The Essential Role of the Crtc2-CREB Pathway in β cell Function and Survival

by

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Abstract:

Immunosuppressants that target the serine/threonine phosphatase calcineurin are commonly administered following organ transplantation. Their chronic use is associated with reduced insulin secretion and new onset diabetes in a subset of patients, suggestive of pancreatic β cell dysfunction. Calcineurin plays a critical role in the activation of CREB, a key transcription factor required for β cell function and survival. CREB activity in the islet is activated by glucose and cAMP, in large part due to activation of Crtc2, a critical coactivator for CREB. Previous studies have demonstrated that Crtc2 activation is dependent on dephosphorylation regulated by calcineurin. In this study, we sought to evaluate the impact of calcineurin-inhibiting immunosuppressants on Crtc2-CREB activation in the primary β cell. In addition, we further characterized the role and regulation of Crtc2 in the β cell. We demonstrate that Crtc2 is required for glucose dependent up-regulation of CREB target genes. The phosphatase calcineurin and kinase regulation by LKB1 contribute to the phosphorylation status of Crtc2 in the β cell. CsA and FK506 block glucose-dependent dephosphorylation and nuclear translocation of Crtc2. Overexpression of a constitutively active mutant of Crtc2 that cannot be phosphorylated at Ser171 and Ser275 enables CREB activity under conditions of calcineurin inhibition. Furthermore, β cells lacking Crtc2 display impaired glucose-stimulated insulin secretion and cell survival. Together, these results demonstrate that phosphorylation of Crtc2 plays a critical role in regulating CREB activity and contributes to β cell dysfunction and death caused by chronic immunosuppression.

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List of Abbreviations:

AKT/PKB	Protein kinase B
AMPK	Adenosine monophosphate kinase
ATF-1	Activating transcription factor-1
Bax	Bcl-associated X protein
Bcl-2	B cell lymphoma 2
bZIP	basic leucine zipper
Brsk2	Brain selective kinase 2
CaMK	Ca^{2+}/Calmodulin dependent kinase
CART	Cocaine- and amphetamine-regulated transcript
Cnb1KO	Calcineurin β subunit knockout
CRE	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator
CRH-1	CREB homologous gene-1
Crtc	CREB regulated transcriptional coactivator
CsA	Cyclosporin A
DN-CREB	Dominant negative CREB
ER	Endoplasmic reticulum
Epac	Exchange protein directly activated by cAMP
G6Pase	Glucose-6-phosphatase
GADA	Glutamic acid decarboxylase antigen
GIP	Glucose-dependent insulinotropic peptide

GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
HEK293T	Human embryonic kidney 293 T-antigen
IAA	Insulin auto-antigen
ICA	Islet cell cytoplasm antigen
ICER	Inducible cAMP early repressor
InsP3	Inositol 1,4,5-trisphosphate
IL-2	Interleukin -2
IRS-2	Insulin receptor substrate
KID	Kinase inducible domain
LKB1	Liver kinase B1
MARK2	Microtubule affinity-regulating kinase 2
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NOD	Non-obese diabetic
NODAT	New onset diabetes after transplantation
PP	Pancreatic polypeptide
PP1	Protein phosphatase1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B/ calcineurin
PPAR	Peroxisome proliferator-activated receptor
Pdx-1	Pancreatic and duodenal homeobox-1
PEPCK	Phosphoenolpyruvate carboxykinase-1

PGC-1a	PPAR gamma coactivator 1 alpha
PKA	Protein kinase A
PKB/AKT	Protein kinase B
PMP	Pancreatic multipotent precursor
PTDM	Post-transplantation diabetes mellitus
TORC	Transducer of regulated CREB activity
SCID	Severe combined immunodeficiency
SIK	Salt-inducible kinase
SNARE	Soluble NSF Attachment Protein Receptor
SUR1	Sulfonylurea receptor 1
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TLR-4	Toll-like receptor 4

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Chapter 1: Introduction

1.1 Diabetes:

Diabetes is characterized by an inability to regulate blood glucose levels due to insufficient insulin secretion or peripheral insulin insensitivity [1, 2]. Insulin dependent type I, and insulin independent type II diabetes are the two major forms of the disease. Development of both forms of diabetes is dependent on genetic and environmental risk factors [2-4]. The incidence of diabetes is increasing and has largely been attributed to changes in environmental factors such as diet [5, 6]. There has been a decline in the average age of onset of diabetes and due to the rise in childhood obesity, diagnosing the form of diabetes in youth has become an additional clinical challenge [7]. Despite current therapies for diabetes, patients still suffer from various complications and have a shorter life expectancy, highlighting the need to improve current treatment strategies [8].

1.1.1 Type I Diabetes:

Type I Diabetes (T1D) or juvenile diabetes results from a T-cell mediated autoimmune destruction of pancreatic insulin producing β cells [9]. T1D makes up 10% of diabetes and onset occurs in children and adolescents [10]. Genetic predisposition to T1D is largely associated with immune-related genes such as the major histocompatibility complex [11]. Environmental factors such as microbial, and dietary antigens have been shown to increase the risk of T1D [12, 13]. The combination of genetic susceptibility and environmental triggers leads to inflammatory state of the immune system that predisposes individuals to an autoimmune reaction against β cell specific antigens with the most common autoantigens being insulin (IAA), islet cell cytoplasm (ICA), glutamic acid decarboxylase (GADA) and aborted tyrosine phosphatase

(IA2) (reviewed in [14]). Risk of T1D correlates with the number of autoantibodies present [15]. Local inflammation caused by early autoimmune destruction of the β cells leads to the release of β cell specific antigens which promote the production of additional autoantibodies that propagate β cell destruction, resulting in increased risk of T1D. Once less than 10% of the β cells remain, the patients become incapable of regulating blood glucose levels and become dependent on regulated insulin injections.

1.1.2 Type II Diabetes:

Type II diabetes (T2D) onset occurs later in life after the development of insulin resistance. β cell dysfunction and failure of peripheral tissues such as the liver, skeletal muscle and adipocytes to respond to insulin underlies T2D [16]. Chronic low-grade inflammation plays a key role in the development of insulin resistance and type 2 diabetes (reviewed in [17]). In fact, blocking this inflammation through transgenic ablation of Toll-like Receptor-4 (TLR-4) has been shown to prevent diet-induced insulin resistance and diabetes in mice [18]. Dysfunction of adipocytes involving hypertrophy, cell death and infiltration of inflammatory immune populations has been shown to underlie the progression of insulin resistance [19]. Furthermore, inflammatory cytokines impair insulin sensitivity through suppression of insulin receptor kinase activity in adipocytes and suppressed expression of adipocyte genes involved in insulin sensitivity such as GLUT-4, long chain fatty acyl synthase and peroxisome proliferator-activated receptor (PPAR)-gamma [20, 21]. In addition, inflammation impairs adipocyte secretion of adiponectin, an adipokine that promotes insulin sensitivity in liver and skeletal muscle [22]. Impaired insulin signaling in peripheral tissues block gluconeogenesis, glucose uptake and fatty acid oxidation and contribute to elevated blood glucose levels. β cells

have some capacity to proliferate and functionally compensate to meet the additional demand for insulin, which manifests as hyperinsulinemia in pre-diabetic patients. Eventual failure of the β cell to meet the elevated insulin requirement results in the onset of diabetes. Interestingly, many of the genes identified as risk factors for T2D are β cell specific predispositions, suggesting that despite complexity of the metabolic syndrome that precedes T2D, the ability of the β cell to compensate for this stress is the determining factor for whether the individual will develop T2D [23, 24].

1.2 The β cell:

β cells are located within the islets of Langerhans, the endocrine tissue of the pancreas, along with alpha, delta and pancreatic polypeptide cells (PP). The islets make up 2% of total pancreas and the rest is predominantly comprised of acinar exocrine tissue that secretes digestive enzymes into the intestinal tract. The ability to secrete insulin is unique to the β cells giving it an indispensible role in whole body glucose homeostasis. β cells are lowly replicating cells making their survival and functional capacity crucial for the prevention of diabetes [24, 25].

1.2.1 β cell function:

Pancreatic β cells are highly responsive to changes in blood glucose levels due to the high K_m (affinity constant) of glucokinase, the enzyme that phosphorylates and traps glucose within the cell [26, 27]. Glucose enters the β cell through the GLUT2 transporter in rodents and GLUT1 in humans [28]. In response to feeding, elevations in glucose concentration increase the ATP:ADP ratio through mitochondrial respiration [29]. This rise in ATP:ADP ratio causes closure of ATP sensitive potassium channels (K^+_{ATP}), membrane depolarization and opening of L-type Ca^{2+} channels [30-32]. The large influx

of Ca^{2+} stimulates exocytosis of insulin granules docked at the plasma membrane through SNARE mediated membrane fusion [33]. Although insulin secretion is predominantly stimulated by glucose, some amino acids (leucine, arginine, glutamine) have also been shown to stimulate insulin secretion [34]. Nervous input, incretin hormones and fatty acids potentiate insulin secretion in high glucose conditions and contribute to glucose clearance in a physiological setting [35-37].

1.2.2 β cell mass:

β cell mass is governed by the processes of hypertrophy, hyperplasia, differentiation and apoptosis (reviewed in [38]). Lineage tracing studies in mice have supported a key role of proliferation of existing β cells as the source of new β cells with little or no contribution from other precursor cells [39]. Formation of new islets, termed islet neogenesis, has also been proposed as a source of new β cells through differentiation of progenitor cells or transdiffentiation of other pancreatic cell types (reviewed in [40]). In addition, recent analysis of dispersed islet cell populations has revealed rare pancreatic multipotent precursor cells (PMP) that retain the capacity to give rise to islet-like structures [41]. Interestingly, these rare insulin positive precursor cells have been shown to give rise to other endocrine cell types, acinar, and neurons *in vivo* [42]. These data suggest that the pancreas and the β cell are highly plastic and have a variety of mechanisms to expand β cell mass.

1.2.3 β cell compensation:

An increase in β cell mass and improved function are observed in humans and to a larger extent in mice in the event of an increased insulin demand [43]. Evidence for β cell compensation has been observed in gestational women and obese individuals that have

greater β cell mass compared with lean individuals [44, 45]. In a T1D setting, pancreatic reorganization generates structures called tubular complexes that promote differentiation of islet precursor cells and neogenesis [46]. Furthermore, drastic loss of β cells promotes β cell function by reducing the glucose threshold required to stimulate insulin secretion and has been attributed to increased glucokinase activity [47, 48]. These data suggest that the β cell compartment can compensate for additional insulin demands in healthy individuals. Understanding the factors that promote β cell survival and function is important for the design of future treatment strategies that aim to restore the β cell compartment of diabetic patients. A variety of signaling mechanisms have been implicated in the regulation of functional β cell mass and are thought to contribute to the β cell compensation observed in healthy individuals.

1.3 β cell signaling:

In addition to the immediate response of insulin secretion, insulin secretagogues and insulin itself induce signaling that is crucial for the maintenance of β cell function and survival (Summarized in Figure I). For instance, the β cell responds to glucose and incretin hormones through Ca^{2+} and cAMP signaling respectively to alter β cell fitness in preparation for subsequent challenges (reviewed in [49]). Furthermore, autocrine insulin signaling and growth factor signaling are crucial for the β cell and the prevention of diabetes (reviewed in [50]) .

1.3.1 Glucose signaling:

Glucose has positive effects on β cell function and survival in both *ex vivo* and *in vivo* studies. For instance, culture of rat islets in moderately elevated glucose concentrations (10 mM) promoted β cell function and survival [51]. Furthermore, glucose

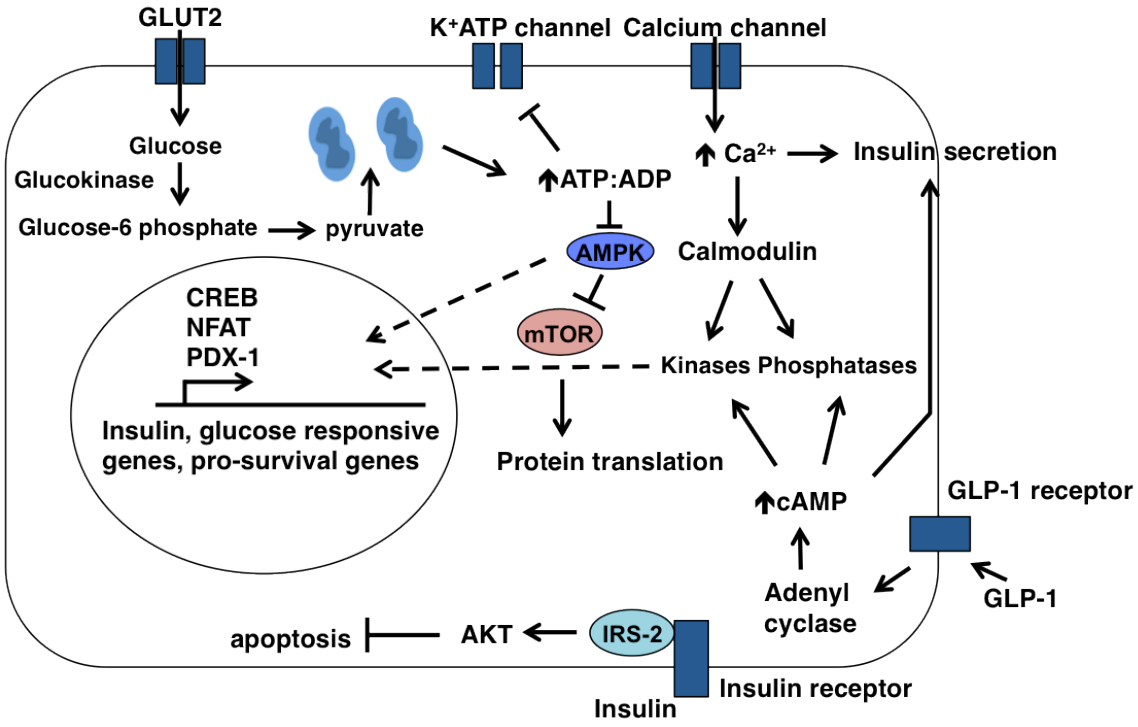

Figure I: Overview of β cell signaling. Glucose is imported through the glucose transporter GLUT2 and becomes phosphorylated by glucokinase, trapping it within the cell. Metabolism of glucose through oxidative phosphorylation in the mitochondria result in an increase in the cellular energy status or ATP:ADP ratio. This change in energy status prompts closure of the K^+_{ATP} channel, membrane depolarization, influx of Ca^{2+} and insulin secretion. The increase of ATP:ADP also deactivates AMPK to relieve inhibition on protein translation through mTOR. Ca^{2+} signaling further regulates the β cell through Ca^{2+}-activated calmodulin regulation of additional kinases and phosphatases. GLP-1 signaling through the GLP-1 receptor induces cAMP signaling, potentiates insulin secretion and regulates kinase/phosphatase activity. Altered kinase/phosphatase activity in response to glucose and cAMP promote transcription of genes involved in β cell function and survival. Insulin signaling through IRS-2 is important for the prevention of apoptosis through activation of AKT.

infusion in mice promoted β cell proliferation [52] and proliferation of human islets transplanted in mice, suggesting that elevations in serum glucose levels act to trigger expansion of β cell mass as a compensatory mechanism [53]. Early studies indicated that glucose stimulation promoted insulin biosynthesis and up-regulated anti-apoptotic proteins [54-56]. Microarray studies have since demonstrated glucose-dependent transcriptional up-regulation of genes that are crucial for β cell function including GLUT2, K^{+}_{ATP} channel subunits and the Ca^{2+} -modulated protein calmodulin among other genes [57, 58].

Glucose stimulation is primarily thought to exert its effect on the β cell through Ca^{2+} signaling. In response to glucose, intracellular Ca^{2+} is elevated due to influx from the extracellular and endoplasmic reticulum compartments. Ca^{2+} signaling activates kinases and phosphatases such as CaM kinase (CaMK) and protein phosphatase 2B (PP2B/calcineurin) by promoting complex formation with calmodulin (CaM) (reviewed in [59]). Both calcineurin and CaMKIV have been implicated in important β cell biology providing mechanisms by which Ca^{2+} signaling positively regulates the β cell [60, 61]. Glucose stimuli can also impact β cell fitness by altering the cellular energy status of the cell. Energy status regulates β cell transcription through allosteric regulation of adenosine monophosphate kinase (AMPK) by AMP and ADP [62]. Under low glucose conditions, AMPK is also thought to attenuate protein translation through negative inhibition of mammalian target of rapamycin (mTOR) [63]. Glucose-dependent inactivation of AMPK could thereby serve as a mechanism to enhance translation observed in β cells cultured in high glucose [64]. These data highlight potential mechanisms by which glucose and Ca^{2+} signaling promote maintenance of functional β cell mass.

1.3.2 cAMP signaling:

Regulation of the β cell compartment has also been demonstrated by incretin-induced cAMP signaling. In response to feeding, incretin hormones glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are secreted from the gastrointestinal tract and promote insulin secretion from the β cell [65]. Incretins bind G-protein coupled receptors on the plasma membrane and induce cAMP signaling through activation of adenyl cyclase [66]. cAMP signaling potentiates insulin secretion through regulation of the K^{+}_{ATP} channel by protein kinase A and the guanine nucleotide exchange factor Epac2 [67-69]. In addition to regulation of insulin secretion, GLP-1 promotes insulin synthesis through increased pancreatic and duodenal homeobox-1 (Pdx-1) expression, a key transcription factor that regulates insulin transcription [70]. Maintenance of glucose responsiveness is also promoted by GLP-1 through enhanced expression of GLUT-2 and glucokinase in mice [71]. Various β cell survival and proliferation pathways are also promoted by GLP-1 (reviewed in [65]). GLP-1 promotes human β cell survival through elevated B-cell lymphoma 2 (Bcl-2) expression and reduced expression of the proapoptotic Bcl associated X protein (Bax) [72]. In addition, cAMP signaling promotes β cell survival through enhanced expression of insulin receptor substrate 2 (IRS-2), a crucial protein for β cell insulin signaling [73].

1.3.3 Insulin signaling:

Although insulin action is usually considered at a hormonal level on peripheral tissues, insulin also acts in an autocrine fashion on the β cell to promote insulin secretion and stimulate signaling that regulates β cell function and survival (reviewed in [50]). β cell ablation of the insulin receptor impaired insulin secretion and induced glucose

intolerance in mice highlighting an important role of insulin signaling for the β cell [74]. In response to insulin binding, insulin receptors become activated through autophosphorylation of tyrosine residues and signals through a family of adaptor proteins called insulin receptor substrates by tyrosine phosphorylation. Of which, insulin receptor substrate-2, IRS-2 has an indispensible role for β cell function and peripheral insulin sensitivity [75]. Loss of IRS-2 resulted in diabetes in mice characterized by both β cell dysfunction and peripheral insulin resistance [75]. Conversely, transgenic expression of IRS-2 protected mice from diet-induced, β cell poison-induced (streptozotocin) and IRS-2 deficiency-induced diabetes by promoting insulin secretion and β cell survival [76]. In addition, low IRS-2 expression has been observed in isolated islets from T2D patients compared with control islets further supporting a crucial role of IRS-2 in the β cell and the pathogenesis of diabetes [77]. Interestingly, cAMP and Ca^{2+} signaling that promote β cell function and survival discussed above also enhance insulin signaling through elevated IRS-2 expression under the transcriptional control of CREB [73]. Insulin signaling and IRS-2 activation have been shown to promote β cell proliferation and survival through phosphoinositide 3-kinase activation of protein kinase B/AKT [73, 78, 79]. β cell responsiveness to various stimuli illustrates the complex concerted mechanisms for adaptation to external stimuli in processes of β cell compensation in healthy individuals.

1.4 β cell dysfunction and death:

The diabetic condition creates an environment that is harmful to the β cell and has been proposed to aggravate genetic susceptibility. Type 2 diabetic individuals have lower β cell mass compared with weight-matched non-diabetic individuals, highlighting the

failure of β cell compensation in this setting [45, 80, 81]. In addition, apoptosis and reduction in β cell mass have been observed in humans at early stages of diabetes suggesting that β cell defects precede chronic hyperglycemia [45]. Endoplasmic reticulum (ER) function is crucial for β cell insulin biosynthesis and has been suggested to be stressed and dysfunctional in a diabetic setting due to high insulin demands [82]. Prolonged ER stress caused by insulin misfolding can promote β cell apoptosis and induce diabetes in mice [83]. In addition, transplantation of an insulinoma in mice demonstrated that prolonged elevations in insulin levels reduced β cell mass and provides a potential mechanism by which hyperinsulinemia, characteristic of insulin resistant individuals, leads to loss of β cell mass [84]. In addition, some data suggest that the β cell becomes resistant to mechanisms that promote functional β cell mass such as incretin and insulin signaling [85, 86]. In later stages of the disease, hyperglycemia and hyperlipidemia characteristic of T2D pathogenesis inflict strain on the mitochondria as they are required to utilize an excess of hydrocarbons [87]. An increase in apoptosis is observed due to mitochondrial dysfunction and an increase in reactive oxygen species [87]. In addition, chronic elevation in glucose results in lipotoxicity in the β cell through prolonged inactivation of AMPK and inhibition of fatty acid oxidation [88]. This "glucolipotoxicity" has been suggested to contribute to the eventual demise of the β cell [89].

1.5 Diabetes therapeutic strategies

1.5.1 Management of diabetes:

Therapeutic strategies for early stages of T2D focus on treating insulin resistance by promoting peripheral glucose uptake and reducing glucose output by the liver through

activation of AMPK using biguanide or thiazolidinedione classes of drugs [90]. Interestingly, exercise has also been shown to activate AMPK and promote insulin sensitivity in peripheral tissue [91]. In addition, sulfonylurea drugs are used to enhance insulin secretion from the β cells by promoting closure of the K^+_{ATP} channel. While these treatments serve to temporarily improve glucose regulation, they require that the patients have remaining functional β cell mass. In addition, there is evidence that chronic treatment with these drugs impair β cell function and survival directly [92, 93]. Loss of functional β cell mass at either late stages of T2D or in the case of T1D renders the patient dependent on exogenous insulin injections for the regulation of blood glucose.

1.5.2 Promotion of functional β cell mass:

Newer strategies to treat diabetes focus on the promotion of functional β cell mass such as GLP-1 therapy but have encountered challenges translating from rodent models to humans with respect to the efficacy of the treatment. It is likely that this is in part due to the significant ability of the rodent β cell to proliferate compared with the human β cell [39, 94]. For instance, partial removal of the pancreas by a pancreatectomy does not induce proliferation of the human β cell as it does in rodent models [95, 96]. Nuclear exclusion of cell cycle machinery in the human β cell, but not the mouse β cell, suggests that the human β cell has an additional hurdle to overcome to enter the cell cycle and proliferate [25]. Currently, the only successful way to replace β cell mass in humans is through pancreas or islet transplantations that are highly limited by the number of human pancreas donors and the need to transplant a large excess of human β cells due to poor survival of the grafts.

1.5.3 Transplantation:

Early attempts to restore β cell mass preceded the discovery of insulin and were performed by whole pancreas transplantation from sheep and human cadavers into severely diabetic individuals [97, 98]. After the discovery of insulin and the subsequent emergence of diabetic complications, pancreas transplantation was first successfully performed as a pancreas kidney co-transplantation in a patient with renal failure [99]. Due to the invasive nature of this procedure, pancreas transplantations are still generally reserved for diabetic individuals with severe complications.

Transplantation of purified islets was later considered as a strategy to reduce the invasiveness and risk of these procedures. However, due to technical challenges isolating pure healthy islet preparations, early attempts of islet transplantations were largely ineffective [100]. Subsequent trials achieved insulin independence in patients, however, only 10% remained insulin independent after one year of the transplant due to poor graft survival of [101]. In 2000, the "Edmonton Protocol" clinical trial was performed in an attempt to improve the success rate of islet transplantation by avoiding the use of glucocorticoid immunosuppression drugs that frequently induce hyperglycemia and diabetes in patients [102]. The Edmonton protocol has become the foundation for the current islet transplantation strategy worldwide and involves the transplantation of islets into the portal vein of the patients coupled with an immunosuppressant regime including tacrolimus (FK506), sirolimus (rapamycin) and anti-IL-2 receptor antibody to prevent immune rejection of the islets [102]. However despite the initial promising results of the Edmonton protocol, 90% of patients became insulin-dependent after 5 years [21]. Poor β cell survival has in part been attributed to graft rejection, immune destruction of the β

cells and poor oxygen supply, however, increasing evidence indicates these immunosuppressants have profound inhibitory effects on β cells [21, 103].

1.6 New Onset Diabetes After Transplantation (NODAT)

Post-transplantation diabetes mellitus (PTDM) or new onset diabetes after transplantation (NODAT) is frequently observed in patients after undergoing renal or liver transplantation with reported incidences ranging from 2-50% depending on the type of transplant and the definition of diabetes used [104-106]. Early documentation of transplantation-induced diabetes was associated with steroid-based immunosuppression regimes that induced hyperglycemia by impairing peripheral insulin sensitivity [107]. Use of corticosteroids also increased risk for cardiovascular complications through hyperlipidemia and hypertension [108]. Due to these unwanted side effects, corticosteroids were replaced by new classes of drugs such as mammalian target of rapamycin (mTOR) inhibitors (rapamycin/sirolimus) and calcineurin inhibitors (cyclosporin A/CsA, tacrolimus/FK506). Unfortunately, NODAT was still encountered with the use of these new drugs and growing evidence suggest that this is due to direct effects on the β cell.

1.6.1 Immunosuppressants and glucoregulation

Due to the clinical association with diabetes, the impact of immunosupressants on glucose regulation was evaluated extensively in animal models. Early studies demonstrated that tacrolimus treatment impaired glucose clearance in rats [109-113], dogs [114] and primates [115-117]. Most studies suggest that these detrimental effects were reversed upon discontinued treatment [109, 115, 116, 118]; however, irreversible impaired insulin secretion was documented in dogs treated with tacrolimus for 4 weeks

[114]. In rats, tacrolimus was shown to impair insulin secretion rather than alter peripheral sensitivity [109-113]. Low doses of sirolimus treatment in rats did not impair glucose clearance; however, rats exhibited elevated serum insulin levels suggestive of insulin resistance [113]. In addition, impaired glucose clearance observed in tacrolimus-treated rats was exacerbated in the presence of sirolimus [113], effects that were irreversible four weeks after discontinued use [119]. Perhaps most interestingly, sirolimus and tacrolimus co-treatment as used in the Edmonton Protocol, impaired β cell regeneration in mice [96]. In this study, β cells were ablated using a doxycycline-inducible β cell specific transgenic diphtheria toxin that specifically killed the β cells [96]. Although control mice were able to regain glucose regulation, mice treated with immunosuppressants remained diabetic [96]. Furthermore, restoration of glucose regulation in non-obese diabetic (NOD) severe combined immunodeficient (SCID) mice by transplantation of human islet grafts was impaired by tacrolimus [120]. These data suggest that immunosuppressants inhibit crucial β cell function, proliferation and survival pathways and contribute to diabetes in a chronic immunosuppression setting.

1.6.2 Immunosuppressants and β cell function and survival:

Further evidence for impaired β cell function and survival has been elucidated by *ex vivo* analysis of human and rodent islets and histological analysis from subjects treated with immunosuppressants. Post-mortem analysis of pancreas sections and isolated islets from tacrolimus-treated rats revealed defects such as an increase in apoptosis, impaired insulin transcription and impaired insulin secretion [113, 117]. Rats treated with sirolimus had increase β cell turnover as indicated by an increase in β cell apoptosis with no change in β cell mass [113]. Furthermore, co-treatment with tacrolimus and sirolimus

increased the number of apoptotic β cells and reduced islet size to greater extents than tacrolimus treatment on its own [113]. Islet defects have also been observed in human patients treated with calcineurin-inhibitors. Presence of apoptotic β cells and abnormal insulin staining was observed in biopsies from patients having undergone pancreas transplantation and being treated with the calcineurin inhibitors [121]. Furthermore, the extent of islet damage correlated with serum concentrations of these inhibitors [121].

Several *in vitro* and *ex vivo* studies in β cell lines and primary β cells have also demonstrated impaired survival and function by calcinuerin and mTOR inhibition. For instance, isolated rodent and human islets cultured with rapamycin for several days had impaired glucose-stimulated insulin secretion and β cell survival [122, 123]. Several studies evaluating the impact of calcineurin inhibitors, tacrolimus and cyclosporin A (CsA) in rodent and human β cells have demonstrated reduced insulin content, glucose-stimulated insulin secretion and impaired β cell survival [109, 110, 113, 120, 124, 125]. Tacrolimus has been suggested to impair insulin secretion through altered Ca^{2+} oscillations [118]. Together, pharmacological data *in vivo* and *ex vivo* suggest that mTOR and perhaps, more importantly, calcineurin are crucial for the maintenance of functional β cell mass and the prevention of diabetes.

1.7 Calcineurin and the β cell

Evidence for the importance of calcineurin in the β cell has been elucidated by genetic means. Knockout of the regulatory unit of calcineurin, Cnb1, in β cells of mice impaired whole body glucoregulation. Cnb1KO mice were hyperglycemic, had impaired glucose-clearance, and had significantly lower insulin secretion and content [61]. Furthermore, these mice displayed impaired β cell proliferation and reduced β cell mass

[61]. Calcineurin is also involved in pancreatic development and is required for maintenance of replication, insulin storage and secretion in mouse and human islets [126]. Interestingly, transgenic overexpression of constitutively active calcineurin also impairs glucose regulation and alters insulin secretion dynamics [127]. These data suggest that calcineurin regulation is crucial for the maintenance of functional β cells. Evaluation of calcineurin targets in the β cell has provided valuable insight into the mechanisms for the important role of calcineurin. Impaired β cell function caused by the loss of calcineurin has largely been attributed to impaired activation of nuclear factor of activated T-cells (NFAT), the same substrate targeted in T-cells to exert immunosuppressive properties [61, 128]. NFAT is activated in the β cell in response to glucose-induced Ca^{2+} signaling by calcineurin dependent dephosphorylation and nuclear translocation [128]. NFAT regulates transcription of insulin and has been implicated in glucose dependent increases in insulin transcription [129]. In addition, overexpression of NFAT has been shown to revert the diabetic effects of calcineurin knockout in the β cell [61]. However, additional substrates may also be relevant to β cell phenotypes observed in the absence of calcineurin. Transcription regulated by cAMP response element binding protein (CREB) is dependent on calcineurin and is crucial for β cell survival thus serving as an additional factor that could contribute to β cell dysfunction and death observed in an immunosuppression setting [73, 130, 131].

1.8 cAMP Response Element Binding protein (CREB):

1.8.1 CREB Regulation:

CREB is a well characterized transcription factor that is a member of the basic leucine zipper (bZIP) superfamily along with cAMP regulated element modulator

(CREM) and activating transcription factor (ATF-1) (reviewed in [132]). Regulated by phosphorylation, CREB responds rapidly to numerous cellular signals and regulates transcriptional programs that vary across cell types [132-134]. CREB has been shown to govern transcription of genes involved in metabolism, neurotransmission, cell cycle and transcriptional regulation (reviewed in [135]). CREB regulates transcription of genes by binding to palindromic sequences termed cAMP response elements, or CREs, that are found within promoter regions and are highly conserved amongst cAMP-responsive genes [136]. As traditionally documented, cAMP signaling activates protein kinase A (PKA), which phosphorylates CREB at Ser133 located within CREB's kinase inducible domain (KID) [137]. In addition to the original association with cAMP, Ca^{2+} signaling and cellular stresses also promote Ser133 phosphorylation of CREB through activation of additional regulatory kinases [138-140]. Ser133 phosphorylation increases CREB binding with the histone acetyltransferase coactivators CREB binding protein, CBP, and CBP paralogue p300 [135, 136]. CBP:CREB association is enhanced in response to cAMP stimuli and promotes CREB-regulated transcription [137, 138, 141, 142]. Conversely, CREB activity can be negatively regulated by phosphorylation at additional sites such as Ser142 [143] and Ser111 and Ser121 [144] within the KID that impair CREB-CBP binding. After stimulation, CREB regulated transcription is inactivated by dephosphorylation of CREB by protein phosphatases PP1 and PP2A [145, 146]. Furthermore, induction of inducible cAMP early repressor (ICER), a direct target of CREB, provides negative feedback by competitive binding to CREB-target gene promoters [147]. While Ser133 phosphorylation is crucial for CREB transcriptional activation, it is not sufficient for CREB activity as demonstrated by a lack of correlation

between phosphorylated CREB occupancy on gene promoters and transcription levels [133]. An additional level of CREB regulation was revealed through a high throughput cDNA overexpression screen that identified the Transducers Of Regulated CREB transcription or TORCs as potent activators of CREB [148]. Now referred to as CREB regulated transcriptional coactivators or CRTCs, these proteins have been shown to respond to various stimuli to contribute to the finely tuned regulation of CREB.

1.8.2 CREB regulated transcription coactivators (CRTCs/TORCs)

The Crtc family has three highly conserved homologues in mammals and a single conserved orthologue in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* [148-150]. Crtcs contain an N-terminal CREB binding domain, a central regulatory domain, an alternate splicing domain and a C-terminal transactivation domain (reviewed in [134]). Crtcs bind CREB as a tetramer, promote CREB occupancy on promoters, enhance CREB activity and are involved in the alternative splicing of target genes [151, 152]. Given that Crtcs serve as an additional level of CREB regulation, understanding Crtc regulation provides further mechanistic knowledge for how CREB is intricately regulated in a variety of physiological settings. In addition, Crtcs regulation by distinct proteins from that which regulate CREB has provided new insight into how CREB is regulated by proteins that seemingly had no impact on classic regulatory mechanisms of CREB.

1.8.3. Regulation of Crtcs:

Crtc regulation of CREB is highly conserved as demonstrated by Crtc regulation of CREB orthologue CRH-1 in *C. elegans* [149]. Crtcs are negatively regulated by phosphorylation at sites that promote cytoplasmic retention of Crtcs through interaction

with 14-3-3 proteins [131, 149, 153]. Crtcs can be activated in response to cAMP and Ca^{2+} stimuli [131]. cAMP-dependent PKA activation promotes Crtc activity by inhibiting kinases that phosphorylate and thereby negatively regulate Crtcs [131, 149, 154]. For instance, cAMP-induced PKA phosphorylation of Salt inducible kinase 2 (SIK2) at Ser587 inactivates SIK2, a negative regulator of Crtc2 and thereby relieves inhibition of the Crtc2-CREB pathway through Crtc2 dephosphorylation and nuclear localization of Crtc2 [131, 155, 156]. Conversely, insulin promotes phosphorylation of Crtc2 and inhibits CREB activity in the liver via PKB/AKT-mediated activation of SIK2 at an additional regulatory site, Ser358 [157]. In addition, Ca^{2+} signaling regulates Crtcs through activation the Ser/Thr phosphatase calcineurin by promoting dephosphorylation and nuclear translocation of Crtcs [131, 149]. A recent study in the liver demonstrated that cAMP signaling triggers Ca^{2+}-signaling events via the InsP3 receptor in response to glucagon, and activates gluconeogenesis via Crtc2-CREB in a calcineurin dependent manner [158]. The finding that Crtcs are regulated by calcineurin provided an elegant mechanism for impaired CREB activity in HIT hamster insulinoma cell line observed in CREB reporter assay in the presence of calcineurin inhibitors that was reported long before the discovery of Crtcs [159, 160]. Furthermore, it highlights Crtcs as targets of calcineurin-inhibition and suggests that Crtcs inactivation could play a role in the phenotypes observed in an immunosuppression setting.

Additional regulation of Crtc2 through O-glycosylation has been proposed to compete with negative phosphoregulation of Crtc2 to promote its activity [161]. In addition, CBP has been shown to acetylate Crtc2 in the liver and promote Crtc2 activation and subsequent ubiquitination and degradation [162]. Interestingly, SIK1 is

transiently expressed under the transcriptional control of CREB and has been shown to regulate CREB activity through negative feedback by phosphorylation and inhibition of Crtc1 in cortical neurons and Crtc2 in the liver [154, 163]. These data highlight an intricate coordinated mechanism of Crtc-CREB regulation in response to various cellular stimuli. The extensive post-translational modifications of Crtcs serve as new potential targets that may be altered in a variety of physiological settings and translate to altered transcription programs that govern cellular function.

Crtc phospho-regulation by the AMPK family of kinases provided a novel mechanism of CREB regulation via hormonal and energy sensing. There are 14 members of the AMPK family of kinases. It is clear from *in vitro* kinase assays and studies in various tissues that many members of the AMPK family can phosphorylate Crtcs and contribute to the regulation of CREB activity [154, 164-166]. For instance, *in vitro* kinase data suggest that SIK2 and MARK2, members of the AMPK family of kinases, can preferentially phosphorylate Crtc2 at regulatory phosphorylation sites Ser171 and Ser275, respectively. In addition, overexpression of SIK2 was shown to reduce cAMP-induced CREB activity through interaction with and phosphorylation of Crtc2 in HEK293T cells [131]. Furthermore, it is established that the global activity of AMP kinases is regulated by phosphorylation at a conserved site in the T-loop region by the upstream kinase Liver kinase B1 (LKB1), suggesting that LKB1 may regulate Crtc2-CREB [167]. Indeed, LKB1 knockout in the liver was shown to impair Crtc2 phosphorylation, promote nuclear entry of Crtc2 and elevate transcription of CREB regulated gluconeogenic genes [90]. LKB1 has also been shown to regulate CREB activity through SIK1 dependent phosphorylation of Crtcs in HEK293T cells [168]. AMPK plays crucial roles in glucose

metabolism by promoting glucose uptake, inhibiting gluconeogenesis, and regulating adipocyte hormone secretion [169-171]. Growing evidence supports a role of Crtc-CREB in cellular biology observed from altered AMPK activity thereby highlighting the existence of a LKB1- AMPK- CRTC- CREB pathway that is involved in energy balance and glucose homeostasis.

1.8.4. Crtc-CREB in energy balance:

Crtcs play a key role in energy balance by regulating glucose metabolism at the level of brain, liver, skeletal muscle, and adipose tissue and potential dysregulation of the Crtc-CREB pathway has been implicated in the development of diabetes. [172-176]. Deletion of the single Crtc orthologue in *D. melanogaster* reduced lipid stores suggesting a conserved role in energy metabolism in simple organisms [150]. Crtc1 is predominantly expressed in the brain and genetic ablation of Crtc1 resulted in hyperphagia and obesity in mice [175]. Crtc1-CREB is involved in leptin-induced satiety by inducing the transcription of the Cocaine- and amphetamine-regulated transcript (CART) [175]. Without Crtc1, mice are leptin resistant and exert similar phenotypes as leptin deficient and leptin receptor deficient mice ob/ob and db/db, respectively. In adipocytes, loss of CREB or Crtc3 prevented weight gain and improved insulin sensitivity through promotion of GLUT4, increased adiponectin secretion and promotion of fatty acid oxidation [174, 177]. Interestingly, a gain of function single nucleotide polymorphism of Crtc3 has been associated with increased adiposity within a Mexican population [174]. Lastly, Crtc2-CREB plays a crucial role in glucose homeostasis by regulating gluconeogenesis in the liver and pancreatic β cell function and survival [73, 130, 151, 178, 179]. The liver and the pancreatic β cell intricately regulate blood glucose through

altered glucose output and insulin secretion, respectively. Under low glucose conditions, alpha cell secretion of glucagon triggers cAMP signaling in the liver and induces Crtc2 activation and CREB regulated transcription of PPAR-gamma and the rate-limiting enzymes for gluconeogenesis phosphoenolpyruvate carboxykinase-1 (PEPCK-1) and glucose-6-phosphatase (G6Pase) to promote glucose output [154]. Conversely, feeding cues such as glucose and incretin hormones activate Crtc2 in the β cell and promote CREB regulated transcription required for β cell survival [73, 131, 164]. Overall, transgenic ablation of Crtc2 results in improved glucose clearance, increased insulin sensitivity, and reduced serum insulin levels in response to a glucose challenge [151]. This phenotype has largely been attributed to impaired gluconeogenic gene expression and reduced glucose output from hepatocytes [151, 178]. Together, Crtc-CREB regulates a variety of systems that contribute to whole body energy metabolism and glucose homeostasis that are required for the prevention of obesity-induced insulin resistance and diabetes.

1.9 LKB1-AMPK-Crtc2-CREB pathway in the β cell:

1.9.1 CREB and the β cell:

In the β cell, CREB is activated by cAMP and glucose stimuli by PKA and CAMKIV-dependent phosphorylation of Ser133 [60]. As previously discussed, cAMP and glucose/Ca^{2+} signaling induce transcriptional changes that promote β cell function and survival. Given that CREB responds to glucose and cAMP stimuli, CREB-regulated transcription was expected to mediate some of these effects. Indeed, disruption of CREB activity in the islet impaired β cell survival and proliferation, demonstrating a crucial role of CREB in the maintenance of functional β cell mass [73, 130]. The first indication that

CREB was crucial for the maintenance of functional β cell mass was observed in a transgenic mouse expressing a dominant negative CREB (DN-CREB) that abolished CREB activity [73]. DN-CREB mice were severely diabetic due to apoptotic loss of β cell mass that was attributed to impaired expression of the CREB target gene insulin receptor substrate 2 (IRS-2) and activation of protein kinase B (AKT) [73]. A subsequent study evaluated the impact of β cell transgenic expression of induced cAMP early repressor (ICER), a potent repressor of CREB-regulated transcription, and reported impaired glucoregulation that was attributed to a reduction in β cell proliferation and mass [130]. Conversely, transgenic expression of a constitutively active CBP that cannot be inactivated at a negative regulatory phosphorylation site had enhanced CREB activity, and an increase in β cell mass and proliferation [179]. However, insulin secretion from isolated islets from these mice was impaired due to elevated levels of PPARgamma coactivator 1 alpha (PGC1a) [179]. CREB has also been implicated in β cell dysfunction in a diabetic setting. Hyperglycemia has been shown to induce impaired β cell function due to inactivation of CREB [180]. This has in part been attributed to prolonged induction of ICER and has been shown to impair transcription of insulin, NeuroD and SUR1 [181]. These data suggest that fine regulation of CREB is crucial for the maintenance of functional β cell mass.

1.9.2 LKB1-AMPK and the β cell

LKB1 and AMPK serve as negative regulators of CREB in various tissues [90, 168]. Loss of LKB1 in the β cell promoted insulin secretion and protected mice from high-fat diet induced diabetes [182, 183]. These affects were attributed to elevated β cell mass, due to an increase in β cell proliferation and β cell hypertrophy, as well as

functional enhancement of insulin secretion from isolated islets [182]. LKB1 knockout mice had impaired AMPK activity and interestingly AMPK alpha1 knockdown phenocopied enhanced insulin secretion observed in LKB1 knockdown MIN6 β cell line [182]. Previous documentation also suggests that maintenance of low AMPK activity promotes β cell survival and function whereas chronic activation is detrimental to the β cell (reviewed in [92]). In the LKB1-AMPK-Crtc-CREB model, LKB1-AMPK would act to impair β cell function by inhibiting CREB activity through phosphorylation of Crtc2.

1.9.3 Crtc2 and the β cell

Crtc2 is highly expressed in the β cell and is required for the induction of CREB activity in response to Ca^{2+} and cAMP signaling [131, 164]. From work in other tissues and studies performed primarily in β cell lines, a proposed model for Crtc2 regulation in the β cell has been elucidated and is summarized in Figure II. Crtc2 phosphorylation at Ser171 and Ser275 has been shown to cause 14-3-3 protein binding and cytoplasmic retention of Crtc2 under basal conditions, thereby preventing activation of nuclear CREB [131, 164]. Ca^{2+} and cAMP signaling activate Crtc2 by causing calcineurin-dependent dephosphorylation and nuclear entry [131]. Overexpression studies suggest that phosphorylation at Ser171 is regulated by cAMP stimuli in a calcineurin-independent fashion by the kinase SIK2 [131, 164]. Identification of a novel glucose-regulated phosphorylation site Ser275 provided a mechanism for Ca^{2+}/calcineurin dependent Crtc2 activation in the β cell [164]. These data suggest that calcineurin is crucial for Crtc2 activation and that Crtc2 is a target by which immunosuppressants impair CREB activity and β cell survival.

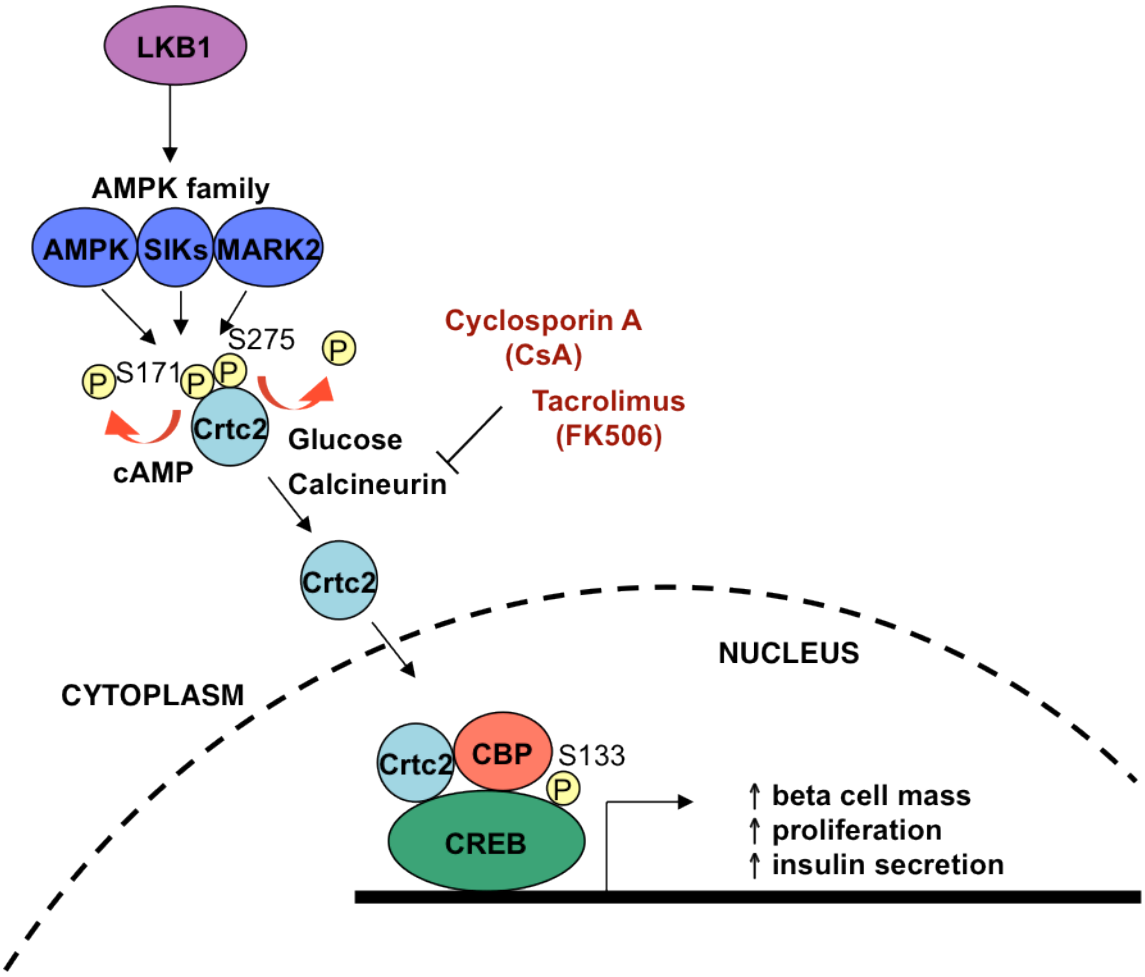

Figure II: Overview of the LKB1-AMPK-CRTC2-CREB pathway. Crtc2 is phosphorylated by kinases of the adenosine monophosphate kinase (AMPK) family; the activity of which is governed by the master kinase LKB1. Under basal conditions, Crtc2 is phosphorylated at regulatory Serine residues Ser171 and Ser275 that enable binding to 14-3-3 proteins and cytoplasmic retention of Crtc2. In response to cAMP and glucose/Ca^{2+} signaling, Crtc2 is dephosphorylated by the phosphatase calcineurin enabling removal of 14-3-3 binding and nuclear relocalization of Crtc2. cAMP and Ca^{2+} signaling also activate CREB by phosphorylation at Ser133. CREB is then activated by recruitment of CREB binding protein (CBP/p300) and Crtc2 to enable transcription of genes such as Irs-2 and Bcl-2 that are important for β cell survival. Immunosuppressants that are known inhibitors of calcineurin such as cyclosporin A and tacrolimus would be expected to impair activation of this pathway through inhibition of Crtc2.

1.10 Hypotheses and Objectives:

Hypotheses:

We proposed that the AMPK family of kinases and calcineurin coordinately regulate Crtc2 in response to glucose and cAMP in the primary β cell. We hypothesized that calcineurin-inhibiting immunosuppressants impair CREB activity and β cell biology by preventing dephosphorylation and nuclear translocation of Crtc2. Furthermore, we expect that constitutive activation of Crtc2 would block CsA/FK506 impairment of CREB activity and β cell function and survival post-transplantation.

Objectives:

To test these hypotheses we evaluated the functional role of Crtc2 in the β cell to determine whether loss of Crtc2 activity through calcineurin inhibition could lead to β cell dysfunction or death. In addition, we evaluated whether previously documented regulatory mechanisms of Crtc2 are relevant to the primary β cell and determined whether a constitutive activation of Crtc2 could restore CREB-regulated transcription in the primary β cell when calcineurin is inhibited.

Specifically the objectives of this study were to:

1. **Evaluate the functional role of Crtc2 in the β cell**

   CREB activity is known to be crucial for β cell survival, however, the importance of its elegant regulation by Crtc2 has not been evaluated. Given that Crtc2 provides an additional level of stimulus dependent regulation of CREB, it may play crucial roles in the maintenance of functional β cells. Furthermore, analysis of the functional role of Crtc2 in the β cell is important for determining whether the β cell defects observed as a consequence of calcineurin inhibition could occur due to inhibition of Crtc2.

2. **Characterize the regulation of Crtc2 in primary β cells**

The majority of our mechanistic understanding of Crtc2 regulation in the β cell has stemmed from studies in other tissues and immortalized β cell lines. Due to potential tissue-specific or cancer cell line-specific differences in Crtc2 regulation, confirmatory studies in primary β cells are required to improve our current understanding of Crtc2 regulation. In addition, several proposed mechanisms of regulation of Crtc2 have been elucidated by *in vitro* and overexpression studies that may not reflect Crtc2 regulation in a physiological setting. While Crtc2 is suspected to be regulated by LKB1-AMPK and calcineurin, novel studies in the islets will determine the relevance of these Crtc2-CREB regulatory mechanisms in the β cell. In addition, these studies will shed light on the mechanism of impaired CREB activity in an immunosuppression setting.

3. **Demonstrate whether constitutively active Crtc2 expression can restore CREB-regulated transcription in the presence of calcineurin-inhibiting immunosuppressants.**

Mutation of regulatory phosphorylation sites on Crtc2 to a non-phosphorylatable residue has been shown to promote nuclear localization in a stimulus-independent fashion in hamster insulinoma cells [164]. Given that calcineurin inhibitors are suspected to impair CREB activation by inhibiting dephosphorylation of Crtc2, constitutively active Crtc2 that cannot be inhibited by phosphorylation will be used to evaluate whether CREB activity can be restored in the presence of calcineurin inhibition.

Chapter 2: Materials and methods

2.1 Islet Isolation:

Mouse islets were harvested from male FVB/n mice of at least 8 weeks of age. Mice were anesthetized using isofluorane and culled by cervical dislocation. Pancreatic drainage to the duodenum was blocked with a clamp to enable inflation of the pancreas. Five mL of 0.5 mg/mL type XI collagenase (Sigma) digestion solution was injected into the pancreatic duct prior to harvest to improve digestion efficiency. The inflated pancreas was incubated for up to 15 min at 37^{o}C in an additional 5 mL of collagenase solution and was monitored visually for completion of digest. Collagenase solution was prepared in Hank's Balanced Salt Solution (From 10X HBSS (Wisent)) plus 5 mM glucose, 1 mM $MgCl_2$ at pH 7.4. After digestion, 10 mL of HBSS described above containing 1 mM $CaCl_2$ was added to inactivate collagenase. Tissue was filtered using a 70 μm filter, rinsed and islets were picked manually from remaining tissue using a dissection microscope.

2.2 Tissue culture:

MIN6 cells (passage 25-35) were cultured in DMEM containing 10% fetal calf serum, penicillin, streptomycin and 100 μM β-mercaptoethanol. shRNAs targeting Crtc2 were packaged in HEK293T cells cultured in 10 cm dishes by reverse transfection of pLKO.1, pCMV8.74 and pDM2.G vectors using 50 μg/mL polyethylenimine (PEI). Seed sequences of shRNAs targeting Crtc2: 1: GACCCATACTATGACCCATTT, 2: GAGGACTCATTCCGTAGTGAT 3: AGCAAGGTGTAGAGGGAAATC, 4: GATGCTAAAGTCCCTGCTATT. At 72 hr post transfection, HEK293T supernatant containing lentivirus was filtered using a 0.2 μm filter and virus was pelleted by

ultracentrifugation at 28000g for 2 hr with a lower 20% sucrose layer. Virus was resuspended to 100X in DMEM at 4 ^{o}C overnight and stored in aliquots at -80 ^{o}C for use. Virus packaged in parallel was assumed to be equal in titer. 1.25x10^5 MIN6 cells were infected with 1 uL of concentrated shRNA lentivirus for 72 hr prior to analysis. For overexpression, Crtc2-GFP constructs were cloned into the pLD puro vector. Virus was packaged as described above and 15 μL of concentrated lentivirus was used to infect 1.25 X10^5 MIN6 cells. Islets were cultured in RPMI containing 10% fetal calf serum and antibiotics. Mouse islets were dispersed by trypsin digest for 1 min and seeded on poly-D lysine coated tissue culture plates.

2.3 Glucose-stimulated insulin secretion (GSIS):

MIN6 cells were equilibrated with Kreb's Ringer buffer (KRB; 128 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES, and 0.1% BSA) plus 1 mM glucose for 30 min. Insulin secretion was measured from supernatant of a 1 hr incubation with 1 mM glucose KRB and from a subsequent 1 hr incubation in 20 mM glucose KRB. For primary cells GSIS experiments, 2.8 mM and 16.7 mM glucose in KRB were used for basal and stimulated insulin secretion respectively. Cells were sonicated for insulin content and DNA determination in 10 mM Tris HCl (pH 7.0) containing 1 mM EDTA, and 1 mg/mL BSA. DNA was determined using Quant-iT PicoGreen kit after 1hr ProteinaseK digestion. Total insulin was harvested from cells by overnight acid-ethanol extraction. Samples were speed vacuum-dried for 2 hr and resuspended in water at 65 ^{o}C for 15 min prior to insulin quantification. All insulin measurements were performed by homogenous time resolved fluorescence (HTRF) analysis using a Synergy2 Biotek plate reader (Cisbio). Sensitivity was set to the

low and high standards for the reading of each experiment. Insulin samples were serially diluted to fall within the standard range of 0.3-10 ng/mL.

2.4 Glucose and exendin-4 cell treatments:

i) Crtc2 phosphorylation analysis*:*

MIN6 cells were seeded at a density of $2x10^5$ cells per 48 well-plate well in 250 μL of medium. The next day, cells were starved in 1 mM glucose KRB for 1 hr and then either starved for an additional hour or treated with 10 nM Exendin-4, 20 mM glucose or both for 1 hr. The same protocol was used for the isolated islet experiments with the exceptions that 2.8 mM glucose was used for starvation and 16.7 mM glucose for treatment. 75 islets/condition were used for analysis. When the cells were treated with cyclosporin A, or tacrolimus/FK506, they were included throughout the duration of the starvation and treatment incubations at 500 nM and 10 nM, respectively and the control cells were treated with equal volume of ethanol or dimethyl sufoxide (DMSO). The PKA inhibitor H89 was used at 20 μM.

ii) mRNA and protein analysis of CREB target gene expression:

MIN6 cells were cultured in 5 mM glucose in DMEM containing 10% fetal calf serum, antibiotics and 100 μM β-mercaptoethanol for 16 hr to reach basal CREB transcription. MIN6 cells were then starved for an additional hour in 1 mM glucose in KRB prior to harvest or subsequent treatment with 20 mM glucose and 10 nM Exendin-4 for 4 hr. Induction of IRS-2 protein was evaluated after 2 hr low glucose in KRB followed by an 8 hr treatment with 20 mM glucose, 10 nM Ex-4 alone or combined. Calcineurin inhibitors were added 24 hr prior to the experiment and kept throughout the treatments.

2.5 RT-qPCR:

RNA was harvested using QIAGEN RNeasy kit. cDNA was prepared from 750 ng of RNA in a reaction containing 1 mM dNTP, 50 μg/mL oligo dT_{18}, 10 mM DTT, 1X First strand buffer and SuperScript™ II Reverse Transcriptase (Invitrogen). qPCR was performed using QuantiTech SYBR Green PCR kits (QIAGEN) using an Eppendorf Mastercycler epgradient S thermocycler. Transcriptional changes in CREB-target genes were normalized to 36B4 by the ΔCT method.

Oligo sequences:

Nr4a2:	Forward: CGACCTATCCGGCTTTTA	
	Reverse: CGCCGAAATCGTTGTCAGTA	
Irs2:	Forward: AGCTTTGATTGGCTGTCCTGGAG	
	Reverse: TGGCGATATAGTTGAGGCCGTTGT	
PEPCK:	Forward: CATATATACAGCTAAGTATGTTTTC	
	Reverse: CTCTGAAGTTTGCATTTGACACC	
G6Pase:	Forward: CAGGGTCTCCAGCAGCAGG	
	Reverse: CATTTCTGTATGGTAGTGGTG	
36B4:	Forward: CCACGAAATCTCCAGAGGCAC	
	Reverse: ATGATCAGCCCGAAGGAGAAGG	

2.6 Western blotting:

Proteins were harvested using Laemmli sample buffer. Samples were run on 6-10% polyacrylamide gels with 4% upper stacking gel. Proteins were transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane using a Tris/glycine/methanol transfer buffer for 80 min at 100 V. Membranes were blocked with 5% skim milk in Tris

buffered saline containing 0.1% Tween-20 (TBS-T). Membranes were incubated for 1 hr at room temperature or overnight at 4 ^{o}C with the primary antibody in 5% milk in TBS-T + 0.05% sodium azide. Membranes were washed 3-5 times with 1X TBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) at 1:5000 for 1 hr at room temperature. Antibodies against phospho (S133)-CREB 1:500 (Cell signaling technology), Irs-2 1:2000 (Millipore), cleaved caspase 3 1:1000 (Cell signaling), LKB1 1:1000 (Santa Cruz), β actin 1:20,000 (Sigma) were used. CREB and Crtc2 antibodies were generated in rabbit hosts and serum was used for Western blot analysis at 1:1000 and 1:1500, respectively. Phospho-specific antibodies were purified before using. Phospho and non-phospho peptides were coupled to sulfolink beads using cysteine HCl coupling buffer in separate columns. Columns were washed with TBS containing 100 mM NaCl and 2.5 mL of serum from a host rabbit was flowed through the column coupled with the phospho-peptide to bind to phospho-specific antibodies. The column was washed with TBS and phospho-specific antibody was eluted with 0.2 M glycine pH 2.5. The eluted antibody was run through the non-phospho-specific column to remove species recognizing non-phospho specific regions of the peptide and the flow through was collected. The specificity of the antibody was tested on protein samples from cells overexpressing either Crtc2-GFP wt or Crtc2 phospho-mutants.

2.7 Fluorescence imaging:

MIN6 cells were infected with Crtc2-GFP lentivirus for 72 hr prior to treatment for GFP analysis. Cells were treated as described for Crtc2 phosphorylation analysis and then fixed with 4% formaldehyde and stained with 5 nM Hoechst in medium for image analysis using an Opera automated confocal microscope. Localization analysis was

performed using Acapella derived algorithm that was run on Columbus analysis software. Nuclear and cytoplasmic intensity of GFP signal was determined on a single cell basis and were averaged within 30 fields/ well and 3 wells/ condition. The contrast ratio was expressed as ((nuclear- cytoplasmic)/ total cell intensity) and was normalized to 200 ng/mL leptomycin B treated MIN6 cells expressing wildtype Crtc2-GFP for 1 hr to obtain complete nuclear accumulation of Crtc2. For endogenous Crtc2 analysis, cells were seeded 48 hr prior to treatment and similar imaging and quantitation was performed on cells stained for endogenous Crtc2 using 1:100 Crtc2 antibody (Bethyl) and rabbit 488 AlexaFluor secondary antibody in 3% bovine serum albumin in phosphate buffered saline (PBS).

2.8 *In vivo* Crtc2 localization:

FVB-n mice from Charles River were fasted for 16 hr. Fasted blood glucose levels were evaluated using an Ultra OneTouch glucometer (Lifescan). Mice were either refed for 30 min, injected interperitoneally with glucose (2 mg/kg) or injected with glucose plus 24 nmol/kg exendin-4 for 15 min. Blood glucose was monitored prior to harvest. Whole pancreas was fixed in 4% paraformaldehyde overnight and sent to the histology lab for sections cut at a thickness of 5 μm. Sections were deparaffinized with xylene and rehydrated with ethanol and then PBS. Microwave treatment for 10 min in citrate buffer (10 mM citric acid, 0.05% Tween 20) was used for antigen retrieval. Sections were permeabilized with 0.2% triton for 5 min and blocked for 1 hr at room temperature with 5% horse serum. Crtc2 primary antibody (1:100) in horse serum plus 0.2% triton was incubated overnight at 4^{o}C. Sections were washed with PBS and then rabbit secondary antibody AlexaFluor 594 (Invitrogen) was used at 1:250 for 1 hr. Insulin co-staining was

performed at 1:300 (DAKO) and using a guinea pig AlexaFluor 488 secondary antibody (Invitrogen). Images were taken on an Olympus FV1000 confocal microscope and image analysis was performed using Olympus Fluoview software.

2.9 FK506 mouse experiment:

FVB-n mice ordered from Charles River were split into control and experimental groups and fasted/fed blood glucose measurements were taken using a OneTouch glucometer (Lifescan) to establish no pre-existing difference between the groups. Mice were injected daily with 1 mg/kg tacrolimus (Sigma) prepared in 5% polyethylene glycol and 5% Tween80 in sterile PBS or vehicle control for 10 days. Mice were fasted for 16 hr and blood glucose levels were monitored. Glucose tolerance test (GTT) was performed by interperitoneal injection with 2 mg/kg glucose in PBS. Blood glucose was monitored in mice 15, 30, 60 90 and 120 min post glucose injection. Two hr later, mice were harvested for liver and islets. Subsets of islets were harvested immediately for protein and mRNA analysis. The following day, insulin secretion was measured from remaining islets.

2.10 Generation of construct for Crtc2DM transgenic mouse:

Crtc2DM-GFP was subcloned from pCDNA vectors into pCall-EGFP by three-way ligation with GFP. Crtc2 was amplified by PCR with oligos containing XhoI (forward oligo) and SalI (reverse oligo) sites using Phusion Taq polymerase (NEB). GFP was amplified from pEGFP vector with oligos containing SalI (forward oligo) and NotI (reverse oligo). pCal2-EGFP was digested with XhoI and NotI and PCR amplicons were digested with their respective restriction enzymes. Three-way ligation was performed with T4 ligase (Invitrogen) for 5 min at room temperature and 2 uL was incubated in 50 uL of XL1-blue *E. coli* cells for 15 min on ice. Bacteria was transformed by heat shock at

45 °C for 45 sec and incubated on ice for an additional 2 min prior to adding SOC and growing for 30 min at 37 °C in a 225 rpm shaking incubator. Bacteria were plated on 50 μg/mL ampicillin LB plates overnight at 37 °C. Colonies were grown overnight in LB media, plasmids were isolated by miniprep (Nucleospin) and screened for insert by restriction digest. Expression of positive plasmids were confirmed by 24 hr reverse transfection of 150 ng of plasmid with lipofectamine 2000 (Invitrogen) in 9.8 $x10^4$ HEK293T cells in 48 well plate. Expected molecular weight was confirmed by Crtc2 and GFP Western blot analyses. Cells were fixed for 5 min at 4 °C with 1 mL of 2% formaldehyde, 0.2% glutaraldehyde in PBS for β galactosidase staining. Cells were stained with 200 μg/mL X-gal in DMF, 400 μM ferricyanide, 400 μM ferrocyanide and 200 μM $MgCl_2$ in PBS for 1 hr. Cells were imaged with an inverted microscope and images taken with Cellsens software.

2.11 Validation of Crtc2DM mice

Genotyping: Genomic DNA was isolated from 2 mm piece of tail from mice by incubation in 75 μL of alkaline buffer containing 0.07% NaOH and 0.7 mM EDTA in water at 95 °C for 30 min followed by 4 °C for 15 min. 75 μL of 40 mM Tris-HCl pH 5 was immediately added to neutralize the samples. DNA was used for genotyping or stored at -20 °C. Mice were genotyped with oligos specific to β galactosidase. Forward: ATCATCCCGAACGCCTTACT, Reverse: CTGTAGCGGCTGATGTTGAA

β galactosidase staining: Mouse tails were cut longitudinally and stained with 200 μg/mL X- gal in DMF, 400 μM ferricyanide, 400 μM ferrocyanide and 200 μM $MgCl_2$ in PBS for 4 hr and were left overnight when no staining was obtained.

Mouse embryonic fibroblast: Ear clippings were cut into 1 mm^2 pieces in PBS in an eppendorf tube and 200 μL of 4 mg/ml collagenase type 1 (Sigma) and 30 μL of 10 mg/ml dispase in DMEM was added and incubated at 37^oC for 45 min to digest. Five hundred μL of DMEM plus pen/strep was added and digestion was continued overnight. The next day, cell clumps of digested ears were triturated until cloudy and transferred to a 48 well plate for seeding. Cells were rinsed, trypsinized and re-seeded with and without adeno-CRE at a multiplicity of infection of 200. Fibroblasts were monitored for GFP fluorescence and were harvested 3 days after seeding for Western blot analysis.

Chapter 3: Results

3.1 Crtc2 is required for β cell function and survival:

Previous studies have shown that β cell function and Crtc2 activation require calcineurin [131]. To evaluate whether Crtc2 inhibition could contribute to impaired β cell function and survival in a calcineurin-based immunosuppression setting, the functional consequence of acute loss of Crtc2 in the β cell was evaluated using RNA interference. Knockdown of Crtc2 in MIN6 mouse insulinoma cells using 4 lentiviral shRNAs reduced insulin secretion in response to glucose by 50% relative to cells infected with a non-silencing (NS) control shRNA lentivirus (Figure 1A). Early signs of cell death were observed after 72 hr infection of MIN6 cells with 2/4 shRNAs targeting Crtc2 and was confirmed by the presence of the apoptotic marker cleaved caspase 3 by Western blot analysis (Figure 1B). Furthermore, impaired cell growth was observed in MIN6 cells lacking Crtc2 and was reflected by a reduction in cell number at 72 hr and 96 hr post infection (Figure 1C). Due to altered glucose responsiveness of β cell lines compared with primary β cells, insulin secretion was evaluated in primary mouse islets 96 hr after lentiviral knockdown of Crtc2. Knockdown of Crtc2 in primary β cells reduced glucose-stimulated insulin secretion by 25% compared with control islets (Figure 1D). These data suggest that Crtc2 is important for the maintenance of functional β cells and that impaired Crtc2 activation could contribute to β cell dysfunction in an immunosuppression setting.

3.2 Crtc2 is required for insulin secretion upstream of membrane depolarization:

There are several steps in the insulin secretion pathway that could be affected by the loss of Crtc2 and contribute to the impaired insulin secretion observed (Figure 2A). To further understand the role of Crtc2 in insulin secretion, cells were treated with the

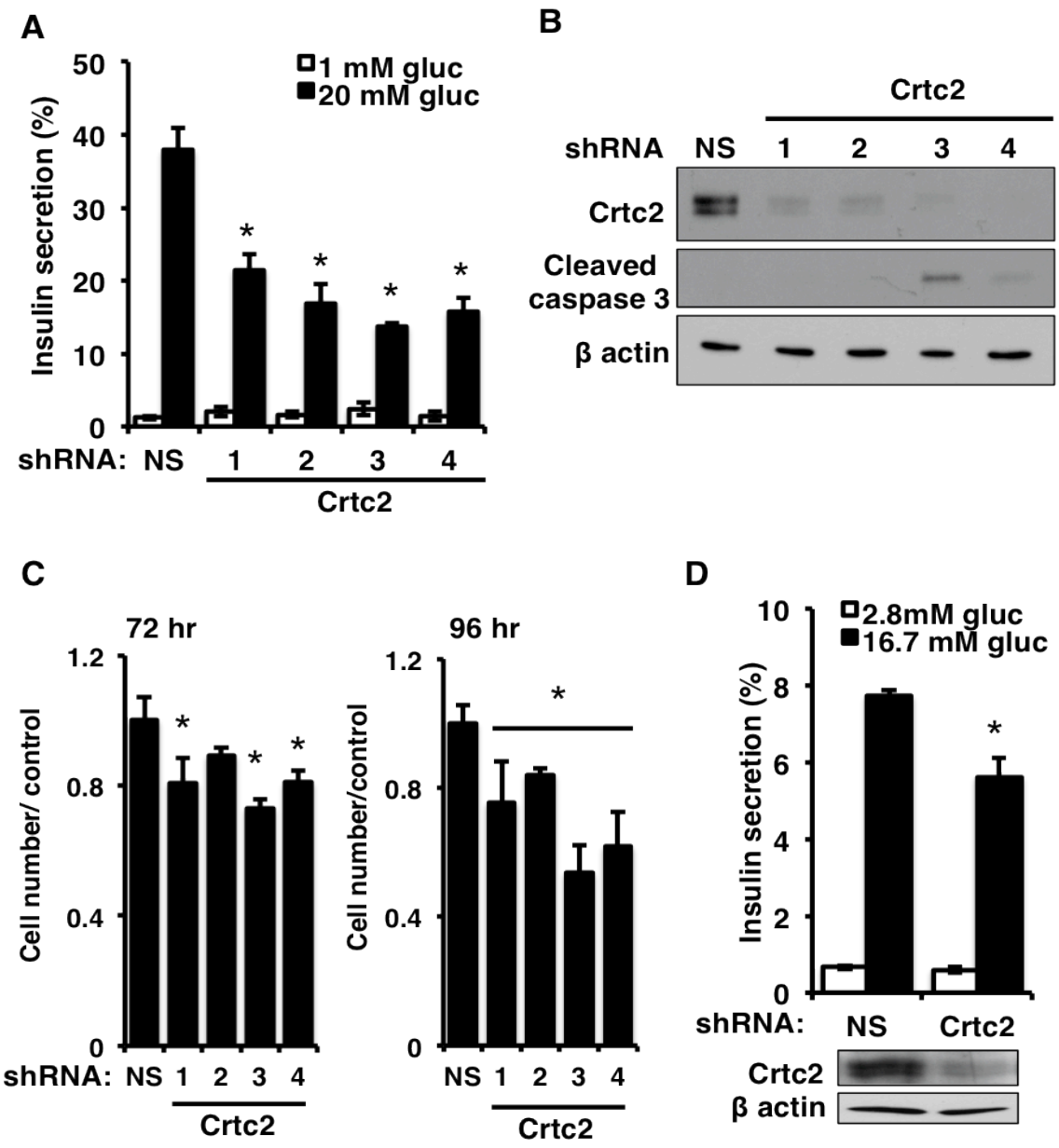

Figure 1: Crtc2 is required for β cell function and survival. A) Loss of Crtc2 impairs glucose-stimulated insulin secretion in MIN6 cells. **B)** Loss of Crtc2 protein by lentiviral mediated shRNA knockdown of Crtc2 caused cell death with 2 out of 4 shRNAs shown by the presence of cleaved caspase 3. **C)** MIN6 cells lacking Crtc2 displayed reduced cell number after 72 and 96 hr. **D)** Loss of Crtc2 impairs glucose-stimulated insulin secretion in primary mouse islets. Data shown are the average of technical triplicates with standard deviation and representative of 3 independent experiments. MIN6 cells were infected with 1 uL of concentrated lenti virus shRNA targeting Crtc2 or NS: non-silencing control. Insulin secretion was measured from MIN6 cells and mouse islets supernatant after 1 hr incubation in indicated glucose concentrations. * $p<0.05$

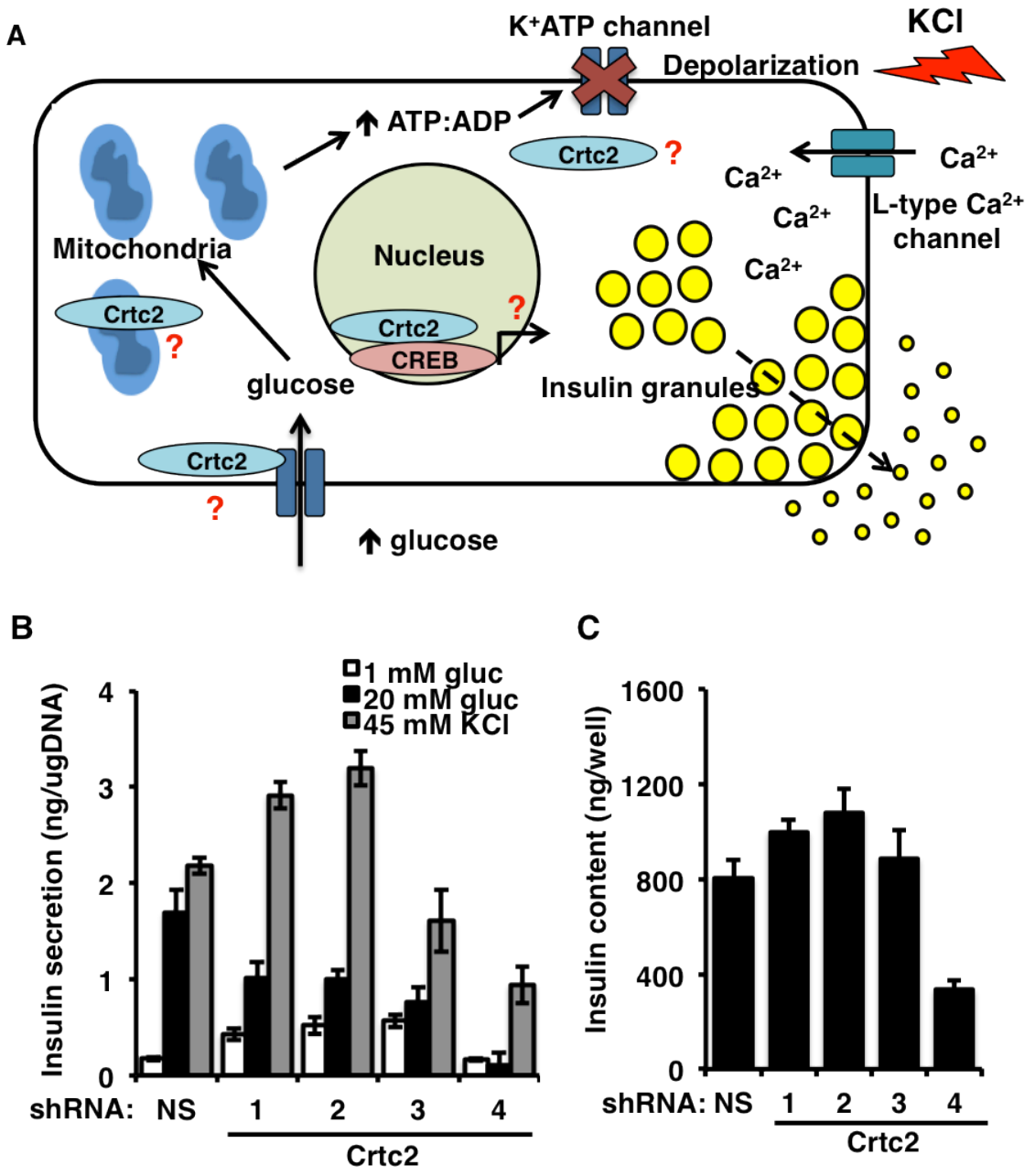

Figure 2: Crtc2 is required for glucose-stimulated insulin secretion upstream of membrane depolarization. A) Steps of the insulin secretion pathway that Crtc2 may be required. **B)** Insulin secretion is restored in β cells lacking Crtc2 by direct KCl-mediated membrane depolarization. **C)** Loss of Crtc2 does not impair insulin content in MIN6 cells. Crtc2 is required for glucose-responsive insulin secretion at steps that are upstream of membrane depolarization. Crtc2 may impact insulin secretion through altered CREB-regulated transcription, impaired glucose import, glucose metabolism or K^{+}_{ATP} channel closure. Insulin secretion was measured as described in figure 1 under the indicated conditions. Insulin content was measured from acid ethanol cell extracts. Data shown are the average of technical triplicates with standard deviation and are representative of 3 independent experiments.

depolarizing agent KCl to trigger late events in the insulin secretion pathway (Figure 2A). Insulin secretion in MIN6 cells lacking Crtc2 was restored by treatment with KCl suggesting that loss of Crtc2 does not impair insulin production or mobilization of insulin granules (Figure 2B). Furthermore, no difference in insulin content was observed with 3 out of 4 shRNAs suggesting that insulin production is not dependent on Crtc2 (Figure 2C). These data suggest that Crtc2 is important for acute responsiveness to glucose and plays a role in early events in the insulin secretion pathway either directly or through transcriptional changes regulated by CREB (Figure 2A).

3.3 Glucose and cAMP-induced CREB-regulated transcription are dependent on Crtc2

Glucose/Ca^{2+} and cAMP have been shown to promote CREB activity in β cell lines in a Crtc2-dependent manner [131, 164]. To confirm this mechanism of regulation in a more physiologically relevant setting, endogenous CREB transcription was evaluated in primary mouse islets treated with glucose and the GLP-1 receptor agonist exendin-4 (ex-4). mRNA levels of the CREB target gene Nr4a2 were elevated by 2 fold in mouse islets treated for 4 hours with glucose or ex-4 alone, and 3-fold in combination (Figure 3A). [131]. To evaluate the extent to which Crtc2 contributes to cAMP and glucose-dependent CREB transcription in the β cell, Crtc2 was knocked down using lentiviral shRNA in MIN6 cells for 72 hr and then stimulated with glucose and ex-4. Glucose and ex-4 treatment, either alone or in combination, increased mRNA levels of CREB targets Nr4a2 and Irs2, consistent with previous findings [131, 164] (Figure 3B,C). In MIN6 cells lacking Crtc2, induction of Nr4a2 and Irs2 mRNA in response to glucose alone or glucose and ex-4 was blocked by 60% and 30% respectively compared with control cells

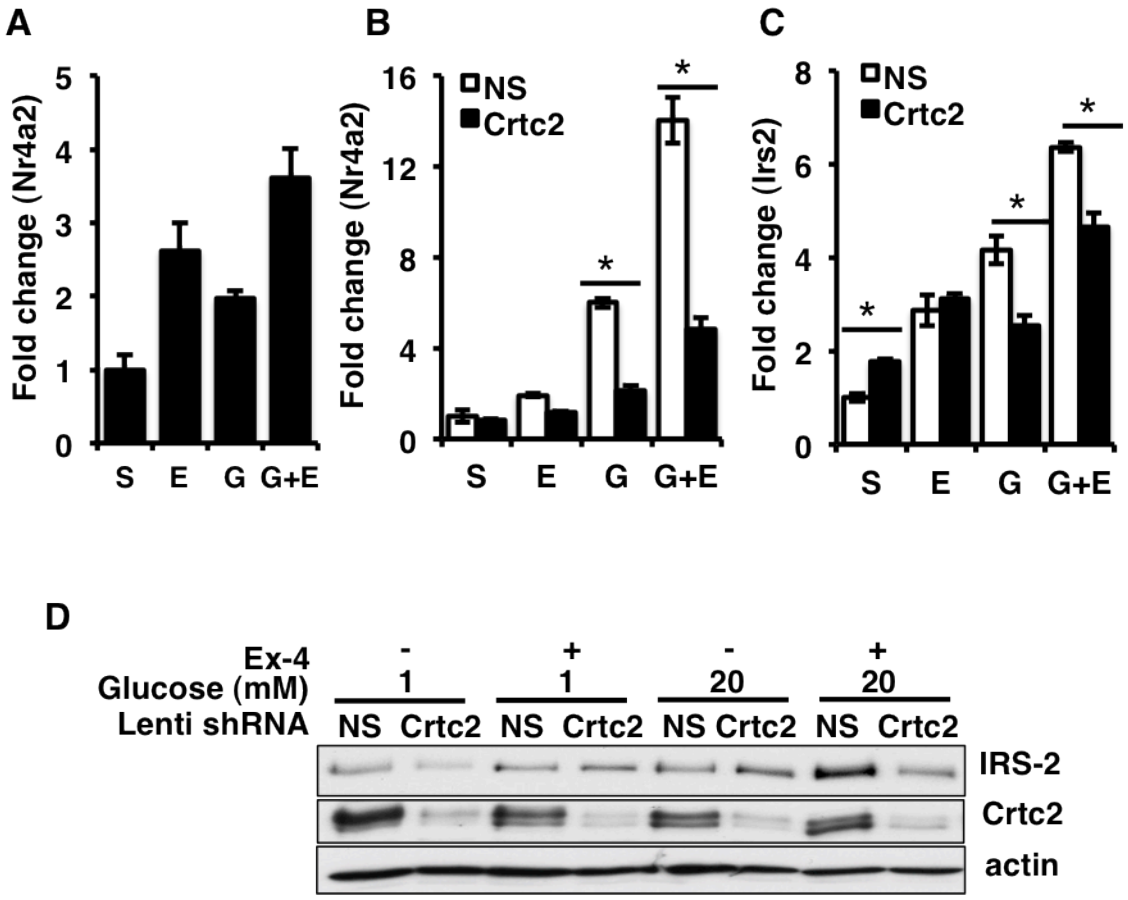

Figure 3: Crtc2 is required for glucose and cAMP activation of CREB in the β cell. **A)** Expression of the CREB target gene Nr4a2 induced in response to glucose and ex-4 in primary mouse islets. **B,C)** Knockdown of Crtc2 reduces glucose and ex-4 induced CREB-regulated transcription of **B)** Nr4a2 and **C)** Irs-2 in MIN6 β cell line. **D)** Knockdown of Crtc2 impairs induction of IRS2 protein in response to glucose and ex-4. For mRNA analysis, MIN6 cells and mouse islets were pre-incubated in 1 mM glucose KRB and 2.8 mM glucose KRB respectively for 2 hr. Cells were treated with ex-4 (10 nM) and glucose (16.7 mM for islets and 20 mM for MIN6 cells) alone or in combination for 4 hr prior to harvest. qPCR was preformed to measure mRNA levels of CREB target genes. For IRS-2 protein analysis, cells were stimulated for an addition 4 hr to allow for protein accumulation prior to harvest for Western bot analysis. Data shown are the average of technical triplicates with standard deviation and representative of 3 independent experiments. * $p < 0.05$

whereas the mRNA levels induced by ex-4 alone were not impacted by the lack of Crtc2 (Figure 3B,C). Furthermore, 8-hour induction of IRS-2 protein levels in response to glucose and ex-4 was inhibited by knockdown of Crtc2 in MIN6 cells (Figure 3D). These data suggest that Crtc2 activation by glucose and ex-4 contributes to the induction of CREB regulated transcription in the β cell.

3.4 Crtc2 localization is regulated by glucose and cAMP

Ca^{2+} and cAMP stimuli are required for Crtc2 nuclear entry in hamster insulinoma cells (HIT-T15 cells) [131, 164]. To further evaluate Crtc2 regulation in the β cell in response to physiological glucose and cAMP stimuli, an automated unbiased approach was taken to detect and quantitate changes in Crtc2 localization. MIN6 cells were infected with lentivirus expressing a Crtc2-GFP to monitor Crtc2 localization by fluorescence imaging. Crtc2 was found to localize to the cytoplasm in the absence of glucose and the nucleus in response to glucose and ex-4 (Figure 4A) as previous documented [131, 164]. Quantitation of Crtc2 localization demonstrated partial nuclear translocation of Crtc2 in response to the individual stimuli (Figure 4B). Crtc2 localization in MIN6 cells and primary dispersed mouse islets was also confirmed by endogenous staining for Crtc2 using a similar quantitation method (Figure 4C,D, Appendix 1). In addition, the partial relocalization of Crtc2 by individual glucose and ex-4 stimuli correlates well with stimulus-dependent CREB target gene expression and further supports that Crtc2 underlies these changes (Figure 3).

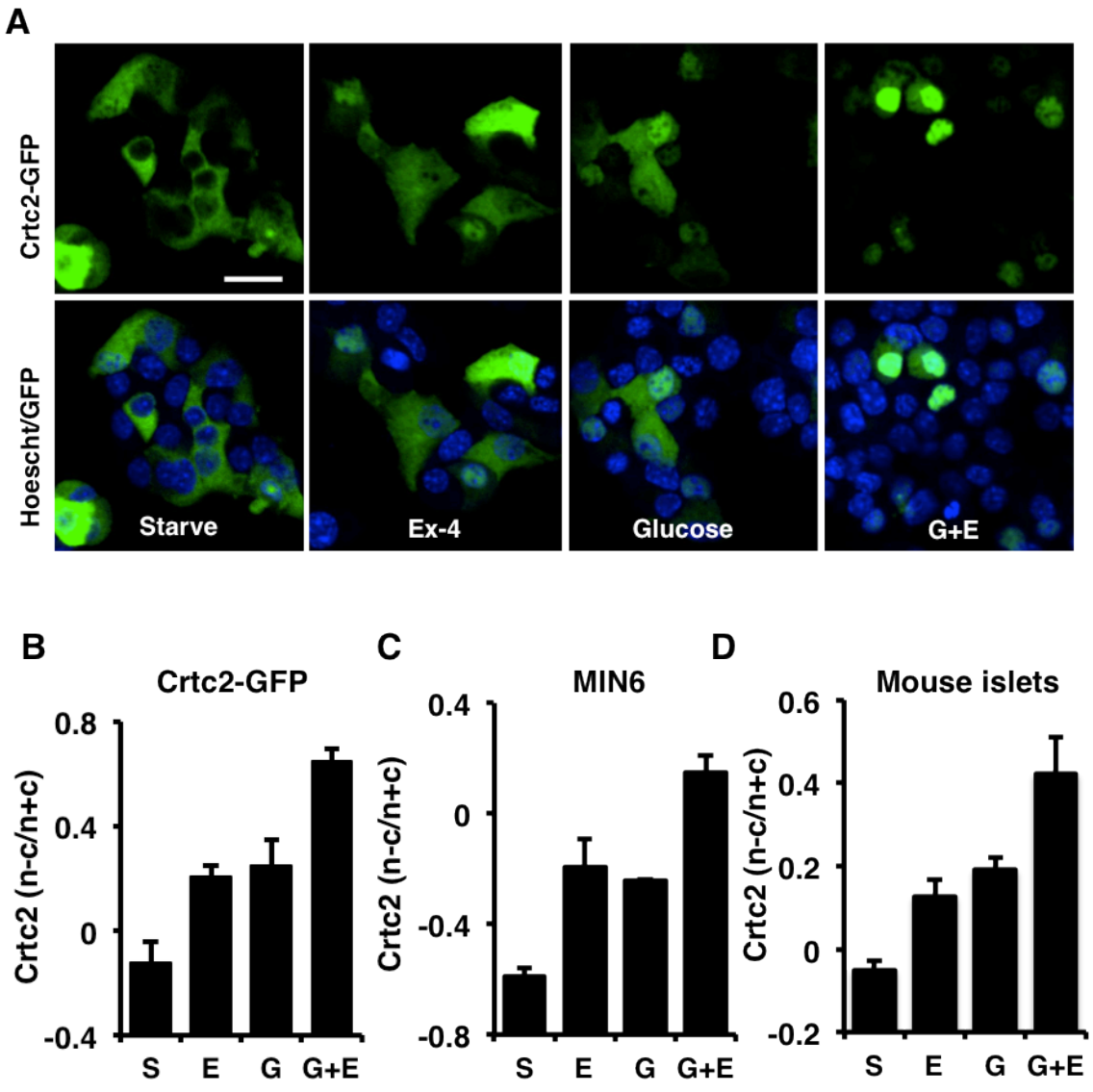

Figure 4: Crtc2 nuclear entry is stimulated by glucose and ex-4 in the β cell. A) Fluorescence images of MIN6 cells expression Crtc2-GFP under glucose-starved or glucose and/or ex-4 treated conditions. **B)** Quantitation of stimulus dependent Crtc2-GFP localization in MIN6 cells. **C,D)** Quantitation of endogenous Crtc2 localization in **C)** MIN6 cells and **D)** dispersed mouse islets. Data shown are the average values from 3 wells per condition with standard deviation. Cells were pre-treated in low glucose for 1 hour and then stimulated as indicated for an hour prior to fixation. 30 fields per well were imaged and analyzed using an algorithm to detect nuclear and cytoplasmic compartments of each cell and express Crtc2 localization as a ratio of the contrast between nuclear and cytoplasmic intensities (n-c) as a fraction of total cellular intensity (n+c). For Crtc2-GFP analysis, a threshold was set to analyze infected cells only. Scale bar 20 μm.

3.5 Crtc2 localization is regulated by glucose and cAMP *in vivo*

To determine whether Crtc2 is regulated in a similar fashion *in vivo*, Crtc2 localization was measured in pancreas sections of fasted versus glucose and cAMP stimulated mice. Blood glucose measurements were taken to ensure the efficacy of the treatments and confirmed a 2-3 fold rise in blood glucose in mice that were fed for 30 min, injected with glucose or injected with glucose and ex-4 for 15 min, as compared to their fasted levels (Figure 5A). Immunohistological analysis of Crtc2 was performed on pancreas sections and Crtc2 subcellular localization was quantified in insulin positive cells. In fasted mice, Crtc2 was predominantly cytoplasmic (Figure 5B,C), consistent with Crtc2 localization in glucose-starved β cells *in vitro*. Treatment of mice with glucose alone, glucose and ex-4 or feeding promoted nuclear entry of Crtc2 that is reflected by a two-fold increase in nuclear intensity of Crtc2 (Figure 5B,C). These data suggest that Crtc2 nuclear entry is regulated by feeding cues *in vivo* by similar regulatory mechanism observed *in vitro*.

3.6 Monitoring Crtc2 activation by endogenous Crtc2 phosphorylation status:

Crtc2 cytoplasmic retention has been shown to be dependent on phosphorylation of Crtc2 at regulatory sites Ser171 and Ser275 through binding to 14-3-3 proteins [164]. Mechanistic studies, predominantly performed in hamster insulinoma cells (HIT cells) led to a proposed model of Crtc2 phosphoregulation by which cAMP promotes Ser171 dephosphorylation, glucose promotes Ser275 dephosphorylation and both events are required for the activation of Crtc2 [164]. Due to previous limitations in reagents, it was not possible to monitor endogenous Crtc2 phosphorylation in the β cell and confirm these findings in the islet. To further evaluate Crtc2 regulation in the β cell, phospho-specific antibodies were generated, purified and optimized to assess Crtc2 phosphorylation on the

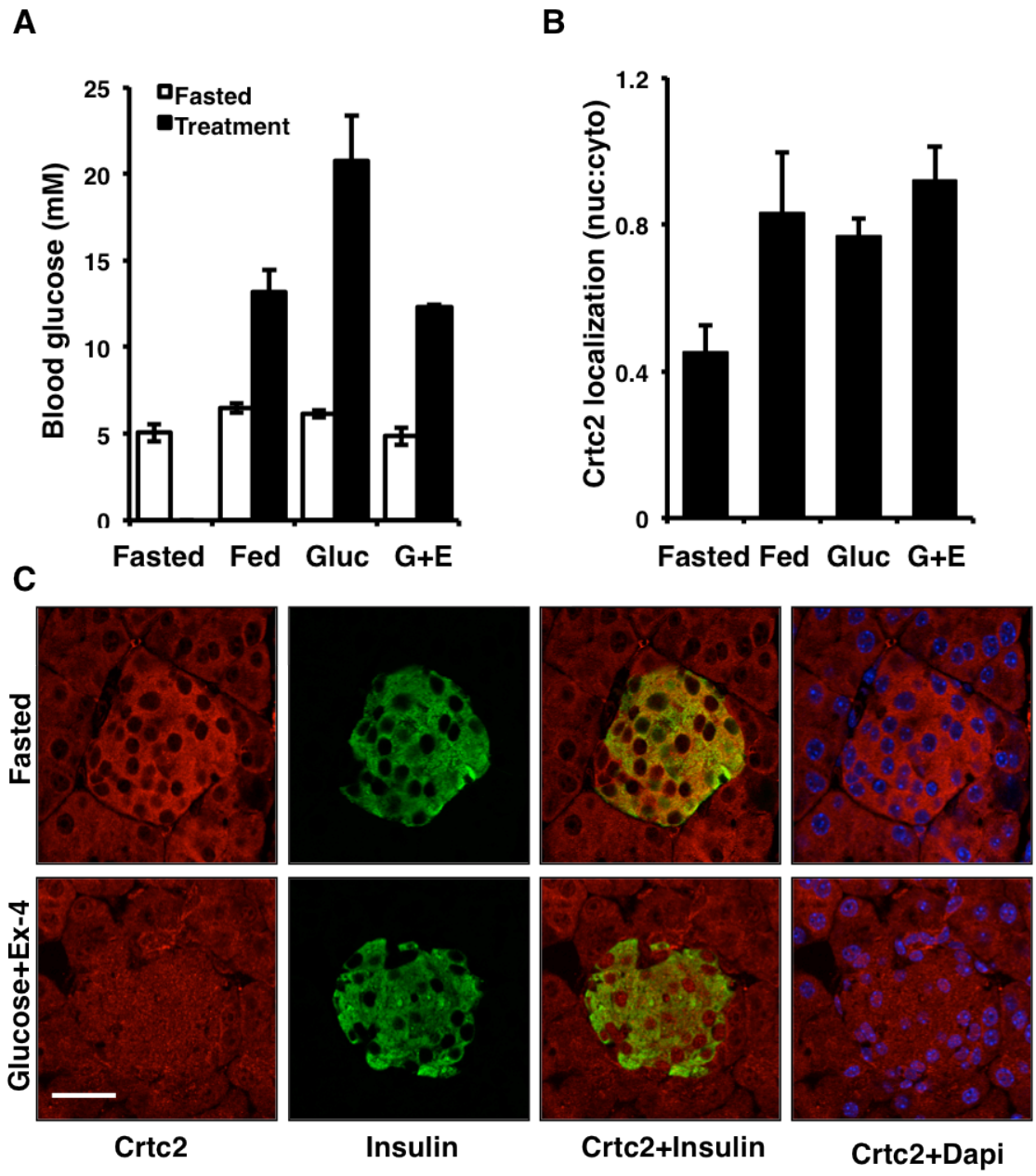

Figure 5: Impact of glucose and cAMP stimuli on Crtc2 localization *in vivo.* A) Blood glucose levels of mice after 16 hr of fasting and after treatment. Mice were sampled 30 min after feeding, or 15 min after intraperitoneal (ip) injection of glucose in the presence or absence of Ex-4. **B)** Quantification of the ratio of nuclear to cytoplasmic Crtc2 signal intensity. Nuclear:cytoplasmic intensities of 10-15 cells per islet were measured, 2 islets / mouse were analyzed, n=2 mice / condition. **C)** Representative images of islets stained with Crtc2, Insulin and Dapi from a 16 hr fasted mouse and 15 min post-IP glucose (2 mg/kg) and ex-4 (24 nmol/kg). Scale bar = 50 μm.

endogenous protein. The resulting antibodies recognized phospho-specific Ser171 and Ser275 as confirmed by recognition of endogenous levels of overexpressed Crtc2-GFP phospho-mutants (Appendix II). The phospho-specific Ser171 antibody detected wildtype and Ser275A Crtc2-GFP, but not S171A and S171,275A Crtc2-GFP proteins (Appendix II A). Conversely the phospho-specific S275 antibody detected wildtype and S171A Crtc2-GFP, but not S275A and S171,275A Crtc2-GFP proteins (Appendix II B).

3.7 Crtc2 phosphorylation is regulated by glucose in the β cell:

Given the novel glucose-dependent dephosphorylation of Crtc2 at Ser275 in the β cell identified by Jansson, et al, 2008, we sought to further characterize glucose regulation of Crtc2 in MIN6 cells and primary mouse islets. β cells were treated with a gradient of glucose concentrations and Crtc2 phosphorylation was evaluated using phospho-specific antibodies in MIN6 cells and mouse islets (Figure 6). Crtc2 was dephosphorylated at Ser275 in response to high concentrations of glucose in MIN6 cells and mouse islets (Figure 6A,B), consistent with previous data [164]. Contrary to previous analysis using overexpressed Crtc2 in HIT cells, glucose also promoted dephosphorylation of endogenous Crtc2 at Ser171 in MIN6 cells (Figure 6A,B) and isolated mouse islets (Figure 6C,D). Interestingly, dephosphorylation of Crtc2 occurs at physiologically relevant glucose concentrations corresponding to the switch between fasted and fed blood glucose levels. These data suggest that phosphorylation of Crtc2 is predominantly regulated by glucose and that Crtc2 may act as a glucose-sensor in the β cell.

3.8 LKB1 regulates Crtc2-CREB activity in the β cell

LKB1 and the AMPK family of kinase regulate Crtc2 in a variety of settings [90, 131, 149, 164]; however, it is unclear whether these regulatory mechanisms translate to Crtc2

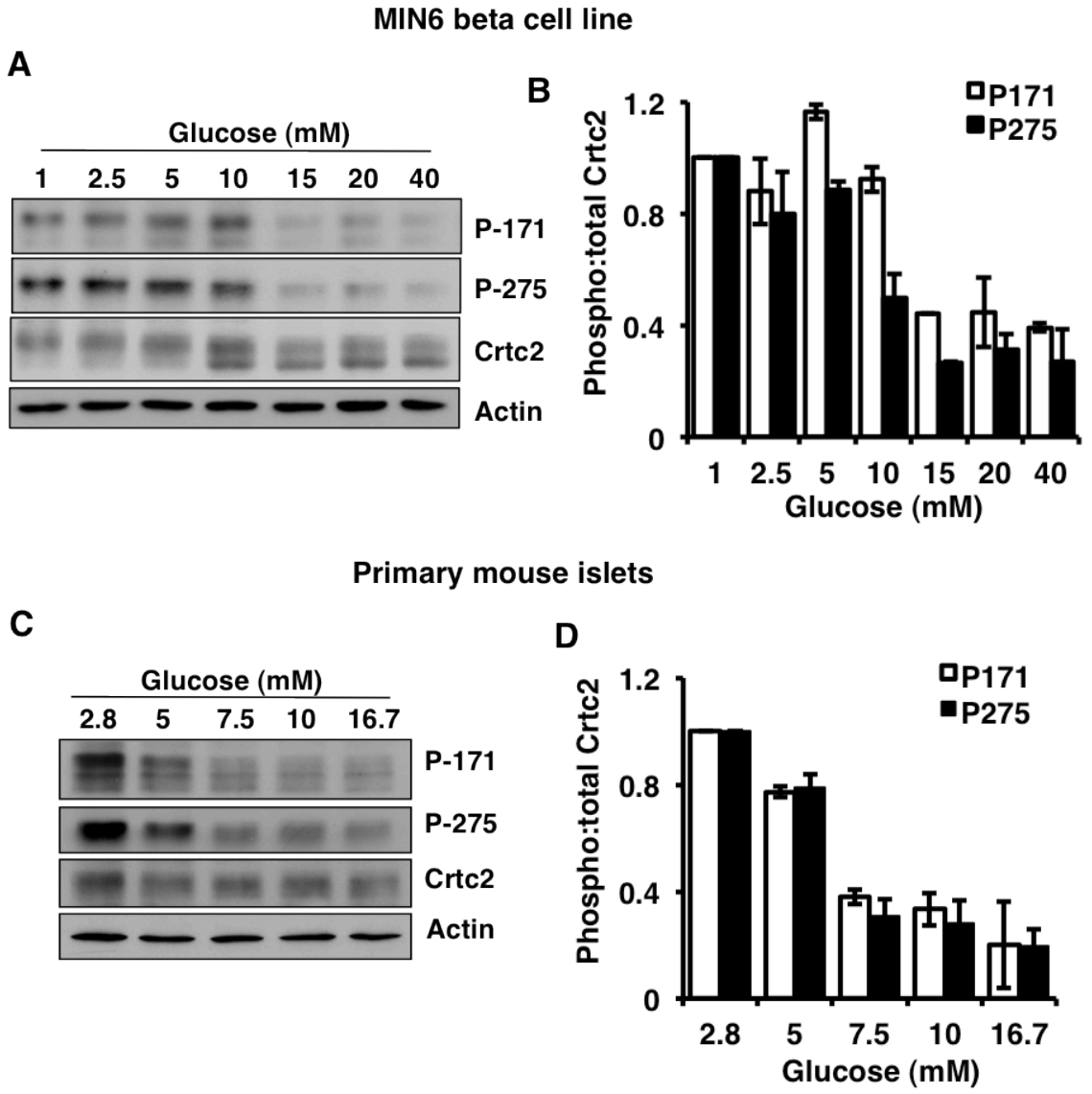

Figure 6: Crtc2 phosphorylation is regulated by physiologically relevant glucose concentrations in the β cell. A) Crtc2 is dephosphorylated in response to elevated glucose concentrations at Ser171 and Ser275 in MIN6 β cell line. **B)** Quantitation of phospho:total intensity in MIN6 cells. **C)** Crtc2 is dephosphorylated in response to elevated glucose concentrations at Ser171 and Ser275 in primary mouse islets. **D)** Quantitation of phospho:total intensity in mouse islets. Western blots shown are representative of 2-3 experiments and quantitation shown is the average of these experiments. MIN6 cells and mouse islets were pre-incubated in 1 mM glucose KRB and 2.8 mM glucose KRB, respectively for 1 hr prior to incubation at the indicated glucose concentration for 1 hr.

in the β cell. To evaluate the role of the AMPK family of kinases in Crtc2-CREB in the β cell, LKB1 knockdown using lenti-shRNA was performed to effectively ablate the activity of all 14 downstream-regulated kinases of the AMPK family [167]. The phosphorylation status of Crtc2 was monitored under starvation as well as glucose and ex-4 stimulated conditions using phospho-specific Crtc2 antibodies. Knockdown of LKB1 reduced phosphorylation of Crtc2 at Ser171 and to a lesser extent at Ser275 in glucose-starved and glucose/cAMP stimulated MIN6 cells (Figure 7A). To evaluate whether this regulation translates to the primary β cell, Crtc2 phosphorylation was monitored in glucose-starved islets from LKB1 adult β cell specific knockout (LABKO) mice compared with L/L control mice. LABKO mouse islets displayed a reduction in phosphorylation at both Ser171 and Ser275 with a more striking reduction in Ser171 phosphorylation (Figure 7B). We next evaluated whether the reduction in Crtc2 phosphorylation in the absence of LKB1 was reflected at the mRNA levels of CREB-regulated genes. Indeed, mRNA levels of Nr4a2 and Irs-2 were elevated in MIN6 cells lacking LKB1 (Figure 7C,D), further suggesting an important role of the LKB1-AMPK pathway in the regulation of Crtc2 in the β cell.

3.9 Crtc2 is regulated by PKA-dependent mechanisms in the β cell:

Given that Crtc2 phosphorylation and CREB activity is regulated by LKB1, we suspected that Crtc2 may also be regulated by downstream kinases of the AMPK family in the β cell. In the liver, glucagon-induced cAMP signaling activates PKA, which phosphorylates and inhibits SIK2, thereby promoting Crtc2 dephosphorylation and Crtc2-CREB activation [154]. To evaluate whether similar PKA-dependent kinase regulation of Crtc2 exists in the β cell, MIN6 cells were stimulated with glucose and ex-4 alone and in

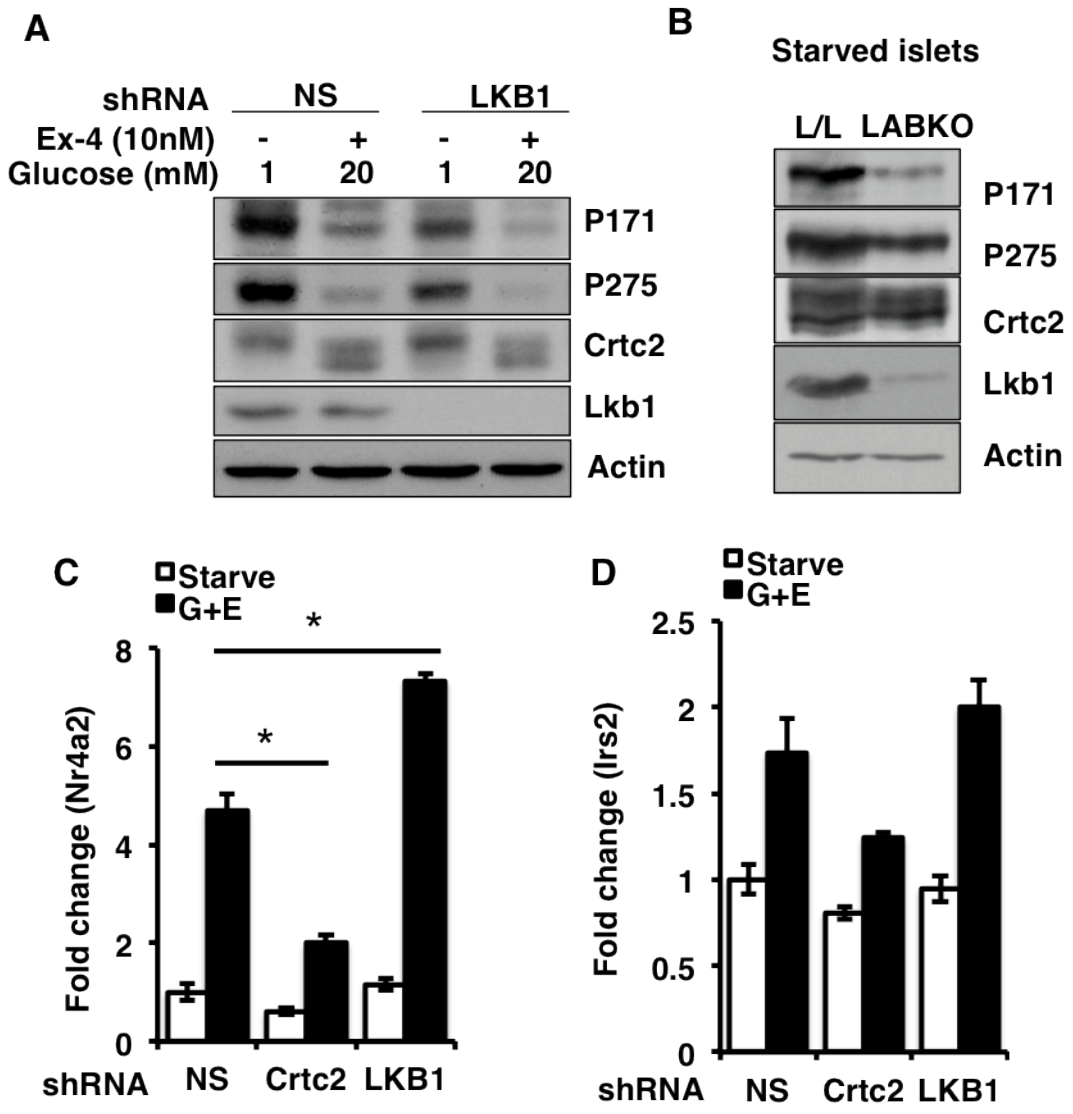

Figure 7: Crtc2-CREB is regulated by LKB1 in the β cell. A) Knockdown of LKB1 in MIN6 cells reduces phosphorylation at Crtc2 negative regulatory phosphorylation sites, Ser171 and Ser275 in glucose-starved and glucose and ex-4 stimulated conditions. **B)** Crtc2 phosphorylation at Ser171 and Ser275 is lower in islets from LKB1 adult β cell specific knockout (LABKO) mice than floxed control (L/L) mice in glucose-starved conditions. **C,D)** mRNA levels of CREB target genes **C)** Nr4a2 and **D)** Irs-2 are dependent on Crtc2 and are enhanced in the absence of LKB1. Data shown are the average of technical triplicates with standard deviation. For Western blot analysis, MIN6 cells were pre-incubated at 1 mM glucose for 1 hr and treated with 20 mM glucose and 10 nM ex-4 for 1 hr. Islets were starved in 2.8 mM glucose KRB for 1 hr. For mRNA analysis, MIN6 cells were pre-incubation in 1 mM glucose KRB for 2 hr. Cells were treated with ex-4 (10 nM) and glucose (16.7 mM for islets and 20 mM for MIN6 cells) alone or in combination for 4 hr prior to harvest.

combination in the presence and absence of the PKA inhibitor H89. In control conditions, glucose, but not ex-4, stimulated Crtc2 dephosphorylation at Ser275 (Figure 8A), consistent with previous studies [164]. Phosphorylation of Crtc2 at Ser171 however was also regulated by glucose, but not ex-4, suggesting co-regulation of Ser171 and Ser275 (Figure 8A), in contrast with previous documentation of cAMP, but not glucose, regulation of Ser171 on overexpressed Crtc2 [164]. Treatment with the PKA inhibitor H89 blocked Crtc2 dephosphorylation in response to glucose alone, and glucose and ex-4 co-treatment suggesting that PKA contributes to Crtc2 activation (Figure 8A). Despite no change in phosphorylation status at Ser171 or Ser175 with ex-4 treatment, a downward shift in the total Crtc2 band was observed in response to ex-4 and glucose alone, and was potentiated by co-treatment with glucose and ex-4 (Figure 8A). Given that PKA is classically activated by cAMP signaling, H89 treatment was expected to block cAMP-dependent mechanisms of Crtc2 activation. Since Crtc2 was only dephosphorylated in response to glucose and yet is still dependent on PKA, a proposed model of Ca^{2+}-activated adenyl cyclases is suggested to be the mechanism of PKA regulation of Crtc2 in the β cell (Figure 8B) [184].

3.10 Crtc2 dephosphorylation requires calcineurin:

The serine/threonine phosphatase calcineurin has been shown to regulate Crtc2 phosphorylation in a variety of settings including the β cell as well as being required for CREB regulated transcription [131, 158, 164]. β cells were treated with pharmacologically relevant low concentrations of calcineurin inhibitors to evaluate whether Crtc2 activation may be impaired in a calcineurin-based immunosuppression setting. Glucose-dependent Crtc2 dephosphorylation at both Ser171 and Ser275 was

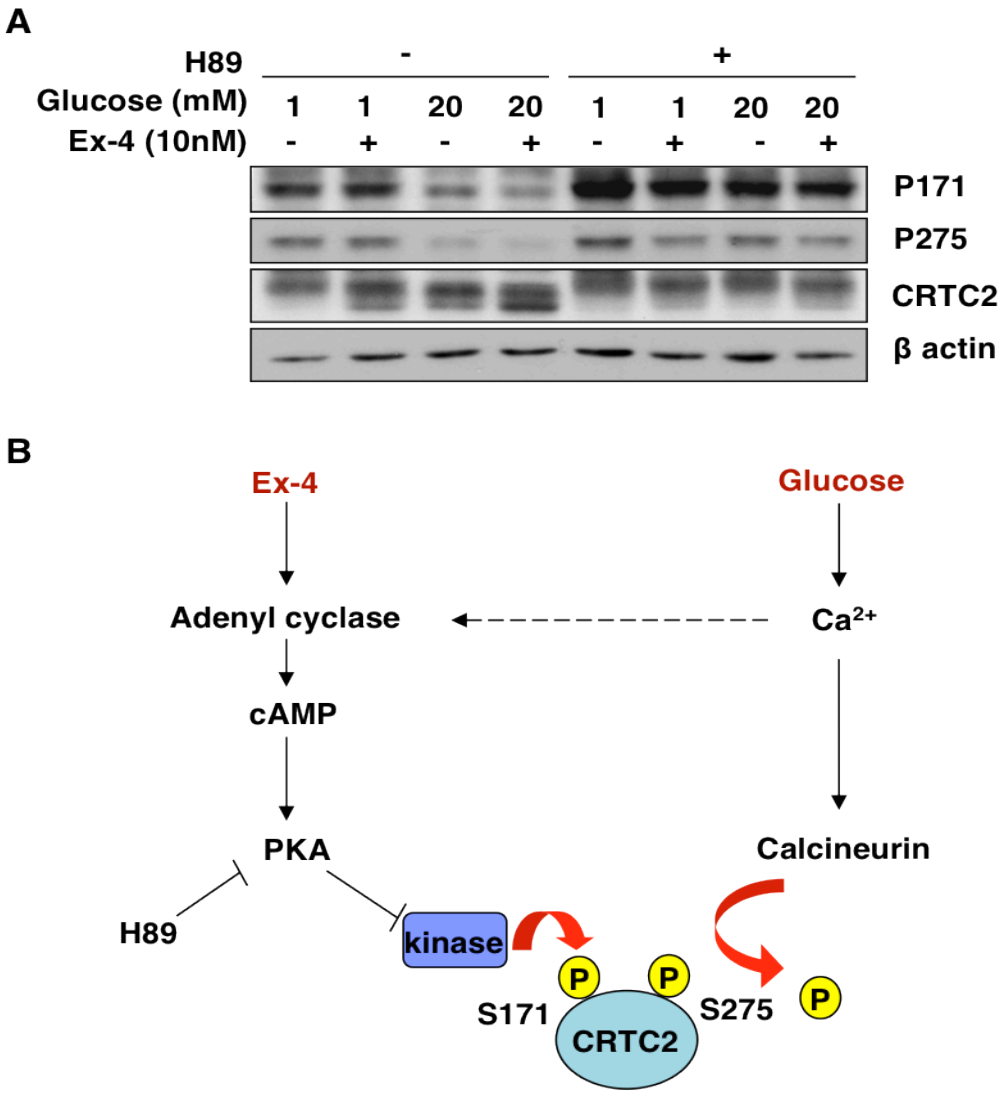

Figure 8: Crtc2 phosphorylation is regulated by protein kinase A. A) Crtc2 dephosphorylation at Ser171 and Ser275 in response to glucose and ex-4 is prevented by inhibition of PKA in MIN6 cells. MIN6 cells were starved for 1 hr in 1 mM glucose in KRB and then treated with glucose (20 mM) or ex-4 (10 nM) alone or in combination for 30 min in the absence or presence of 20 μM H-89. Western blots shown are representative of 3 independent experiments. **B)** Model demonstrating how glucose may regulate Crtc2 phosphorylation through PKA. Classically, PKA activation is induced through cAMP signaling that is stimulated by incretin hormones such as ex-4 in the β cell. Ca^{2+} activated adenyl cyclases in the β cell provide a mechanism by which glucose can regulate Crtc2 through PKA. Since PKA has been shown to inhibit kinases that phosphorylate Crtc2, activation of PKA could lead to dephosphorylation of Crtc2 through reduced kinase activity.

blocked by the calcineurin inhibitors CsA and FK506 in MIN6 cells while having no impact on the phosphorylation status of CREB at Ser133, the key regulatory site for CREB activation through enhanced CBP binding (Figure 9A, Appendix III). These data confirmed previously documented calcineurin regulation of Ser275 in the β cell [164] and also demonstrated for the first time that Ser171 phosphorylation is dependent on calcineurin. Due to differences between cancer cell lines and primary tissue, we sought to evaluate Crtc2 phospho-regulation in the primary β cell. Indeed, Crtc2 was dephosphorylated at both sites in response to glucose, but not ex-4 stimuli in mouse islets (Figure 9B) as shown in MIN6 cells (Figure 8A, 9A). Furthermore, inhibition of calcineurin with FK506 impaired Crtc2 nuclear translocation in response to glucose in MIN6 cells (Figure 9C) and dispersed mouse islets (Figure 9D). These data suggest that Crtc2 activation is dependent on glucose/ Ca^{2+} activation of calcineurin and is impaired in the presence of low dose of FK506 and CsA.

3.11 β cell function and CREB-regulated transcription are impaired by FK506 and CsA:

Calcineurin inhibitors have been shown to impair insulin secretion in a variety of species and experimental conditions. For comparison of β cell function in the presence of calcineurin inhibitors with the loss of Crtc2 in the β cell, we assessed insulin secretion in MIN6 cells and mouse islets in the presence of CsA and FK506. MIN6 cells were treated overnight with FK506 and CsA at a range of low doses sufficient to inhibit Crtc2 and insulin secretion was measured in low glucose and high glucose conditions 48 hr later. In this setting, both CsA and FK506 impaired insulin secretion in MIN6 cells by 20-30% (Figure 10A). A similar reduction in glucose stimulated insulin secretion was observed

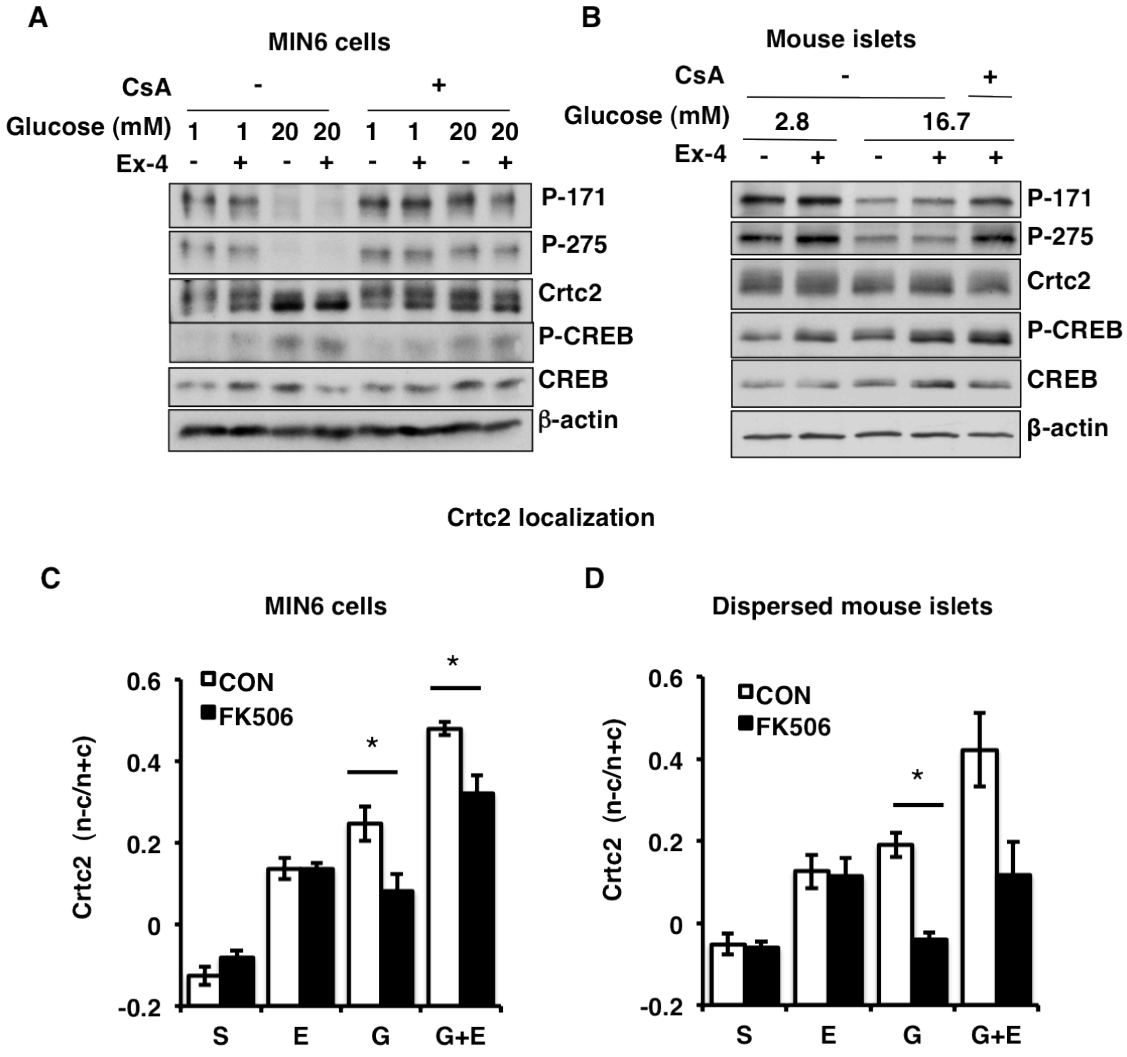

Figure 9: Glucose-dependent Crtc2 activation is dependent on calcineurin. A,B) CsA blocks Crtc2 dephosphroylation but do not impact on phosphorylation of CREB in **A)** MIN6 cells and **B)** primary mouse islets. **C,D)** Glucose-dependent Crtc2 nuclear translocation in MIN6 cells is impaired by FK506 in **C)** MIN6 cells and **D)** dispersed mouse islets. MIN6 cells and islets were starved in 1 mM and 2.8 mM glucose in KRB respectively prior to incubation at the indicated conditions for 1 hr. Ex-4 was used at 10 nM. CsA and FK506 were added during the 1 hr pre-incubation at 500 nM and 10 nM respectively. Data shown are representative of 2-3 experiments. * p <0.05

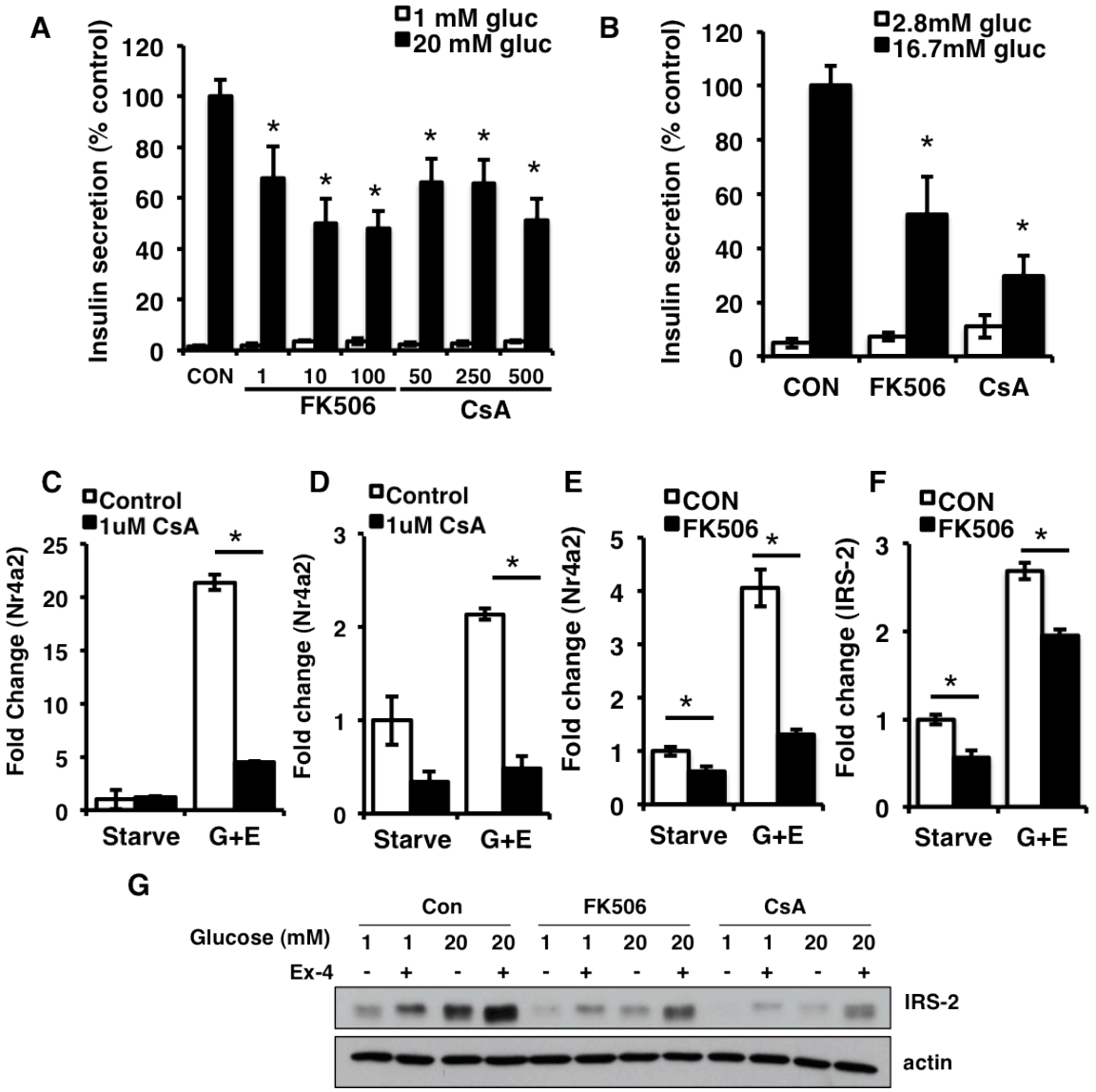

Figure 10: Insulin secretion and CREB-regulated transcription are impaired by calcineurin-inhibiting immunosuppressants. A,B) Insulin secretion is impaired in **A)** MIN6 β cell line and **B)** isolated mouse islets treated with calcineurin inhibitors FK506 and CsA for 2 and 5 days respectively. Insulin secretion values are normalized to DNA content and expressed as % of control. Concentrations of drugs are indicated in nM. **C,D)** Transcription of the CREB target genes **C)** Nr4a2 and **D)** Irs-2 are impaired by FK506 in primary mouse islets. **E,F**) Transcription of Nr4a2 is impaired by CsA in **E)** MIN6 cells and **F)** mouse islets. **G)** Induction of IRS-2 protein in response to glucose and ex-4 is impaired by FK506 and CsA. For mRNA analysis, MIN6 cells and mouse islets were pre-incubated in 1 mM glucose KRB and 2.8 mM glucose KRB respectively for 2 hr. Cells were treated with ex-4 (10 nM) and glucose (16.7 mM for islets and 20 mM for MIN6 cells) for 4 hr prior to harvest. For IRS-2 protein analysis, cells were stimulated for an addition 4 hr to allow for protein accumulation. Data shown are the average of technical triplicates with standard deviation. For 10B, data from 3 experiments were pooled to obtain significance * p <0.05.

in isolated mouse islets cultured for 5 days in the presence of CsA (500 nM) or FK506 (10 nM) (Figure 10B). Various studies have shown that calcineurin is important for CREB activation [131, 159]. Here we provide additional evidence for requirement of calcineurin for endogenous CREB-regulated transcription in the β cell by monitoring gene expression at the mRNA and protein level. qPCR analysis of mRNA levels revealed that CsA impairs glucose and ex-4 induction of Nr4a2 mRNA levels in MIN6 cells and islets by 75% (Figure 10C,D). Similarly, FK506 treatment reduced levels of Nr4A2 and Irs2 mRNA by 75% and 25%, respectively, in mouse islets after a 4 hr treatment with glucose and ex-4 (Figure 10E,F). These data suggest that while calcineurin is important for CREB activation, the extent to which it is required may vary depending on the gene. In addition, Western blot analyses indicate that protein levels of IRS-2 were reduced under glucose-starved and glucose/ex-4 stimulated conditions after overnight treatment with FK506 and CsA (Figure 10G). These data support an important role for calcineurin in CREB activation and the induction of IRS-2, an important protein for insulin signaling and β cell survival.

3.12 Impact of FK506 treatment on glucose regulation in mice:

In an attempt to translate the impact of calcineurin inhibitors on β cell function and CREB activity observed *in vitro* to an *in vivo* model, mice were treated with FK506 with the expectation of observing impaired glucose regulation as documented in other model organisms [113, 116, 185]. In addition we expected to observe impaired CREB activity and β cell function. Mice were intraperitoneally injected with FK506 at 1 mg/kg/day for 10 days. After 10 days of FK506 treatment, glucose regulation was tested using a glucose tolerance test in which the clearance of 2 mg/kg of glucose was monitored over 2 hr.

Unexpectedly, FK506 treated mice had enhanced glucose clearance compared with vehicle treated mice (Appendix IV A), a phenotype likely due to improved peripheral insulin sensitivity rather that enhanced insulin secretion. Nonetheless, islets were isolated from these mice to determine whether *in vivo* FK506 treatment introduced a defect within the β cell compartment. However, no difference in insulin secretion was observed between islets from control and FK506 treated mice (Appendix IV B). FK506 reduced mRNA levels of CREB target genes Irs-2 and Nr4a2 in the β cell and increased mRNA levels of Pepck and G6Pase in the liver (Appendix IV C,D). These observations reflect changes that would be predicted by the difference in serum glucose of mice, rather than FK506 treatment itself (Appendix IV C,D). Thus further interpretation of the impact of FK506 treatment in mice of CREB regulated transcription could not be made under these tested conditions.

3.13 Constitutively active Crtc2 maintains CREB activity in the presence of calcineurin inhibitors.

Previous work indicated that mutation of Crtc2 phosphorylation sites Ser171 and Ser275 impaired binding to 14-3-3 proteins, promoted nuclear localization of Crtc2 and increased CREB activity monitored by a CREB reporter assay in HIT cells [164]. Since Crtc2S171,275A or Crtc2DM (double mutant) cannot be inhibited by phosphorylation at these sites we sought to evaluate the extent to which Crtc2DM could promote endogenous mRNA levels of CREB targets in the β cell and maintain them in the presence of CsA/FK506. Here we generated lentiviral GFP-tagged Crtc2 single and double phosphorylation mutants in order to monitor subcellular localization of the constructs and evaluate the impact of Crtc2 overexpression on endogenous CREB-

regulated transcription. In the absence of glucose, conditions that promote wildtype Crtc2 cytoplasmic retention, mutation of S171A and S275A each promoted nuclear localization of Crtc2 and together as a double mutant (Crtc2DM) resulted in complete nuclear localization (Figure 11A,B). Transcription of the CREB target gene Nr4a2 was monitored to evaluate the impact of Crtc2 wt and DM overexpression on endogenous gene transcription in MIN6 cells. Overexpression of constitutively active Crtc2DM induced basal mRNA levels of Irs-2 and Nr4a2 in MIN6 cells (Appendix V) and dispersed mouse islets in culture medium (Figure 11C,D). Furthermore, Crtc2DM maintained mRNA levels at higher levels in the presence of CsA for 24 hr when compared with wildtype Crtc2 or GFP control (Figure 11C,D, Appendix V). Of note, partial inhibition of CREB-regulated transcription was observed in Crtc2-DM infected MIN6 cells and isolated mouse islets treated with CsA. This is likely a reflection of incomplete infection efficiency or inhibition of remaining endogenous Crtc2 protein within the cell, however it is possible that additional calcineurin regulation of CREB exists. To further evaluate the impact of Crtc2 overexpression on Irs-2 regulation, IRS-2 protein levels were monitored by Western blot analysis in the absence and presence of calcineurin inhibitors. Protein levels of IRS-2 were also induced by Crtc2DM in MIN6 cells (Figure 11E) and mouse islets (Figure 11F) and were maintained in the presence of calcineurin inhibitors in MIN6 cells (Figure 11E). These data suggest that impaired CREB-regulated transcription in the presence of CsA/FK506 is in part due to inhibition of Crtc2 in the β cell and that Crtc2DM overexpression could serve as a strategy to promote maintenance of CREB-regulated transcription in an immunosuppression setting.

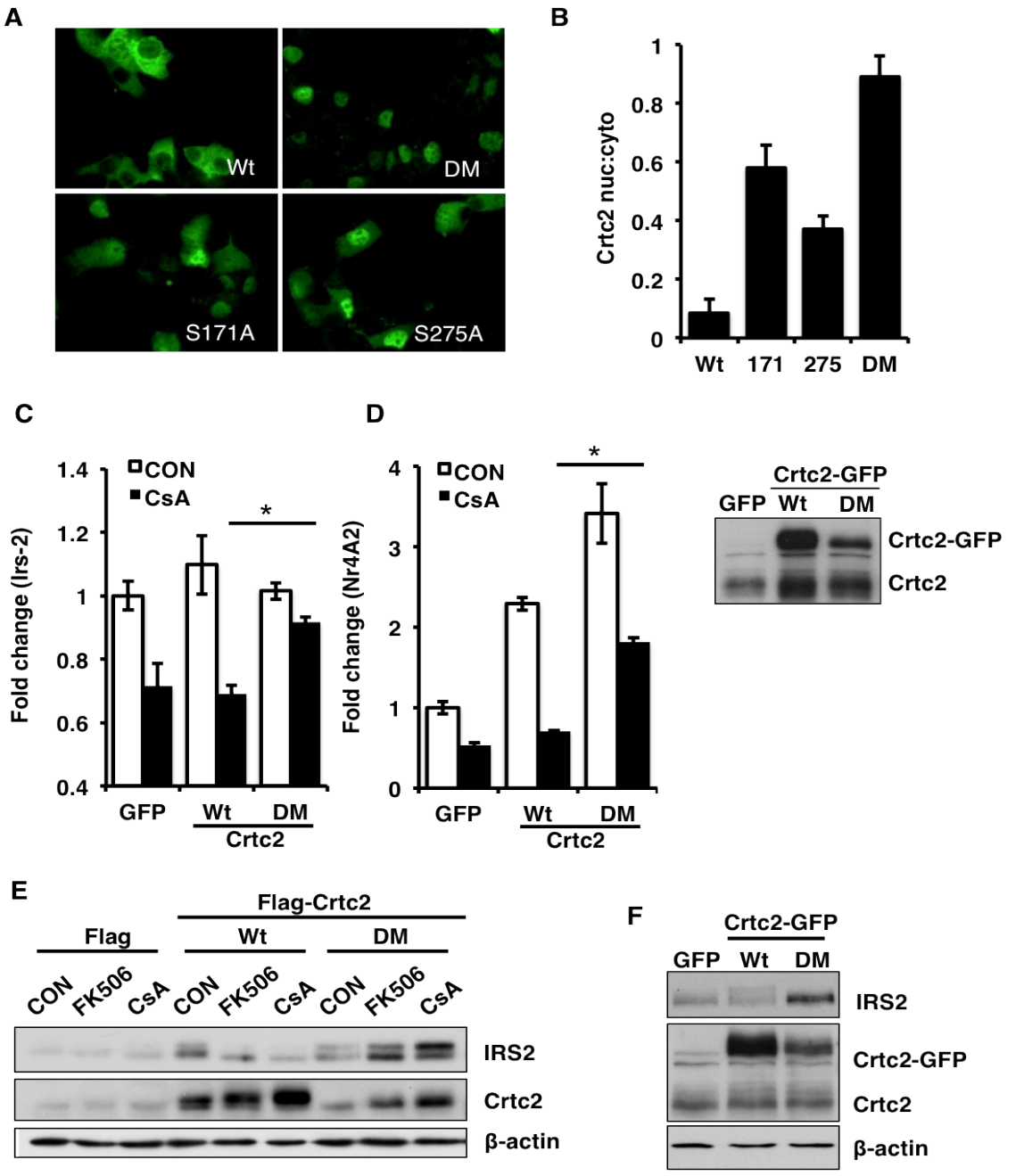

Figure 11: Constitutively active Crtc2 maintains CREB activity in the presence of calcineurin inhibitors. A,B) Crtc2 phospho-mutants at Ser171 and Ser275 localize to the nucleus whereas wildtype Crtc2 is primarily cytoplasmic in low glucose conditions. **C,D**) Constitutively active Crtc2 maintains CREB-regulated transcription of **C)** Irs-2 and **D)** Nr4a2 in the presence of CsA in dispersed mouse islets. **E)** Expression of constitutively active Crtc2 increases protein levels of IRS-2 in MIN6 cells in the presence of calcineurin inhibitors. **F)** IRS-2 protein levels are elevated in islets expressing constitutively active Crtc2. mRNA and protein levels were measured from cells in culture medium. Data shown are the average of technical triplicates with standard deviation. * p <0.05.

3.14 Constitutively active Crtc2 *in vivo*.

From these studies in cell lines and primary β cells, indication of an important role of Crtc2 in the β cell has been gained. In order to better understand the implication of altered Crtc2 activation in the β cell and its protective effects against immunosuppression-induced inhibition of β cell function through CREB, we sought to generate a Crtc2DM-GFP β cell specific inducible transgenic mouse. Crtc2DM-GFP was cloned into a vector containing a lox-P flanked upstream β galactosidase (β gal) reporter with a stop codon to prevent translation of the Crtc2-GFP construct in the absence of CRE recombinase (Appendix VI). In the presence of CRE recombinase, the β gal reporter and stop codon is expected to be removed, resulting in expression of Crtc2-GFP. β cell specific expression of Crtc2DM-GFP could thereby be obtained by mating Crtc2DM-GFP transgenic mice with mice expressing CRE under a β cell specific Pdx promoter. CRE-dependent regulation of this construct was confirmed in HEK293 T cells (Appendix VI A). In the absence of CRE, HEK293T cells expressed β gal confirmed by X-gal staining and did not express GFP. In the presence of CRE, β gal expression was lost and expression of Crtc2-GFP was observed. The plasmid was then linearized and sent for pronuclear injections at IRCM in Montreal. We received tail clips of pups from several litters and used a portion of each to genotype and the other portion to stain for β gal. The mice were genotyped using β gal specific oligos. 40% of the pups were positive for β gal but none of the mouse-tails stained positive β gal (Appendix VI B). In order to rule out the possibility of a dysfunctional β gal reporter, fibroblasts from the ear clippings of mice were cultured and infected with an adenovirus expressing CRE to evaluate whether or not Crtc2DM-GFP could be expressed. Western blot analysis revealed that no

Crtc2-GFP was expressed despite successful CRE activity in this experiment using a floxed SIK2 mouse as a positive control (Appendix VI C,D). Due to the lack of Crtc2-GFP expression, no further experiments were performed on these mice and the role of constitutive Crtc2 activity could not yet be evaluated *in vivo*.

Chapter 4: Discussion

Evaluation of the proposed model of Crtc2 regulation in the β cell

New insight into the regulation of Crtc2 has been gained by evaluating previously shown regulatory mechanisms in a physiological setting in primary β cells. Differences observed in this setting include the degree to which CREB regulates endogenous transcription, the relative impact of glucose and cAMP stimuli on CREB activation and the contribution of these stimuli to Crtc2 activation. From CREB reporter assays, CREB activity has been shown to increase 50 fold in response to glucose and cAMP and through expression of Crtc2DM [131, 164]. While these factors translate to endogenous gene transcription, the fold increase is much smaller and varies depending on the specific target, indicative of additional levels of regulation. In addition, it was previously shown that Crtc2 activation requires both glucose and cAMP through overexpression studies but here we show that each stimuli contributes to Crtc2 nuclear translocation and Crtc2:CREB activation. This alters our understanding of Crtc2:CREB activation and suggests that activation can be promoted without necessitating that both signaling pathways be restored. It also suggests that the β cell has established potentially redundant mechanisms for Crtc2:CREB activation. Furthermore, by monitoring endogenous Crtc2 protein and CREB-regulated transcription in primary β cells in the presence of clinically relevant concentrations of immunosuppressants, we were able to better evaluate their implication on activation of Crtc2:CREB in a physiological setting. From this work, it is clear that immunosuppressants impair activation of CREB at least in part through Crtc2.

The role of Crtc2 in the β cell

The biological roles of the Crtcs and CREB in energy balance are becoming increasingly evident in various tissues (reviewed in [134]). We have demonstrated for the first time, an important role for Crtc2 in insulin secretion. Interestingly, β cells lacking Crtc2 have a defect in glucose-responsiveness, however, maintain the capacity to produce and secrete insulin. Given that glucose dependent induction of CREB-target genes requires Crtc2, it is likely that Crtc2 plays an important role in glucose-dependent CREB-regulated transcriptional changes that may contribute to the up-regulation of glucose-responsive genes. For instance, glucose has been shown to promote gene expression of enzymes involved in cataplerosis, metabolic processes that promote energy production, which have been shown to couple glucose to insulin secretion thereby promoting β cell fitness [58]. Further experiments are required to determine whether β cells lacking Crtc2 have impaired glucose uptake or glucose-dependent ATP production. In addition, loss of Crtc2 may affect non-canonical ATP-independent regulation of insulin secretion by altering other metabolites that have been shown to couple glucose to insulin secretion [186].

An important consideration for this work is that functional experiments performed using RNAi technology should be interpreted with caution due to the possibility of off-target effects, particularly when a loss of function or cell death phenotype is observed [187]. Given that insulin secretion defects were observed with multiple shRNAs targeting Crtc2, it raises the likelihood that this effect is on-target compared with experiments using fewer reagents. Conversely, the cell death phenotype was only observed with a subset of shRNAs targeting Crtc2 raising the probability that this effect was off-target.

However, it is also possible that Crtc2 knockdown predisposes β cells to apoptosis and that off-target effects merely contribute to the eventual cell death observed rather than cause it. In addition, the inconsistent cell death observed with Crtc2 knockdown could be a result of a threshold issue in which a more complete knockdown of Crtc2 progresses the phenotype from dysfunction to cell death. Evaluation of insulin secretion from islets isolated from Crtc2-/- mice or genetic rescue through overexpression of shRNA-resistant Crtc2 cDNA is required to confirm the role of Crtc2 for β cell function.

How does Crtc2 mediate its effects on the β cell?

The findings of this work open another question of whether the requirement of Crtc2 for insulin secretion is through CREB-regulated transcription or a yet unidentified cytoplasmic role of Crtc2. Given that insulin secretion occurs in the presence of glucose, conditions in which Crtc2 is localized to the nucleus, it is difficult to rationalize a direct role for Crtc2 in the cytoplasmic process of insulin secretion. On the other hand, under low glucose conditions, cytoplasmic Crtc2 may act to inhibit insulin secretion and thereby prevent unwanted basal insulin secretion. In fact, insulin secretion was elevated in MIN6 cells lacking Crtc2 under low glucose conditions supporting a role for cytoplasmic Crtc2 in the prevention of insulin secretion. The use of transcriptionally inactive Crtc2 constructs will help clarify whether the requirement of Crtc2 for insulin secretion occurs through the activation of CREB.

Identification of the specific CREB targets that are important for β cell function will improve our understanding of the role of Crtc2-CREB in the β cell. Impaired expression of the CREB target gene IRS-2 in the β cell can induce diabetes in mice and is observed in diabetic patients [75, 77]. Here, we demonstrated impaired induction of IRS-

2 in the absence of Crtc2 and in the presence of calcineurin inhibitors providing a potential mechanism for the impaired β cell function and survival observed. On the other hand, it is unknown whether impaired IRS-2 expression in a diabetic setting is due to impaired Crtc2:CREB activation. Firstly, transcriptional control of IRS-2 is also regulated by NFAT, an additional target of calcineurin [128]. In addition, post-translational modifications of IRS-2 can alter its activity and lead to its degradation, suggesting that several mechanisms can contribute to impaired IRS-2 levels [188]. While IRS-2 serves as a good candidate target gene that is important for β cell function and survival, other Crtc2-CREB regulated genes may also contribute the phenotypes observed. Interestingly, genes regulated by CREB have been shown to have varied dependence on Crtc2 suggesting that Crtc2 provides an additional level of specificity in gene transcription [189]. In addition, it raises the possibility that altered gene expression from loss of Crtc2 is merely a subset of the transcriptional changes observed in cells lacking CREB. In a microarray screen performed by our lab to identify glucose and ex-4 responsive genes in MIN6 cells, tachykinin-1 (tac-1) was identified (Depatie et al., unpublished data). Interestingly, tac-1 expression was highly induced by Crtc2DM overexpression and the loss of tac-1 impaired insulin secretion in MIN6 cells (Depatie et al., unpublished data). Further work is required to validate and characterize the role of tac-1 and other potentially relevant CREB targets that are important for β cell function and survival that may mediate the effects of loss of Crtc2 in the β cell.

Whole body impaired calcineurin-Crtc2 activation

Despite a potentially important role shown for Crtc2 in the β cell, further work is required to determine the role of Crtc2 expression in the β cell on whole body glucose

metabolism. Whole body Crtc2 knockout mice have been generated by two groups independently and present conflicting data in some respects [151, 178]. Wang et al., 2010 demonstrate that Crtc2 -/- mice have improved glucose clearance that was attributed to enhanced insulin sensitivity and reduced gluconeogenesis in the liver. On the other hand, Le Lay et al., 2009 showed that Crtc2-/- mice had reduced glucose output from the liver, but no overall improvement in glucose regulation [178]. The β cell compartment was not evaluated in either of these studies, but could contribute to the whole body glucose metabolism observed and potentially explain the discrepancy between the glucose metabolism phenotypes documented. Our data demonstrate that Crtc2 is important for β cell function and suggest that these mice may exhibit impaired insulin secretion. Small differences between studies such as the age of the mice may be sufficient to exacerbate a β cell defect and thereby mask the liver-driven improved glucose clearance phenotype observed [151]. Interestingly, Crtc2-/- mice that demonstrate improved glucose clearance also had reduced whole body serum insulin levels that may indicate impaired insulin secretion; however, it can also possibly be a mere reflection of lower blood glucose levels of these mice [151]. β cell specific ablation of Crtc2 will be necessary to clarify its role in whole body glucose metabolism.

Similar to whole body Crtc2 ablation, the net impact of pharmacological calcineurin inhibition on glucose metabolism is variable. In healthy humans, short-term treatment (5 hours) with FK506 has been shown to improve glucose clearance, potentially due to a increased insulin sensitivity or a reduction in glucose output from the liver via inhibition of CREB [190]. These results are similar to our observation of improved glucose clearance in FK506-treated mice after 10 days. A more chronic

treatment with FK506 could be required to induce a glucose intolerance phenotype in mice. However, studies in rats have elucidated severe glucose intolerant phenotypes in as little as two weeks [113]. In addition, it is important to note that immunosuppressants only induce diabetes in a subset of patients, thereby suggesting that they aggravate a pre-existing susceptibility to β cell defects and diabetes rather than being sufficient to induce diabetes on their own. Thus the diabetogenic effect of chronic calcineurin-based immunosuppression is likely due to failure of the β cell compartment to meet the insulin requirements in the presence of the additional stress that calcineurin-inhibition places on the β cell. Calcineurin is required for β cell compensation mechanisms as demonstrated by impaired proliferation and regeneration in the absence of calcineurin activity [61, 96, 126]. Individuals predisposed to diabetes may rely more heavily on these compensatory mechanisms due to impaired β cell survival or function and thus be more susceptible to new onset diabetes after transplantation.

Crtc2 as a therapeutic intervention for islet transplantation

Similar to inducing diabetes post-transplantation, calcineurin inhibition also impairs efficacy of transplanted islets [120]. A therapeutic strategy using the knowledge gained from this study would be to express constitutively active Crtc2DM (calcineurin insensitive) *ex vivo* in human islet preparations from cadavers prior to islet transplantation into diabetic individuals. Given that Crtc2DM can maintain CREB-regulated transcription in the presence of calcineurin inhibitors, it is expected to improve islet graft survival and function. Several functional studies would be required before this could be seriously considered as a therapeutic approach. Firstly, the consequence of constitutive Crtc2 activation in the β cell is still unknown. Prior attempts to generate

non-inducible Crtc2DM mice under the control of the PDX-1 promoter resulted death at the age of weaning suggesting that Crtc2 regulation is important for development. In addition, constitutive activation of CBP in the β cell resulted in impaired insulin secretion from isolated islets [179]. These data suggest that chronic promotion of CREB activity is also detrimental to the β cell. Importantly, analysis of CREB-regulated transcription under low glucose conditions demonstrated that cells overexpressing Crtc2-wt or Crtc2DM can still down-regulate CREB-regulated transcription suggesting that Crtc2 enhances CREB activity without overriding other CREB regulatory mechanisms such as phosphorylation of CREB and CBP recruitment that are inactive in the absence of glucose and cAMP signaling (Appendix VII). Thus Crtc2DM may serve as a better approach to promote CREB activity *in vivo*.

Interestingly, Crtc2DM protein was consistently found to accumulate to lower levels than wildtype Crtc2 suggestive of a negative feedback mechanism that stimulates Crtc2 degradation. This finding may be due to enhanced Crtc2 ubiquitination and proteasomal degradation [162]. It has been shown that induction of a CREB-target gene SIK1 promotes Crtc2 phosphorylation at Ser171, cytoplasmic relocalization and subsequent degradation [154]. However, given that Crtc2DM cannot be phosphorylated at this site, other mechanisms must exist to enable Crtc2 turnover. In the liver, Crtc2 is acetylated by CBP at Lys628 and leads to subsequent ubiquitination and degradation of Crtc2 [154]. Mutation of Lys628Arg on Crtc2 may facilitate the maintenance of higher levels of Crtc2 protein accumulation by blocking Crtc2 ubiquitination [154]. Further work will be required to determine whether these kinds of regulatory mechanisms of Crtc2 exist in the β cell.

From this work, Crtc2 dephophorylation at Ser171 and Ser275 is clearly inhibited by CsA and FK506 thereby connecting calcineurin inhibitors with impaired CREB-regulated transcription in the primary β cell. Interestingly, inhibition of Crtc2-CREB activation by calcineurin inhibitors, at the step of Crtc2 localization or CREB-regulated gene expression, showed some signs of improvement with glucose and ex-4 treatment compared with glucose alone. These data suggest that incretin therapy such as GLP-1 receptor agonists, may serve as a simple treatment strategy for patients post-transplantation in attempt to partially overcome the effects of calcineurin inhibition. In fact, a recent study demonstrated that mouse and human islets cultured in the presence of tacrolimus had improved proliferation and survival, respectively, when treated with ex-4 [191]. It is possible that these effects were in part due to promotion of Crtc2-CREB activation.

cAMP versus glucose regulation of Crtc2-CREB

It remains unclear how cAMP and glucose signaling converge to regulate Crtc2 in the β cell. In addition, evidence for cross talk between cAMP and Ca^{2+} signaling in the β cell has been observed further complicating the regulation of Crtc2 by these two distinct pathways [192]. It is possible that redundancy between Ca^{2+} and cAMP signaling has evolved in the β cell because of how important it is that β cells respond to these cellular signals. Interestingly, cAMP signaling in the liver induces Ca^{2+} signaling through the I3P receptor as an important regulatory step of Crtc2 activation [158]. Thus distinct cell types may have adapted mechanisms to regulate Crtc2 by utilizing their well-established signaling pathways such as glucagon-induced cAMP in the liver to elicit the Ca^{2+} signals that regulate Crtc2. Uncoupling between the effects of glucose and ex-4 on Crtc2

phosphorylation, Crtc2 localization and CREB activity has been observed. At the level of Ser171 and Ser275 phosphorylation, Crtc2 activation is predominantly regulated by glucose in the β cell, however, a contribution of additional Crtc2 regulation by ex-4 has been observed. Despite no alteration in Crtc2 phosphorylation at Ser171 and Ser275, ex-4 alters the total Crtc2 mobility pattern to a downshifted position that generally correlates with Crtc2-CREB activation. Interestingly, a similar mobility shift in total Crtc2 protein is observed by S275A mutation, but not S171A mutation. Surprisingly, Ser275 phosphorylation status does not correlate directly with Crtc2 mobility, suggesting that phosphorylation at Ser275 is uncoupled with other post-translational modifications responsible for this shift. It is possible that additional phosphorylation sites contribute to Crtc2 regulation and could account for the partial activation observed with ex-4. In fact, Ser307 has been shown to contribute to Crtc2 subcellular localization in COS-7 cells and should be evaluated in the β cell in future experiment [193]. In addition, Ser369 was shown to contribute to Crtc2 14-3-3 binding, however, no functional consequence of mutating this site was observed [164]. Alternatively, cAMP may merely enable Crtc2 dephosphorylation in a transient fashion, sufficient to destabilize its interaction with 14-3-3 proteins and allow for Crtc2 nuclear entry. In addition, cAMP may promote Crtc2-CREB-CBP interaction thereby impairing Crtc2 nuclear export. Complex formation has been shown to enhance CREB activity through acetylation of Crtc2 by CBP and may not necessitate complete Crtc2 dephosphorylation at Ser171 or Ser275 for enhanced CREB activity [162]. It is important to consider Crtc2 localization as a process of constant shuttling involving several steps of regulation, all of which contribute to the net

localization of Crtc2 in a physiological setting. In addition, CREB regulated transcription is impacted by multiple factors other than Crtc2.

Kinase regulation of Crtc2 by the LKB1-AMPK pathway

The role of Crtc2 in mediating hormonal and energy sensing is well established through regulation by the AMPK family of kinases predominantly in the liver and is conserved in *C.elegans* [90, 149, 154, 157]. Here for the first time, we demonstrate a link between LKB1, the master regulator of AMPK-related kinases, and Crtc2 in the β cell, suggesting that similar Crtc2-CREB regulation exists. Pharmacologically targeting upstream kinases that regulate Crtc2 could serve as a therapeutic in diabetes by decreasing Crtc2 phosphorylation and thereby enhancing CREB activity. Furthermore, LKB1 is elevated in ob/ob mice suggesting it is a relevant target in the pathogenesis of T2D [182]. One approach for the treatment of diabetes would be to promote the Crtc2-CREB pathway in the β cell by pharmacological inhibition of LKB1. In addition, there is precedent for this approach as loss of LKB1 enhanced functional β cell mass in mice [131]. However a subsequent study by Gan, et al (2010) demonstrated that LKB1 is required for hematopoietic stem cell survival [194]. These data suggest that targeting LKB1 directly could not serve as a therapeutic approach due to the whole body nature of pharmacological interventions and the likelihood of severe side effects.

Regulation of Crtc2 through PKA in the β cell shown here further confirms an important role of kinase regulation of Crtc2. SIK2 was a candidate kinase which we thought may regulate Crtc2 in the β cell since it has been shown to physically interact with and regulate Crtc2 in other settings in a cAMP/PKA-dependent fashion [131, 164, 166]. However, our lab demonstrated no change in Crtc2 phosphorylation in SIK2-

knockdown MIN6 cells or in islets from SIK2 knockout mice (Sakamaki et al., unpublished data). It is possible that either compensation or redundancy of kinases may render this approach challenging. However, further investigation into which kinases predominately regulate Crtc2 in the β cell is worth pursuing due to the inability to target LKB1. The impact of loss of LKB1 on various tissue specific cell biology has been attributed to different kinases of the AMPK family suggesting tissue specific regulation and the potential for tissue specific drug targets [195]. Recently, Brain selective kinase 2 (Brsk2) was shown to have a functional role in the β cell by negative regulation of insulin secretion [196]. To shed light on whether Brsk2 could exert its effects on insulin secretion in part through inhibition of Crtc2, an *in vitro* kinase assay was performed to test whether Brsk2 can phosphorylate Crtc2 S171 and S275 peptides. Brsk2 phosphorylated Crtc2 peptides to a similar degree as Sik2 and Mark2, kinases of the AMPK family previously shown to phosphorylate Crtc2, highlighting Brsk2 as a potential regulator of Crtc2 (Appendix VII) [164]. Further studies are required to determine the importance of Brsk2 in Crtc2 regulation in the β cell. AMPK is another potentially interesting kinase that may regulate Crtc2 in the β cell. Firstly, AMPK is active under low glucose conditions, the same conditions in which Crtc2 is heavily phosphorylated and becomes inactivated by elevations in glucose, conditions in which Crtc2 is dephosphorylated. Secondly, chronic pharmacological activation of AMPK has been shown to impair β cell function [92], a result that may be in part due to inhibition of Crtc2-CREB through chronic phosphorylation and inactivation of Crtc2. Certainly, additional studies are required to further characterize kinase regulation of Crtc2 in the β cell and to determine the functional consequence of promoting Crtc2 activation.

Crtc2-CREB and the progression of diabetes

The maintenance of β cell fitness is highly dependent on adaptation to external stimuli via various cell-signaling pathways. Given that glucose and cAMP signaling are essential stimuli that regulate β cell growth and function it can be rationalized that failure of central cAMP/ Ca^{2+}-regulated transcription factor, CREB to respond properly to these cues can elicit diabetes. Furthermore, Crtc2 may serve an important role in contributing to the fine regulation of CREB in the β cells. A recent study demonstrated that chronic hyperglycemia induced prolonged CREB activation, expression of ICER and subsequent reduction of insulin, NeuroD and SUR1 expression, effects that were not observed under moderate glucose conditions in which CREB activation was transient [181]. Crtc2 may serve as a modulator for glucose-regulated CREB activation as we have demonstrated that Crtc2 is dephosphorylated at physiologically relevant glucose concentrations. In this model, prolonged hyperglycemia may impair Crtc2 phosphorylation through inactivation of AMPK and would result in enhanced CREB-regulated transcription that would lead to impaired β cell function through induction of ICER. In addition, O-glycosylation is elevated in a hyperglycemia setting and has been proposed as a mechanism that induces β cell dysfunction by impairing IRS-2/AKT signaling among other effects [43, 197]. Interestingly, O-glycosylation has been shown to promote Crtc2-CREB activation through competitive binding to Crtc2 negative regulatory phosphorylation sites in the liver [161]. If this mechanism translates to the β cell, O-glycosylation of Crtc2 could lead to chronic activation of CREB and β cell dysfunction. Further studies in diabetes models will determine whether Crtc2 is a central mediator of hyperglycemia-induced β cell dysfunction.

Conclusion:

Crtc2 plays an important role in maintaining glucose-stimulated insulin secretion in the β cell. Crtc2 contributes to the regulation of CREB in the β cell and is regulated by LKB1, PKA and calcineurin. β cell function and CREB-regulated transcription are impaired by calcineurin-inhibiting immunosuppressants. Glucose dependent activation of Crtc2 requires calcineurin and is impaired in an immunosuppression setting. CREB-regulated transcription can be restored by expression of constitutively active Crtc2, and may serve as a potential genetic intervention in transplanted islets.

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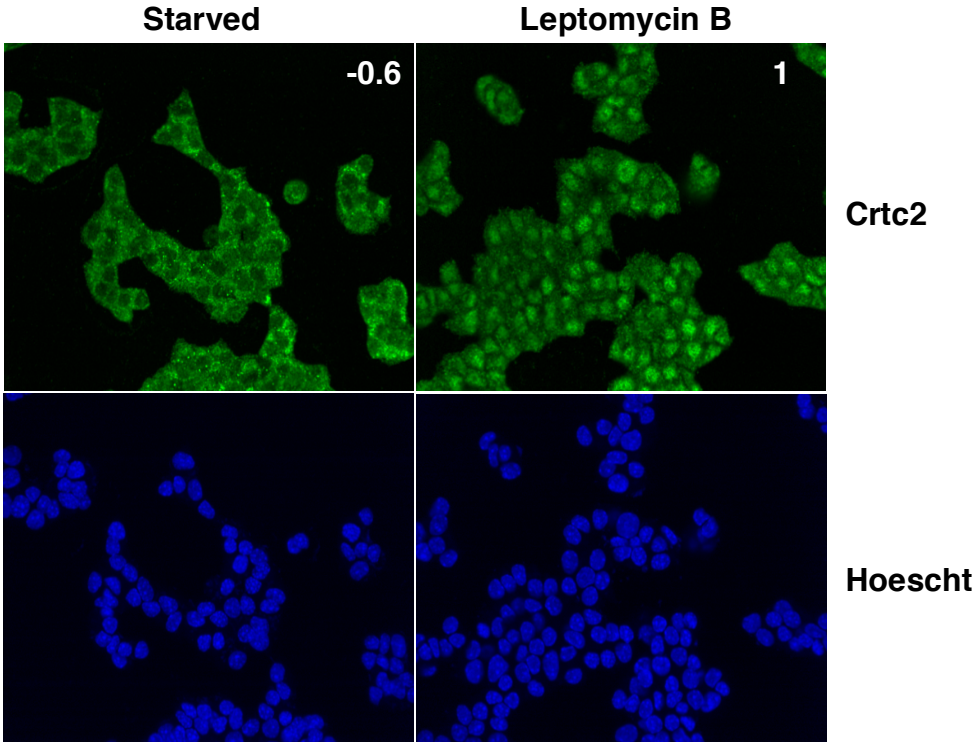
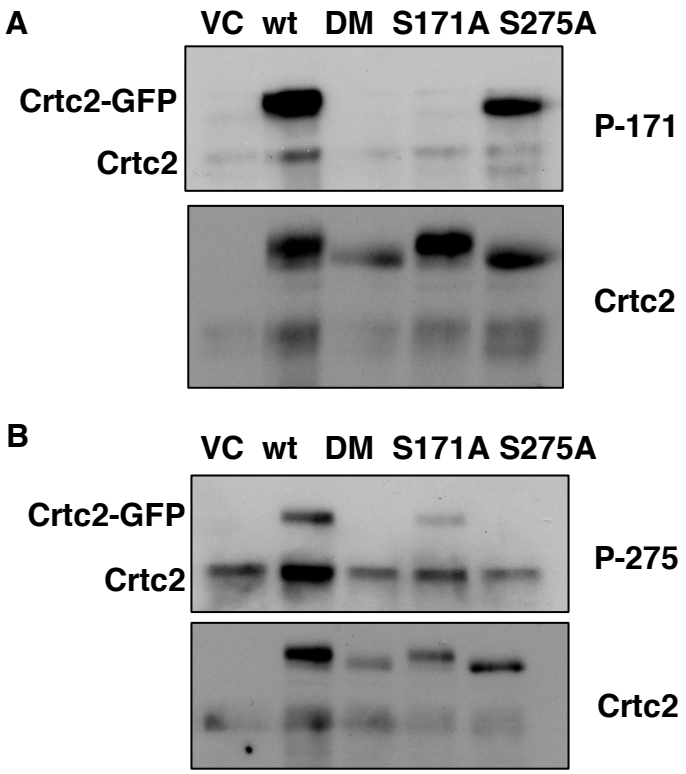
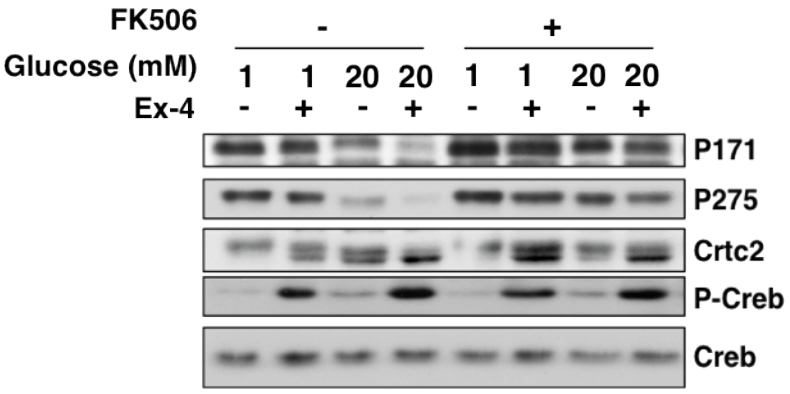


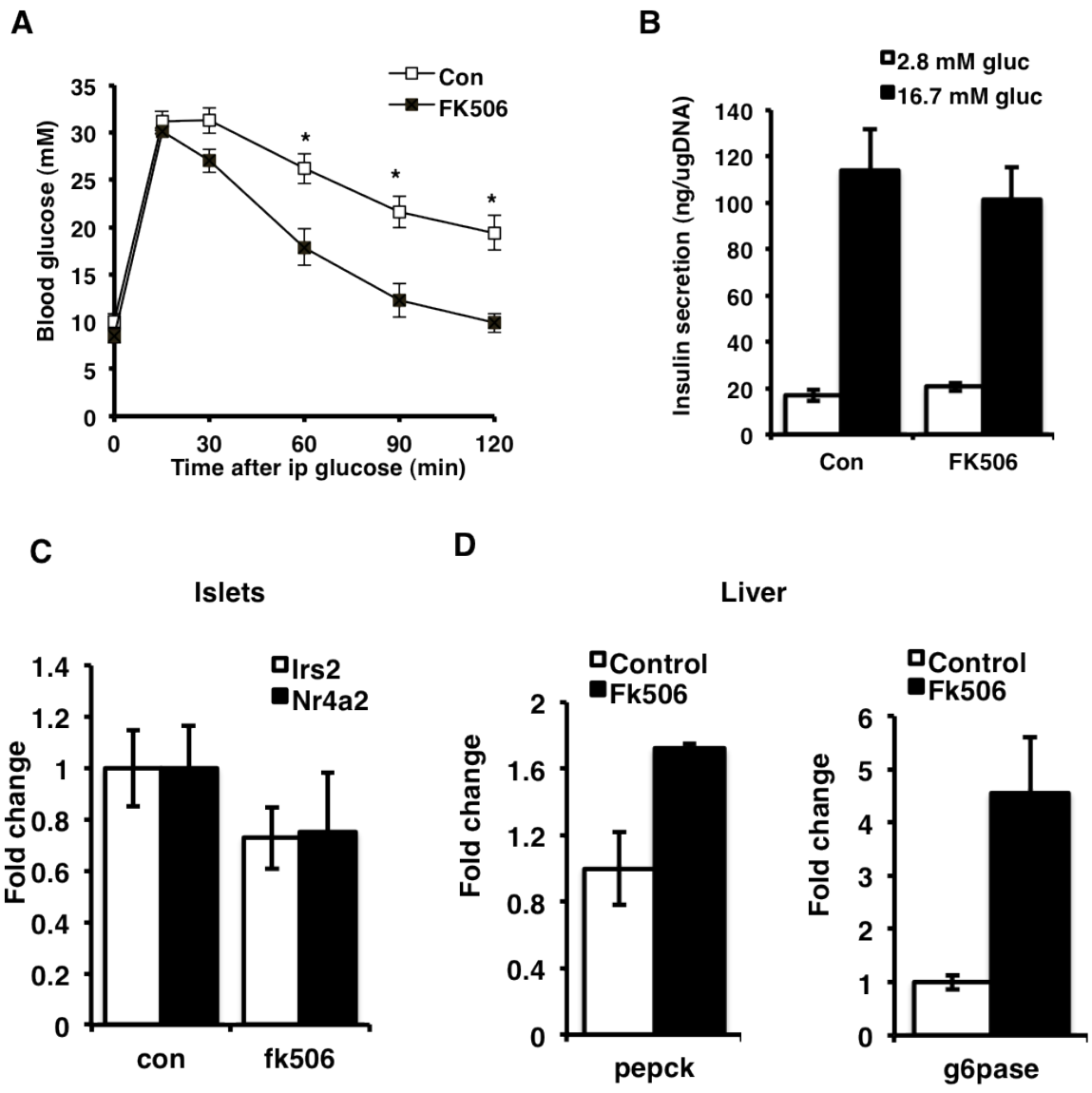
Appendix I: Endogenous Crtc2 immunofluorescence. Visual representation of quantified endogenous Crtc2 staining in MIN6 cells starved or treated with leptomycin B for 1 hr. Crtc2 localization was expressed as (nuclear intensity –cytoplasmic intensity)/ total intensity and expressed relative to 1 hr leptomycin B treatment. Localization shown quantifies as a shift from -0.6 in the starved cells to 1 in the leptomycin B treat cells.



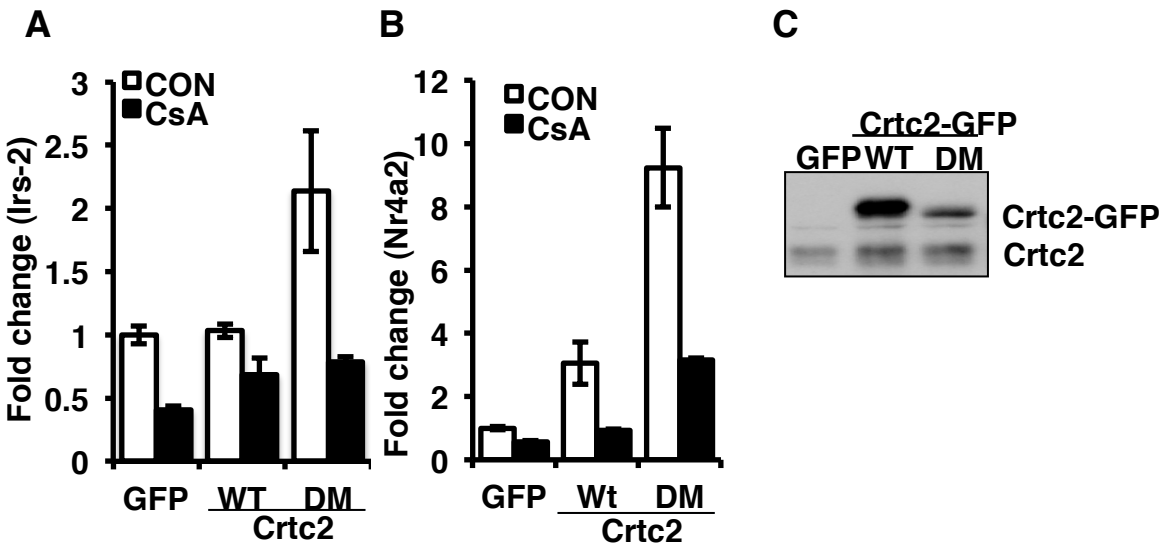
Appendix II: Specificity of Crtc2 phospho-specific antibodies. Crtc2 phospho-specific antibodies probed against MIN6 cells expressing Crtc2-GFP constructs. **A)** Phospho-specific antibody for S171 detects overexpressed wt and S275A mutant but not S171A mutant or S171,275A mutant (DM, double mutant). **B)** Phospho-specific antibody for S275 detects overexpressed wt and S171A mutant but not S275A mutant or S171,275A mutant (DM, double mutant).



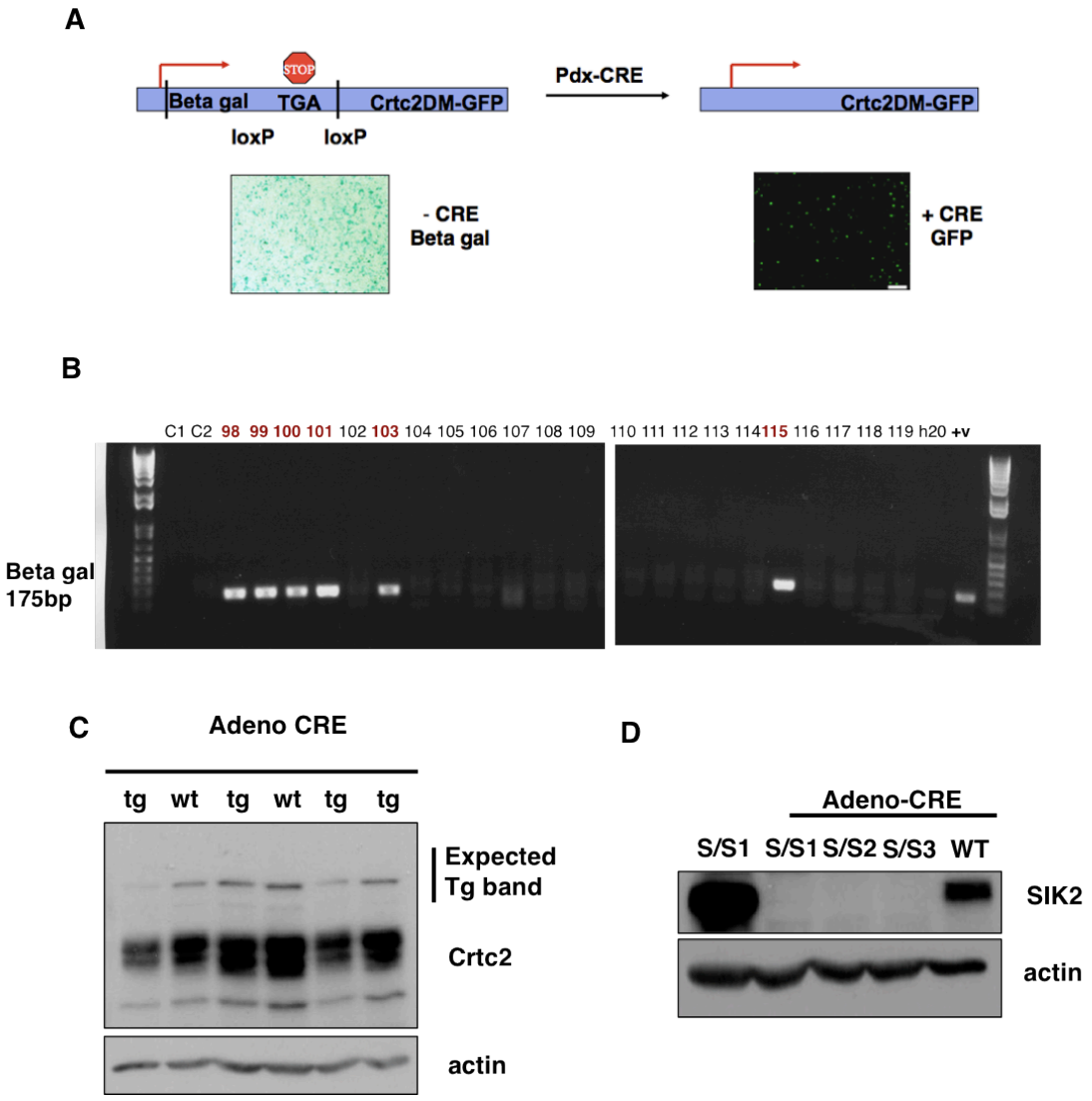
Appendix III: FK506 blocks Crtc2 dephosphorylation but does not impact phosphorylation of CREB in MIN6 cells. MIN6 cells were starved in 1 mM glucose in KRB prior to incubation at the indicated conditions for 1 hr. Ex-4 was used at 10 nM. FK506 was added during the 1 hr pre-incubation at 10 nM. Data shown are representative of 3 experiments.



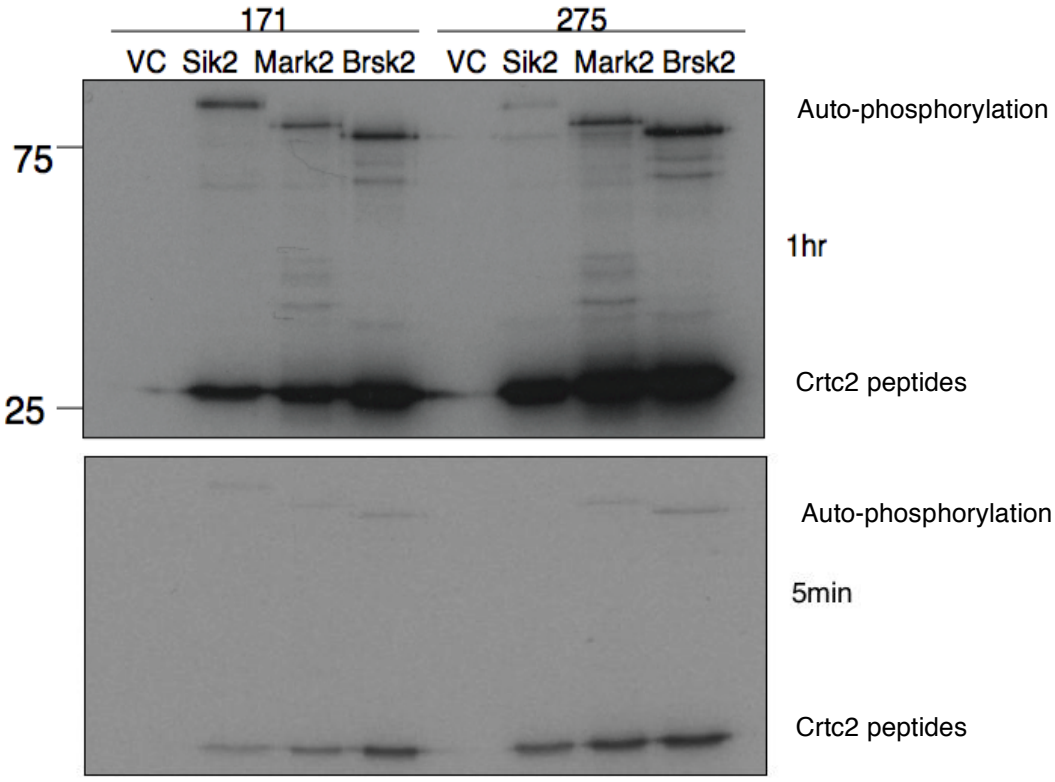
Appendix IV: Impact of FK506 treatment in mice. A) Glucose tolerance test demonstrating that FK506-treated mice have improved glucose clearance compared with control mice. **B)** Insulin secretion from isolated islets from FK506-treated mice was not impaired. **C)** mRNA expression of IRS-2 and Nr4a2 in islets from control and FK506 treated mice. **D)** mRNA expression of CREB target genes pepck and g6pase in the liver of control and FK506 treated mice. Mice were treated for 10 days with 1 mg/kg/day FK506 or vehicle control. Islets from mice were harvested 4 hr after glucose injection. RNA was harvested immediately from a portion of islets and glucose stimulated insulin secretion was measured the following day from remaining islets.



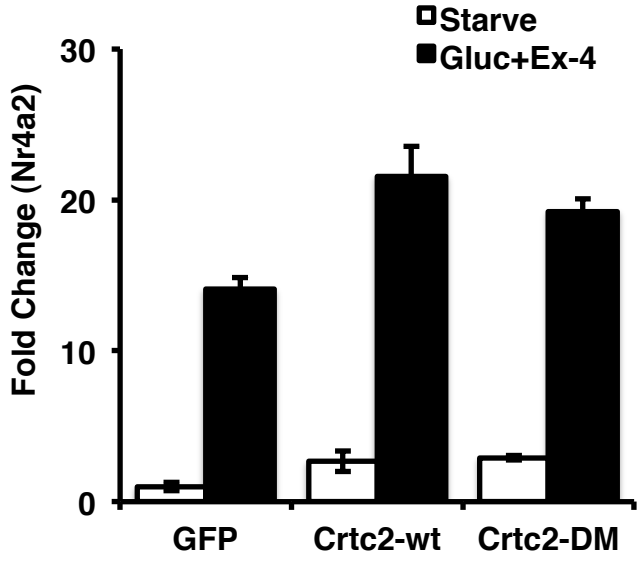
Appendix V: Constitutively active Crtc2 increases mRNA levels of CREB target genes and maintains them in the presence of CsA in MIN6 cells. MIN6 cells were infected with lentivirus overexpression constructs for 72hr. Cells were treated with 500 nM CsA in regular culture medium for 24 hr prior to harvest for mRNA analysis. Western blot analysis was performed to evaluate the levels of Crtc2 overexpression.



Appendix VI: Constitutively active Crtc2 transgenic mice. A) Schematic of expression control of the pCall2-Crtc2DM-GFP vector used to generate a transgenic mouse with representative β gal and GFP images in 293T cells transfected with the generated construct -/+ CRE. **B)** Sample genotyping of pups from CrtcDM-GFP positive founders. **C)** Western blot of ear fibroblasts infected with CRE recombinase expressing adenovirus show no distinct Crtc2DM-GFP band in mice that were positive by genotyping (Tg) compared with wildtype (wt) mice. **D)** Confirmation of successful CRE expression by loss of SIK2 expression in mice containing floxed SIK2 alleles performed in parallel with the Crtc2DM-GFP fibroblasts.



Appendix VII: Brsk2 *in vitro* kinase assay of Crtc2 peptides. Autoradiogram of phosphorylated Crtc2 171 and 275 peptides by overexpressed GST-tagged members of the AMPK family of kinases Sik2, Mark2 and Brsk2. Brsk2 phosphorylates Crtc2 peptides to similar levels as Sik2 and Mark2 (previously shown [164]). GST- kinases were immunoprecipitated from mammalian cell extracts and were incubated with recombinant 171 and 275 peptides with P^{32}. Samples were run on a SDS-PAGE and transferred to a PVDF membrane. Autoradiation exposed film for 1 hr and 5 min for the exposures above.



Appendix VIII: Crtc2 overexpression enhances CREB activity but does not abolish regulation of CREB. MIN6 cells were infected with lentiviruses expressing GFP, Crtc2wt-GFP or Crtc2DM-GFP. mRNA levels of Nr4a2 were monitored under glucose-starved and glucose and ex-4 treated conditions.