution Program (IIDP; <https://iidp.coh.org/>). Donor information is listed in Table S3.

Insulin Secretion

INS-1 cells or primary islets were starved 2 hr in Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 0.2% BSA and exposed to 2.8 mM or 20 mM glucose with or without FSK (10 μ M) or exendin 4 (10 nM) for 1 hr. Insulin

release and content were measured using the ultrasensitive insulin ELISA kit (Mercodia). Results are presented as insulin secretion (ng/ml) per hour normalized to insulin content. Insulin content is normalized to DNA. Insulin dosage in vivo was assayed using the ultrasensitive mouse insulin ELISA (Crystal Chem).

Adenoviruses

For adenoviruses construction, cDNAs were subcloned in the pAdTRACK vector. Rat PKIB cDNA was obtained by PCR using primers 5'-CATCTCGAGAT GAGGACAGATTC (sense) and 5'-CATGGTACCTATTGTCTTCGTCTAG (anti-sense), which introduces XhoI and KpnI sites (underlined), respectively. Complete viral vectors were generated by homologous recombination with the AdEASY vector as described (Koo et al., 2005). The MafA adenovirus was a gift from Dr. T.A. Matsuoka. Adenoviruses were then produced in MGH cells and purified using CsCl gradients. dsAAV-MIP-EGFP was a gift from Dr. Paul D. Robbins of the University of Pittsburgh. Mouse PKIB cDNA was obtained by PCR using primers 5'-CATACCGGTATGAGGACAGATTCAT CAGA (sense) and 5'-CATGCGGCCGCTCATTTCCTTCATTAG (anti-sense), which introduces AgeI and NotI (underlined), respectively. The dsAAV virus expressing mouse PKI β was generated by excising EGFP with restriction enzymes AgeI and NotI. Recombinant dsAAV vectors were generating according to the triple transfection protocol using AAV8 serotype.

Real-Time qPCR and RNA-Seq

Total RNAs from cells or primary islets were extracted using Trizol, and cDNA was generated using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). cDNAs were quantified on a Lightcycler 480 instrument (Roche Applied Science). Gene expression data were presented relative to the expression of housekeeping gene *L32* (rat samples) and *18S* (mouse and human islets). Primer sequences are listed in Table S4. RNA-seq libraries were prepared using the mRNA isolation protocol and the NEBNext-Ultra kits from New England Biolabs following the manufacturer's protocols. Libraries were quantitated by Qubit (Invitrogen) and run on a MiSeq instrument with paired-end 75-bp reads using v3 chemistry (Illumina). Data were analyzed by tophat2 and cuffdiff against the mouse mm10 genome build.

Chromatin Immunoprecipitation

INS-1 cells were plated on 15-cm dishes and exposed to FSK as specified. Chromatin immunoprecipitation with HIF-1 α , CREB, P-CREB, and *CRTC2* antisera was performed as described (Ravnskjaer et al., 2007). Oligonucleotides used for ChIP analysis are listed in Table S5.

Protein Analysis

Total protein from cultured cells or primary islets was extracted in a Tris-HCl buffer containing 0.5% NP40, protease, and phosphatase inhibitors. Proteins were quantified using Bradford reagent and separated using SDS-PAGE. For cellular fractionation, cells were resuspended in hypotonic lysis buffer (40 mM TrisHCl [pH 7.4], 10 mM NaCl, 1 mM EDTA with DTT, and protease inhibitors). Cells were lysed using a dounce homogenizer and centrifuged. Cytosolic supernatants were collected. Nuclear pellets were washed three times and resuspended in nuclear extraction buffer (40 mM TrisHCl [pH 7.4], 420 mM NaCl, 10% glycerol, and 1 mM EDTA). Samples were sonicated and centrifuged. Nuclear supernatants were collected.

Histology

After antigen retrieval, 10- μ m frozen pancreatic sections were incubated with the indicated antibodies overnight and with fluorophore-conjugated secondary antibody and DAPI for 1 hr. Sections were mounted with PBS 70% glycerol and analyzed in a Zeiss VivaTome.

Antisera

Antibodies used for immunoblotting, ChIP, and Immunofluorescence are indicated in alphabetical order: glucagon (G2654; Sigma-Aldrich); HIF-1 α (10006421; Cayman Chemical); insulin (180067; ZYMED Laboratories); MafA (NB400-137; Novus Biologicals); PKA α cat (sc-903; Santa Cruz Biotechnology); PKIB (NBP1-74255; Novus Biologicals); PKIB (IF

(NBP1-55720; Novus Biologicals); pLKB1 (pSer428) (C67A3; Cell Signaling Technology); phospho PKA substrates (100G7E; Cell Signaling Technology); and tubulin (05-829; Millipore). For CREB, pCREB (pSer133), and CRT2 detection, rabbit polyclonal antibodies were raised against their respective antigens.

Statistical Analysis

All mice used in experiments were around 10–12 weeks old except mice in HFD or HCD. Whenever possible, littermates of appropriate genotype were used as age-matched controls. The number of mice per experiment was limited by the availability of the required genotype and age. Criteria of exclusion were (1) gender and age, (2) evident signs of disease, and (3) spontaneous natural death during the experiment. Sample size (number of mice, islets, and cells) is within the range of published literature. All results are presented as means \pm SEM. Statistical analysis was performed with unpaired Student's *t* test. Differences were considered statistically significant at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$).

ACCESSION NUMBERS

The NCBI GEO accession number for RNA-seq studies is GSE60158.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.01.046>.

ACKNOWLEDGMENTS

We thank Mark Huising for helpful discussions. This work was supported by NIH grants R01-DK049777, R01-DK083834, and R01-DK091618 (to M.M.) and P01-DK049210 (to K.K.); the Clayton Foundation for Medical Research; the Kieckhefer Foundation; and the Leona M. and Harry B. Helmsley Charitable Trust grant no. 2012-PG-MED002.

Received: November 5, 2014

Revised: December 24, 2014

Accepted: January 17, 2015

Published: February 19, 2015

REFERENCES

Abdulhad, W.H., Kroese, F.G., Vissink, A., and Bootsma, H. (2012). Immune regulation and B-cell depletion therapy in patients with primary Sjögren's syndrome. *J. Autoimmun.* *39*, 103–111.

Belyamani, M., Gangolli, E.A., and Idzerda, R.L. (2001). Reproductive function in protein kinase inhibitor-deficient mice. *Mol. Cell. Biol.* *21*, 3959–3963.

Buyschaert, M., Paris, I., Selvais, P., Oriot, P., and Preumont, V.; UCL Exenatide Group (2012). Glycemic control and weight changes in patients with type 2 diabetes intensified to three insulin regimens after therapeutic failure to exenatide. *Acta Clin. Belg.* *67*, 250–254.

Fantozzi, D.A., Harootyan, A.T., Wen, W., Taylor, S.S., Feramisco, J.R., Tsien, R.Y., and Meinkoth, J.L. (1994). Thermostable inhibitor of cAMP-dependent protein kinase enhances the rate of export of the kinase catalytic subunit from the nucleus. *J. Biol. Chem.* *269*, 2676–2686.

Gunton, J.E., Kulkarni, R.N., Yim, S., Okada, T., Hawthorne, W.J., Tseng, Y.H., Roberson, R.S., Ricordi, C., O'Connell, P.J., Gonzalez, F.J., and Kahn, C.R. (2005). Loss of ARNT/HIF1 β mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* *122*, 337–349.

Hall, G.C., McMahon, A.D., Dain, M.P., Wang, E., and Home, P.D. (2013). Primary-care observational database study of the efficacy of GLP-1 receptor agonists and insulin in the UK. *Diabet. Med.* *30*, 681–686.

Holst, J.J., Knop, F.K., Vilsbøll, T., Krarup, T., and Madsbad, S. (2011). Loss of incretin effect is a specific, important, and early characteristic of type 2 diabetes. *Diabetes Care* *34* (2), S251–S257.

Iezzi, M., Kouri, G., Fukuda, M., and Wollheim, C.B. (2004). Synaptotagmin V and IX isoforms control Ca²⁺-dependent insulin exocytosis. *J. Cell Sci.* *117*, 3119–3127.

Iezzi, M., Theander, S., Janz, R., Loze, C., and Wollheim, C.B. (2005). SV2A and SV2C are not vesicular Ca²⁺ transporters but control glucose-evoked granule recruitment. *J. Cell Sci.* *118*, 5647–5660.

Irwin, N., Hunter, K., Frizzell, N., and Flatt, P.R. (2009). Antidiabetic effects of sub-chronic activation of the GIP receptor alone and in combination with background exendin-4 therapy in high fat fed mice. *Regul. Pept.* *153*, 70–76.

Jhala, U.S., Canettieri, G., Srean, R.A., Kulkarni, R.N., Krajewski, S., Reed, J., Walker, J., Lin, X., White, M., and Montminy, M. (2003). cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes Dev.* *17*, 1575–1580.

Kaihara, K.A., Dickson, L.M., Jacobson, D.A., Tamarina, N., Roe, M.W., Philipson, L.H., and Wicksteed, B. (2013). β -Cell-specific protein kinase A activation enhances the efficiency of glucose control by increasing acute-phase insulin secretion. *Diabetes* *62*, 1527–1536.

Koo, S.H., Flechner, L., Qi, L., Zhang, X., Srean, R.A., Jeffries, S., Hedrick, S., Xu, W., Boussouar, F., Brindle, P., et al. (2005). The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* *437*, 1109–1111.

Lee, Y., Collins, M., and Kuchroo, V.K. (2014). Unexpected targets and triggers of autoimmunity. *J. Clin. Immunol.* *34* (1), S56–S60.

Martin, F., Apetoh, L., and Ghiringhelli, F. (2012). Controversies on the role of Th17 in cancer: a TGF- β -dependent immunosuppressive activity? *Trends Mol. Med.* *18*, 742–749.

Park, S., Dong, X., Fisher, T.L., Dunn, S., Omer, A.K., Weir, G., and White, M.F. (2006). Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. *J. Biol. Chem.* *281*, 1159–1168.

Preumont, V., Hermans, M.P., Bergman, M., and Buyschaert, M. (2012). Predictive factors associated with primary failure to exenatide and non goal attainment in patients with type 2 diabetes. *Acta Clin. Belg.* *67*, 411–415.

Ravnskjaer, K., Kester, H., Liu, Y., Zhang, X., Lee, D., Yates, J.R., 3rd, and Montminy, M. (2007). Cooperative interactions between CBP and TORC2 confer selectivity to CREB target gene expression. *EMBO J.* *26*, 2880–2889.

Srean, R.A., Conkright, M.D., Katoh, Y., Best, J.L., Canettieri, G., Jeffries, S., Guzman, E., Niessen, S., Yates, J.R., 3rd, Takemori, H., et al. (2004). The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. *Cell* *119*, 61–74.

Selmi, C. (2012). Autoimmunity in 2011. *Clin. Rev. Allergy Immunol.* *43*, 194–206.

Shapiro, A.M., Hao, E., Rajotte, R.V., and Kneteman, N.M. (1996). High yield of rodent islets with intraductal collagenase and stationary digestion—a comparison with standard technique. *Cell Transplant.* *5*, 631–638.

Shin, S., Le Lay, J., Everett, L.J., Gupta, R., Rafiq, K., and Kaestner, K.H. (2014). CREB mediates the insulinotropic and anti-apoptotic effects of GLP-1 signaling in adult mouse β -cells. *Mol. Metab.* *3*, 803–812.

Song, W.J., Seshadri, M., Ashraf, U., Mdluli, T., Mondal, P., Keil, M., Azevedo, M., Kirschner, L.S., Stratakis, C.A., and Hussain, M.A. (2011). Snapin mediates incretin action and augments glucose-dependent insulin secretion. *Cell Metab.* *13*, 308–319.

Taylor, S.S., Kim, C., Vigil, D., Haste, N.M., Yang, J., Wu, J., and Anand, G.S. (2005). Dynamics of signaling by PKA. *Biochim. Biophys. Acta* *1754*, 25–37.

Tu, E., Ang, D.K., Bellingham, S.A., Hogan, T.V., Teng, M.W., Smyth, M.J., Hill, A.F., and van Driel, I.R. (2012). Both IFN- γ and IL-17 are required for the development of severe autoimmune gastritis. *Eur. J. Immunol.* *42*, 2574–2583.

Van de Velde, S., Hogan, M.F., and Montminy, M. (2011). mTOR links incretin signaling to HIF induction in pancreatic beta cells. *Proc. Natl. Acad. Sci. USA* *108*, 16876–16882.

Wen, W., Harootunian, A.T., Adams, S.R., Feramisco, J., Tsien, R.Y., Meinkoth, J.L., and Taylor, S.S. (1994). Heat-stable inhibitors of cAMP-dependent protein kinase carry a nuclear export signal. *J. Biol. Chem.* *269*, 32214–32220.

Wen, W., Meinkoth, J.L., Tsien, R.Y., and Taylor, S.S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell* *82*, 463–473.

Xie, T., Chen, M., Zhang, Q.H., Ma, Z., and Weinstein, L.S. (2007). Beta cell-specific deficiency of the stimulatory G protein alpha-subunit Gsalpha leads to reduced beta cell mass and insulin-deficient diabetes. *Proc. Natl. Acad. Sci. USA* *104*, 19601–19606.

Zhao, J., Cannon, B., and Nedergaard, J. (1998). Thermogenesis is beta3- but not beta1-adrenergically mediated in rat brown fat cells, even after cold acclimation. *Am. J. Physiol.* *275*, R2002–R2011.