

LKB1-AMPK-SIK2-CRTC2 pathway in beta cells

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Abstract

In 2011, Diabetes and prediabetes affected 9 million Canadians and 366 million people worldwide (Canadian Diabetes Website). The underlying pathophysiology of diabetes is beta cell dysfunction leading to loss of appropriate insulin secretion and resulting in hyperglycemia. I have focused on identifying critical molecular regulators of beta cell function and insulin secretion. The CRTC2-CREB pathway is required for maintaining beta cell mass and insulin secretion. I propose that identifying kinases that regulate CRTC-CREB activity will identify other important regulators of pancreatic beta cell survival and function. First, I have identified several AMP kinases as inhibitors of CRTC2-CREB that are activated by an upstream kinase, LKB1. I then went on to generate mice with a beta cell-specific deletion of LKB1 during adulthood. Loss of LKB1 increased insulin secretion and glucose clearance through enhanced beta cell mass and proliferation. The increased insulin secretion was largely the result of loss of AMPK activity and consequent constitutive mTor activity. AMPK is activated under starvation conditions and as such is thought to be a critical regulator of beta cell function. However, the decrease of AMPK activity in high glucose has been a strong argument against it being a critical effector of insulin secretion. I provide genetic evidence supporting the idea that AMPK activity attenuates insulin secretion. During periods of starvation where AMPK activity is high there is a chronic dampening effect on events that prepare beta cells for the next round of insulin secretion. Surprisingly, another downstream kinase of LKB1, SIK2, has opposing functions in the beta cell. I present evidence that the LKB1-AMPK axis attenuates beta cell functions and that targeting this pathway in beta cells may be of therapeutic benefit for T2D.

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List of Abbreviations: 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR), Activator protein-1 (AP-1), Adenosine monophosphate, adenosine monophosphate activated kinase (AMPK), adenosine diphosphate (ADP), action potentials (APs), adenosine triphosphate (ATP), basic leucine zipper domain (bZIP), Brahma-related gene 1 (Brg1), brain-derived neurotrophic factor (BDNF), Ca^{2+} /Calmodulin-dependent Kinase Kinase 2 (CAMKK2), cyclic AMP (cAMP), cAMP response element (CRE), CRE binding protein (CREB), CREB binding protein (CBP), Cre recombinase-estrogen receptor tamoxifen-inducible (Cre-ERT2), CyclosporinA (CsA), G-protein coupled receptor 40 (GPR40), glucagon-like peptide-1 (GLP-1), glucose-stimulated insulin secretion (GSIS), glucose transporter 2 (GLUT2), guanine-nucleotide exchange factors (GEFs), Harvey rat sarcoma viral oncogene homolog (Ha-Ras), inducible cAMP early repressor (ICER), insulin receptor substrate 2 (IRS2), Liver kinase b1 (LKB1), LKB1 adult beta cell knockout (LABKO), mammalian target of rapamycin (mTor), maternal embryonic leucine zipper kinase (MELK), MAP/microtubule affinity-regulating kinase 2 (MARK2), mouse protein 25 (Mo25), neurogenin3 (Ngn3), pancreatic duodenal homeobox 1 (Pdx1), Peutz-Jeghers Syndrome (PJS), protein kinase A (PKA), protein kinase B (AKT), readily releasable pool (RRP), salt-induced kinase 2 (SIK2), SIK2 adult beta cell knockout (SABKO), soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), Ste20-like adapter protein (STRAD), Transforming growth factor beta-Activated Kinase (TAK1), Transducers of regulated CREB activity 2 (TORC2, now called CRTC2), tuberous sclerosis 2 (TSC2), Type 1 diabetes (T1D), Type 2 diabetes (T2D).

Chapter 1. Introduction

Diabetes pathophysiology.

Diabetes mellitus occurs when the body cannot metabolize serum glucose due to the lack of the hormone insulin. Following a meal, blood glucose level increases. Subsequently, insulin is released by the beta cells found in spheroid structures called the Islets of Langerhans within the pancreas, and is absolutely required to maintain glucose homeostasis. The insulin that is released into the blood stream then stimulates peripheral tissues (liver, fat, muscle) to activate signaling pathways that initiate glucose uptake and usage as building blocks in anabolic processes.

In the absence of sufficient insulin secretion in diabetes, the body cannot use the excess energy and excess glucose is retained in the blood stream. Thus, a hallmark of the disease is having high fasting blood glucose levels. In fact the persistence of high fasting glucose levels (≥ 5.5 -7 mM) is one of the diagnostics of prediabetes [1]. Many of the pathologies of diabetes result from having toxic levels of circulating glucose. There are two types of diabetes, distinguished by their underlying causes for insufficient insulin release: Type 1 diabetes mellitus results from autoimmune-mediated destruction of the beta cell population; and Type 2 diabetes results from insulin resistance in the peripheral tissues such as muscle, fat and in the liver and a loss of beta cell compensation. The importance of beta cell function has been debated in the past by proponents of the belief that insulin-resistance is causal to diabetes. This belief stemmed largely from the observation that some T2D

patients have extremely high insulin levels thus making it paradoxical that the disease be caused by an insufficient amount of insulin. However, when considering that the increased degree of demand for insulin in the context of elevated glucose is not met, there is a severe relative deficiency in insulin secretion and beta cell dysfunction in T2D [2, 3]. Further to a loss of function there is also loss of beta cell mass in T2D patients likely due to increased beta cell apoptosis in a high glucose-toxic environment [4]. *Understanding beta cell biology and defining ways to improve beta cell function through loss of function studies is the major focus of my thesis.*

Beta cell biology.

Islets of Langerhans are infrequent size-restricted structures of ~500-2000 cells that comprise only 1-2% of the total mass of the pancreas. The remaining tissue of the pancreas is composed of acinar cells termed exocrine pancreas which aids in digestion of fats and protein by secreting lipases and proteases into the duodenum [5]. Most of the islet is composed of beta cells in both humans and mouse pancreas. The remaining cells are also specialized endocrine cells: alpha, delta and F cells, which each secrete the peptide hormones glucagon, somatostatin, and pancreatic polypeptide, respectively. Glucagon is secreted by alpha cells during low/fasting blood glucose periods and helps stimulate hepatic breakdown of glycogen and glucose production to maintain a normal level of circulating glucose [6]. While the function of somatostatin remains unclear, it is thought to be required to inhibit glucagon release in high glucose and to transiently inhibit insulin release following a response [7].

In mice, islets are composed of ~80% beta cells with the remainder being alpha cells, delta cells and rarely F cells in order of descending abundance (Figure 1) [8]. Interestingly the size distribution of islets remains the same from mouse to human islets even though pancreas sizes differ greatly [8]. In humans, however beta cells comprise only ~60% and there is a complementary increase in the proportion of alpha cells [8, 9]. An increased relative abundance of alpha cells is also seen in ob/ob mice that have a higher insulin demand, thus it is unclear if the increase in alpha cells in humans is due to a higher insulin demand from diet or a fundamental species difference [9]. It would be interesting and biologically advantageous if islet architecture is regulated by islet cell functional demand. Supporting this idea are numerous studies where glucose was observed to stimulate beta cell proliferation [10-12].

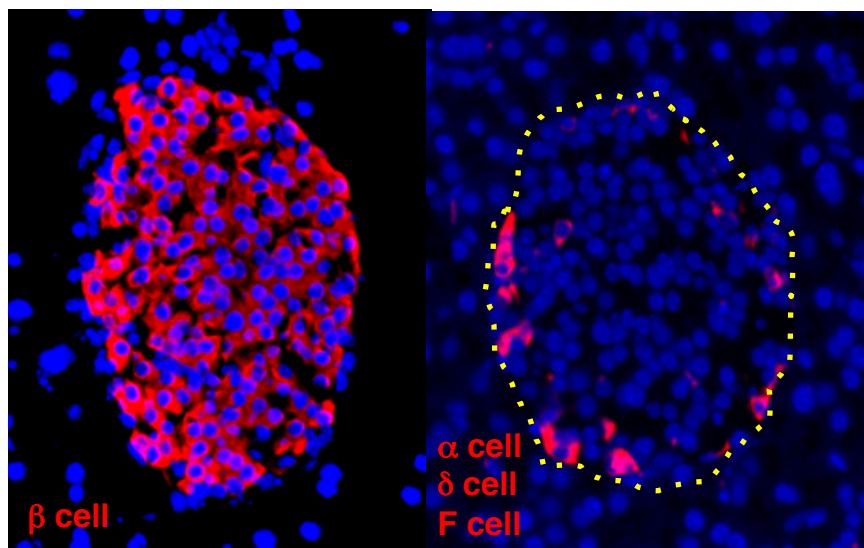


Figure 1. Immunofluorescent staining of a mouse islet revealing dynamic islet cell architecture. Antibody against insulin (beta cells) in red (left) and a cocktail antibody staining against glucagon (alpha cells), somatostatin (delta cells) and pancreatic polypeptide (F cells) in red (right) and hoescht (nuclei) in blue.

Beta cell mass.

Beta cell mass is reduced in diabetes contributing in part to insufficient circulating insulin levels [4, 13]. It was originally thought that an individual was born with all the beta cells they would have for life, now that techniques have improved we are able to identify and measure the production of new beta cells in adult humans. The knowledge that beta cells can renew sparked the next logical question for debate among beta cell biologists: do beta cells come from replication of existing beta cells or from differentiation of a beta cell progenitor?

Strong evidence from lineage tracing studies revealed that in adult mice and humans, the majority of beta cells arise from preexisting beta cells and do not involve a specialized progenitor cell [14-16]. Following removal of pancreas in a 70% pancreatectomy there was a major spike in beta cell proliferation and no appearance of beta cells from non-beta cell origins [14]. It is, however, difficult to empirically prove that something does not exist, and the possibility remains that there are beta cell progenitors in adult pancreas. Another study revealed that a 90% pancreatectomy was required in order to detect progenitors proposed to exist in ductal regions that contribute to islet/beta cell neogenesis [17]. In this study, ductal cells were found to express the islet cell marker pancreatic duodenal homeobox 1 (Pdx1) [17]. Using ductal ligation to induce beta cell proliferation, another group found potential progenitors in ductal cells that had high neurogenin3 (Ngn3) expression and gave rise to endocrine cells *in vitro* [18]. In humans, embryonic islet cell progenitor cells have also been identified but as yet a specific beta/islet cell progenitor cell in adult humans or rodents has not been clearly identified [19, 20].

Many studies that include measurement of beta cell mass do not take a side either way and measure all possible sources of beta cells, thus beta cell mass can be considered as the combined effect of beta cell proliferation, neogenesis, cell size and cell turnover. Beta cell turnover only significantly contributes to beta cell mass under disease conditions such as immune-cell mediated or glucolipotoxic-environment destruction [4, 21, 22] and is not readily observed in normal lean or non-diabetic obese human adults [23]. Another difference between mouse and human islets is the relatively frequent beta cell proliferation of mice in contrast to the rarely observed human beta cell proliferation. In contrast to mice, regeneration of the human pancreas is not observed following a 50% pancreatectomy [24]. These differences could be due to age differences where studies on mice are performed in young adults vs. many similar procedures only occurring in older humans, nonetheless, these differences highlight the importance of confirming discoveries made in rodent models in human islets.

Glucose-stimulated insulin secretion.

As mentioned above, the major function of beta cells is to respond to glucose by secreting insulin to maintain glucose homeostasis. Insulin is synthesized and then processed and stored within granules inside beta cells. The classical pathway for glucose-stimulated insulin secretion (GSIS) has been well studied and was found to involve several key events (Figure 2). Insulin secretion profiles reveal biphasic release of insulin from islets with 1) a rapid and high amplitude initial phase and 2) a prolonged lower amplitude phase of release.

Beta cells are specialized glucose sensors and as such have evolved methods to respond to glucose and coordinate insulin secretion accordingly. As such, beta cells have higher thresholds for responding to glucose stimulation to prevent hypoglycemia by ensuring that they do not release insulin at low fed glucose levels (6-7 mM) in order to prevent inappropriate glucose usage by peripheral tissues.

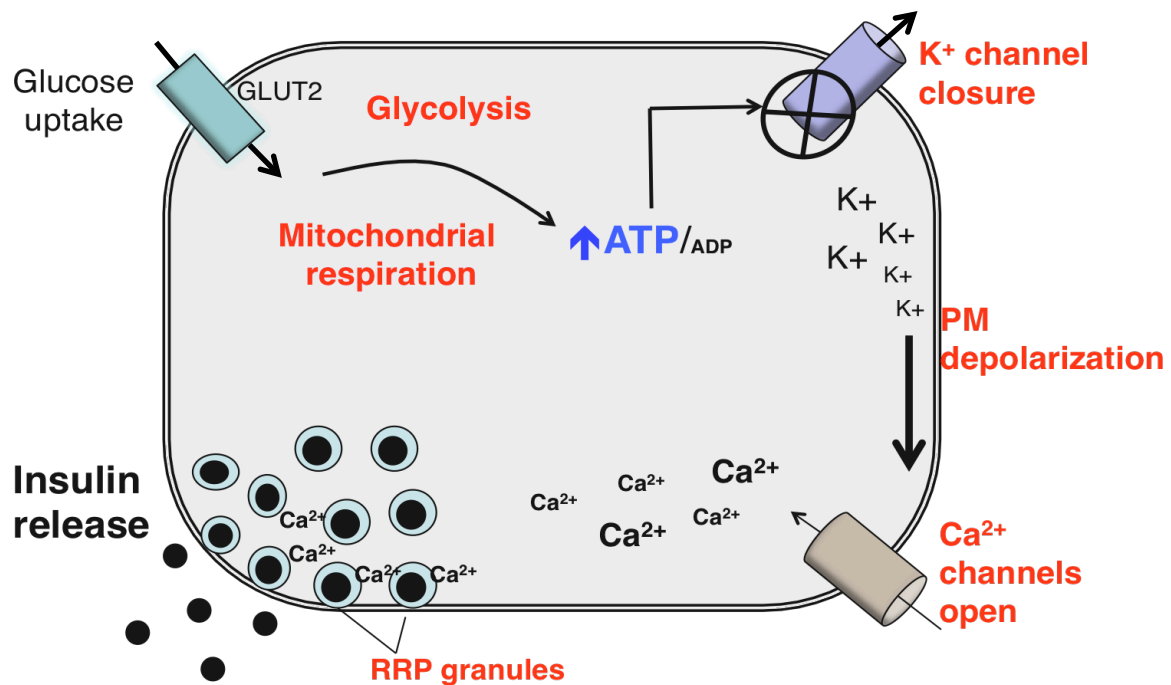


Figure 2. Glucose-stimulated insulin secretion (GSIS) pathway in beta cells. First, glucose is taken up by GLUT2 transporters and metabolized by glycolysis and mitochondrial respiration resulting in an increased ATP/ADP. The rise in ATP causes closure of K⁺_{ATP} channels and depolarization of the plasma membrane. The loss of membrane potential leads to opening of voltage-sensitive L-type Ca²⁺ channels and a major influx of Ca²⁺ triggers the exocytosis of the readily releasable pool (RRP) of insulin granules.

Glucose uptake and metabolism.

First, glucose is taken up by beta cells through glucose transporter 2 (GLUT2) which has a high Michaelis constant (~17 mM) compared to the other GLUT isoforms (1-5 mM). The high Michaelis constant means that it has a lower affinity for glucose and only imports glucose into beta cells at higher concentrations giving them the ability to act as feeding glucose sensors [25]. Next, the internalized glucose is metabolized leading to an increase in energy production and the ratio of adenosine triphosphate to adenosine diphosphate (ATP/ADP), which is thought to be the major coupling factor for stimulation of insulin secretion [26]. Another specialized glucose sensing beta cell feature comes from the use of glucokinase to phosphorylate glucose into glucose-6-phosphate and initiate glycolysis. Glucokinase, like GLUT2 has a lower affinity for glucose and in addition is upregulated by glucose metabolism [27]. Thus initiation of the glycolytic process feeds forward to drive itself further [27]. The end product of glycolysis is pyruvate, which acts as a substrate for mitochondrial respiration and is the major source of ATP production leading to the rise in ATP/ADP [28].

Beta cells are electrically excitable cells.

The increase in ATP/ADP is sensed by the sulfonyl urea receptor 1 (Sur1) subunit of K^+_{ATP} channels on the plasma membrane and leads to their inactivation and closure [29, 30]. As such, Sur1^{-/-} mouse islets have impaired first and second phase insulin secretion [31]. Furthermore, there are many mutations in K^+_{ATP} channel subunits that lead to either a loss of channel activity causing congenital hyperinsulinemia, or an increase in channel activity

causing neonatal diabetes in humans [32-34]. Closure of K_{ATP}^+ channels leads to depolarization of the plasma membrane (from $\sim -60\text{mV}$ to $\sim -30\text{mV}$) triggering opening of voltage-sensitive L-type Ca^{2+} channels and the firing of action potentials (APs) [35]. The stimulation with glucose leading to AP firing in beta cells gives them a unique property and for this reason they are called excitable cells, similar to neurons. The rise in intracellular Ca^{2+} is the final event required for both membrane fusion and exocytosis of insulin granules [36]. Furthermore increased intracellular Ca^{2+} stimulates multiple pathways in beta cells that not only trigger release of insulin and prepare them for the next rounds of secretion but can also promote beta cell proliferation and survival [37].

The first phase of insulin secretion is derived from the readily releasable pool (RRP) of granules, these granules are docked at the plasma membrane prior to stimulation through membrane fusion mediated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes [38-40]. Recruitment of insulin granules during second phase requires small GTPase-dependent cytoskeletal remodeling making it a slower process with lower amplitude secretion than first phase [41]. This is important since the first phase of insulin release is significantly reduced in prediabetes and T2D [42]. A reduction in second phase insulin secretion is also observed in diabetes, however it is not as broadly accepted as an indicator of diabetes [43].

Nutrient-dependent stimulation of GSIS.

Although glucose is the major stimulus of insulin secretion there are several other nutrients and ligands that can stimulate beta cells to secrete insulin alone and/or potentiate GSIS. Essential amino acids, such as leucine and nonessential amino acids, such as arginine and glutamine can potentiate GSIS and stimulate insulin release in the absence of glucose, and have been used to help regulate glycemia in patients with T2D [44, 45]. Fatty acids are also known to amplify GSIS through activation of G-protein coupled receptor 40 (GPR40) and possibly from intracellular lipolysis [46]. However, like glucose, an excess of fatty acids from chronic exposure becomes toxic to beta cell function and survival [46].

The peptide hormone glucagon-like peptide-1 (GLP-1), secreted by the gut following nutrient stimulation, enhances GSIS in beta cells by increasing cyclic AMP (cAMP) levels [47, 48]. cAMP also enhances beta cell survival and can protect against fatty acid induced toxicity. This effect is thought to be mediated via activation of protein kinase A (PKA) and Guanine-nucleotide exchange factors (GEFs) [49]. Furthermore, cAMP is required for activating the transcription factor cAMP response element (CRE) binding protein (CREB) to promote beta cell survival [50]. Thus appropriate nutrient levels and metabolic processes both stimulate beta cells to secrete insulin and as well drive signaling processes, such as gene transcription, required for beta cell survival.

Beta cell gene transcription.

In addition to stimulation of insulin secretion, glucose enhances cell survival and function through initiation of several important transcriptional programs in beta cells.

Together, glucose and cAMP signaling complete the physiological feeding stimulus that stimulates insulin secretion as well as activates several transcription factors and their targets. Following long-term (24 hr) treatment of mouse insulinoma cells (MIN6), an immortalized beta cell line, with high glucose (25 mM) there is an increase in a number of secretory and metabolic genes required for insulin secretion [51]. This long-term treatment however, included the potential for identifying secondary changes in the transcriptome and did not necessarily identify genes that were responsible for initiating the response to glucose.

In order to distinguish early immediate genes that were upregulated from the secondary targets of those genes, Glauser et al. used microarray analysis +/- cyclohexamide to block translation and secondary target activation. In this study the transcription factor activator protein-1 (AP-1) was found to be an immediate early gene that appropriately enhanced transcription of a number of other genes involved in metabolic processes in response to glucose [52]. These studies were aimed at identifying key transcription factors that respond to the functional stimuli and are required for islet cell survival. Another such transcription factor is CRE binding protein (CREB), which responds to both glucose and cAMP via PKA-mediated phosphorylation and CRTC2 co-activation in order to initiate a transcriptional program required for beta cell survival [50, 53, 54].

Kinase signaling mediates cellular processes.

The Nobel-prize winning discovery of Protein kinase A (PKA) by Krebs described for the first time a specific enzyme driving cellular signaling from an external cell stimulus

to an intracellular target [55]. In general, 2 molecules of cAMP bind to the regulatory subunits of PKA and release the kinase catalytic domain making the kinase active and able to phosphorylate its cellular targets [56]. In this case PKA was found to propagate a signal starting with increased cytosolic cAMP initiating downstream catabolic processes in muscle. This discovery lead to the identification of a new class of important signaling molecules called the kinome that consists of 518 kinases in the human genome [57].

Protein kinases have the ability to transfer phosphate from ATP to a serine, threonine or tyrosine residue on a substrate protein. This post-translational modification alters the state of the protein and can make it inactive, active or sometimes prime it for other post-translational modifications such as ubiquitination. In general, protein kinases have a flexible catalytic lobe consisting of many domains in addition to regulatory domains that determine substrate specificity and cofactor binding sites [58]. The catalytic site is the most highly conserved whereas there are major sequence variations in other domains thus providing potential for specific substrates and regulation of distinct molecular pathways [58]. Identification of the kinase(s) responsible for phosphorylating a substrate of interest is key in understanding molecular pathways and can reveal important biological functions for kinases. With the potential for identification of druggable targets, to modify the pathway in translational applications. **I predict that in using an in vitro high throughput screen I will identify kinases that regulate the CRTIC2-CREB signaling pathway (subject of Chapter 3) and that loss of these kinases in vivo will enhance pancreatic beta cell function and survival (subject of Chapter 4).**

Regulation of gene transcription by PKA-CREB pathway.

CREB was first identified as a nuclear protein that was capable of binding the palindromic cAMP response element (CRE) sequence TGACGTCA in somatostatin after getting phosphorylated by PKA in response to cAMP [54]. Active PKA phosphorylates CREB at serine133 in the nucleus and can bind to CREs present in the promoter of target genes to activate their transcription [59]. CREB binds to CREs directly through its basic leucine zipper domain (bZIP) [60]. Other than somatostatin, about 100 out of the total 4,000 genes identified that contain CREs are cAMP-inducible and are bound by CREB in the human genome [61].

In addition, CREB binding protein (CBP) is recruited to DNA via binding phosphorylated CREB to promote transcription of CRE containing genes [62, 63]. CBP is thought not only to act as a scaffold for the CREB transcriptional complex but also as a direct mediator of CREB-regulated transcription in that it can acetylate histones to relax DNA and enhance transcription directly [64]. Surprisingly, the levels of induction of CRE containing genes have been found to correlate with the recruitment of CBP and not with the degree of serine133 phosphorylation of CREB [61]. This suggests that while serine133 phosphorylation of CREB is required for transcription is it not the rate limiting step, and that CBP or another coactivator may be involved.

CRTC coactivates CREB gene transcription.

An overexpression screen of cDNAs that could enhance CREB-regulated gene transcription identified two homologs as the most potent CREB gene coactivators in the absence of cAMP, which were named Transducers of regulated CREB activity (TORCs now called CREB-regulated transcription coactivator or CRTC) [65]. In this study 293 cells were co-transfected with a library of cDNAs and a plasmid containing a CRE promoter driving expression of luciferase thus expression of luciferase correlated with CRE promoter activity. Importantly, overexpression of CRTC1 synergized with forskolin treatment (increasing cAMP) to enhance CREB-target gene transcription whereas no further enhancement was seen with overexpression of CBP, suggesting that CRTCs are the rate limiting regulatory component of CREB-regulated transcription [65].

The CRTC gene family consists of 3 closely related genes CRTC1, 2 and 3 which all share 31-32% identity in amino acid sequences [65]. In a subsequent overexpression screen, CRTC1 was also found to coactivate CREB-transcription of the CRE-containing interleukin 8 promoter [66]. CRTC1 is highly expressed in brain where it is required for CREB-mediated transcription of brain-derived neurotrophic factor (BDNF) and plays a role in long-term memory [67]. In the liver, CRTC2 is expressed to the highest extent, where it is activated by glucagon to stimulate CREB-dependent transcription of gluconeogenic genes [68]. In addition, CRTC3 promotes obesity by attenuating beta-adrenergic stimulation of lipolysis and fatty acid oxidation: fat-burning processes in adipocytes [69]. From studies in peripheral tissues, liver and adipose, it is clear that the CRTC-CREB pathway promotes an energy conservation and catabolic program in response to starving stimuli and increases in cAMP signaling in these tissues. In contrast, cAMP signaling in beta cells is a feeding cue

that promotes insulin secretion, insulin then targets peripheral tissues to activate energy producing and storing, anabolic programs.

CREB-CRTC2 pathway in beta cells.

Repression of CREB activity in beta cells leads to diabetes through a loss of insulin receptor substrate 2 (IRS2) expression which increases beta cell apoptosis, loss of beta cell mass and reduced insulin secretion [50]. Furthermore, overexpression of inducible cAMP early repressor (ICER) in mice leads to severe diabetes through loss of beta cell proliferation and mass [70]. Thus CREB is important for maintenance of a healthy beta cell population, and appropriately CREB is activated by two major stimuli for beta cell function and survival: glucose (Ca^{2+}) and cAMP. Initial evidence for Ca^{2+} -dependent CREB activation came from the use of CREB fusion proteins that deduced the bZIP domain is required to respond to Ca^{2+} following membrane depolarization in PC12 cells [71]. The molecular details for the observation of Ca^{2+} -stimulated CREB activation were not described until 13 years later in another excitable cell type: beta cells.

First, it was confirmed that the bZIP domain is essential for glucose (Ca^{2+} and exendin-4 (cAMP) stimulation of CREB-regulated gene transcription in beta cells [53]. Then it was found that the Ca^{2+} -dependent increase of CREB activity requires CRTC2 and not CBP, and a model for CRTC2 activation in coordinating Ca^{2+} and cAMP-dependent CREB activation was formed (Figure 3). Following glucose and exendin-4 stimulation, CRTC2 is dephosphorylated and translocates into the nucleus [53]. Peptide mapping revealed that

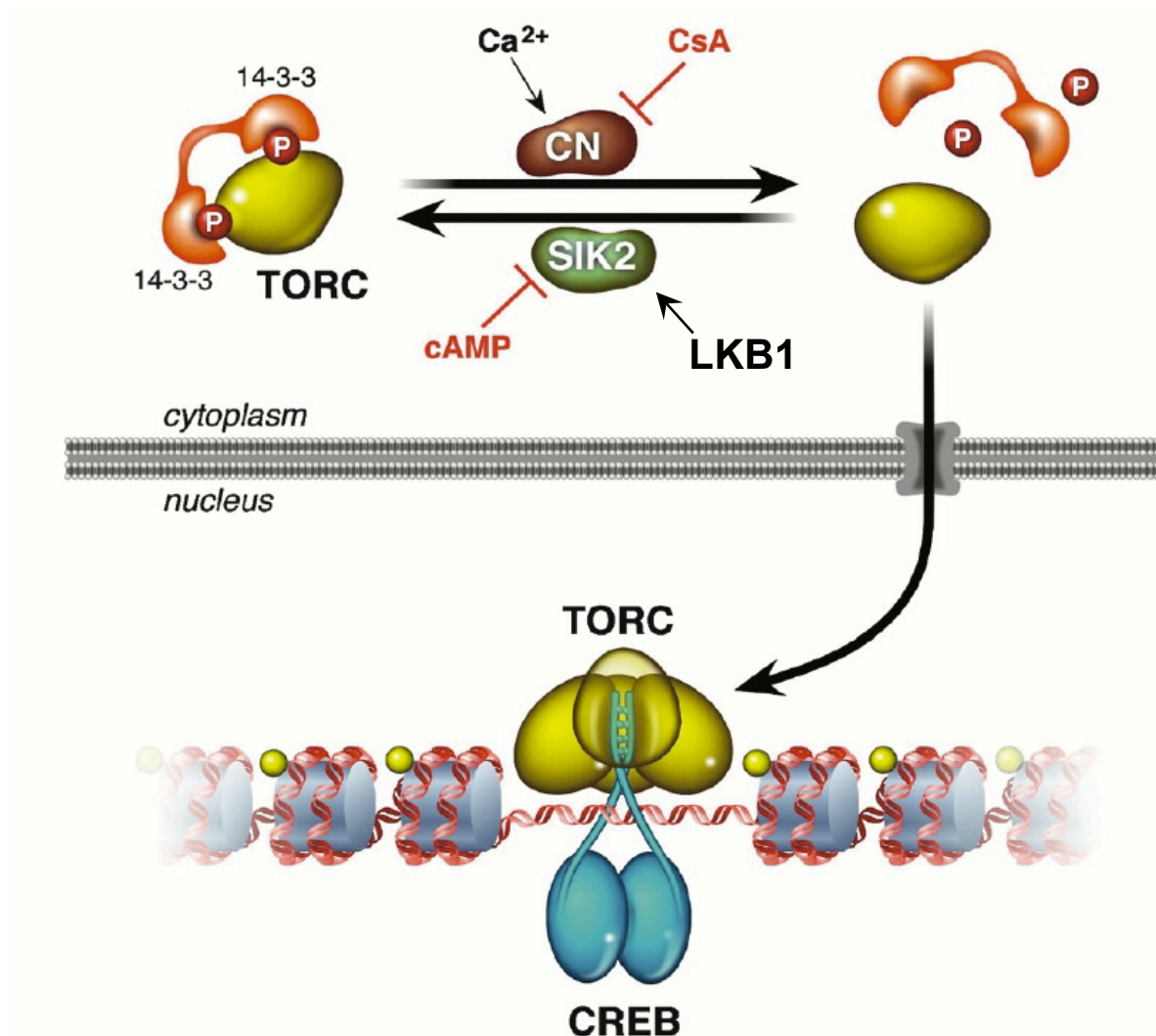


Figure 3. TORC, now called CRTC functions as a coincidence detector of calcium via CN and cAMP via SIK2 to activate CREB gene transcription. cAMP inhibits SIK2 phosphorylation of CRTC and calcium activates CN dephosphorylation of CRTC resulting in dephosphorylation of CRTC, loss of 14-3-3 binding and nuclear translocation. On the other hand, in the absence of these feeding cues, SIK2 is active and phosphorylates CRTC to retain it in the cytoplasm and attenuate CREB gene transcription. Interestingly, the

serine/threonine kinase LKB1 activates SIK2 and is known to coordinate cellular responses to low energy status. Model adapted from Figure 7d in [53].

phosphorylation of serine171 of CRTC2 is a regulatory site for CRTC2 localization and mediated through binding of 14-3-3 proteins and cytoplasmic retention of CRTC2 [53]. Dephosphorylation of CRTC2 by the phosphatase calcineurin is critical for nuclear entry of CRTC2, as treatment with CyclosporinA (CsA), a calcineurin inhibitor, retains CRTC2 in the cytoplasm [53]. Furthermore salt-inducible kinase 2 (SIK2) is able to phosphorylate CRTC2 in vitro and is inhibited by cAMP treatment suggesting that it is an important inhibitor of CRTC2-dependent CREB activation [53]. **Thus I predict the SIK2-CRTC2 pathway is likely to be important for beta cell function and survival.**

Importantly, however, when serine171 is mutated to a non-phosphorylatable alanine residue, 14-3-3 binding to CRTC2 is still detectable in beta cells, suggesting that there is an additional regulatory phosphorylation site on CRTC2 [53]. **Identification of the second regulatory site of CRTC2 will reveal the beta cell-specific mechanism of its activation and may help identify other upstream kinases that are important for beta cell function and survival (subject of Chapter 2 and 3).**

LKB1-AMPK/SIK2 inhibition of CRTC-CREB pathway.

Interestingly, in the liver SIK1 is induced during fasting and attenuates CRTC2 activation as a negative feedback mechanism of gluconeogenesis [68]. In addition, AMPK

also phosphorylates and inhibits CRTC2 coactivation of CREB gluconeogenic genes [68]. The identified CRTC kinases SIK1, SIK2 and AMPK are actually closely related kinases and are members of the AMP kinase family. Furthermore, all members except maternal embryonic leucine zipper kinase (MELK), are directly phosphorylated at a conserved threonine residue by Liver kinase b1 (LKB1 or STK11) which leads to >50-fold increase in their kinase activity [72].

Importantly this pathway was found to be the mode of action of Metformin, a drug given to T2D patients to reduce hyperglycemia. Metformin treatment works by activating LKB1-AMPK which inhibits CRCT2-CREB and blocks gluconeogenesis in the liver thereby lowering glucose output from the liver [73]. Interestingly, as beta cells of the pancreas have an opposing function to the liver, one might predict the same pathways may have opposing actions in these tissues as well. **By association to CRTC-CREB inhibition, I hypothesize that LKB1 activity in beta cells inhibits beta cell survival and function thereby impeding upon whole body glycaemia (subject of Chapter 4). Furthermore, SIK2, a downstream effector of LKB1, in beta cells should similarly inhibit beta cell function and survival (subject of Appendix 1).**

LKB1 biological functions.

The serine/threonine kinase LKB1 was first described as a causal mutation in Peutz-Jeghers Syndrome (PJS) that predisposes patients to developing benign intestinal polyps. This was also the first example of an inactivating kinase mutation leading to a cancer

predisposition [74]. In fact there are many sporadic mutations in LKB1 in human tumors, most of which are inactivating loss of function mutations suggestive of LKB1 functioning as a tumor suppressor [75]. Furthermore, LKB1 was found to block cell growth by causing G1 cell cycle arrest [76].

In mice, heterozygous deletion of LKB1 leads to the development of gastrointestinal hamartomas (benign polyps) as seen in the human disease PJS [77]. Homozygous deletion of LKB1 or its ortholog XEEK1 leads to severe developmental abnormalities in mouse and frog embryos, respectively, which cannot form a normal axis and through defective dorsoanterior patterning are eventually aborted at E13.5 [78]. The requirement of LKB1 for embryo development presented the problem of how to look at LKB1 function using loss of function studies. A solution came from newly available genetic tools in which one could measure effects of gene knockout in a tissue specific and/or time-dependent manner using the Cre recombinase/LoxP systems. Table 1 summarizes the important discoveries made with respect to LKB1 functions in various tissues.

Regulation of LKB1.

LKB1 is ubiquitously expressed however it is enriched in epithelia and in the seminiferous tubules of the testis and expression is higher in fetal versus adult tissues [79]. Regulation of LKB1 has not been classically associated with its expression however, there are promoter binding sequences for FOXO, NF-Y and Sp1 transcription factors and deletion of these sequences reduced LKB1 expression [80].

Table 1. Summary of tissue-specific knockout models of LKB1 in mice. As LKB1

homozygous knockout mice die at E13.5, loss of function studies have been aided in the use of Cre/LoxP recombinase systems which allow tissue-specific and timing specific deletion of genes of interest. Adapted from Figure 1 in [87].

Knockout tissue	Phenotypic effect	Reference
Pancreatic epithelium	Disrupted acinar cell polarity, acinar to ductal cell metaplasia, pancreatic cysts as seen in PJS	[88][89]
Small intestine epithelium	Loss of secretory cell lineages paneth and goblet, disrupted gastrointestinal barrier	[90]
MEFs	Loss of myofibroblast differentiation through attenuated TGF beta signalling	[91]
T cell progenitors	Defective t-cell survival, activation, differentiation and metabolism	[92, 93]
Endothelial cells	Embryonic lethal vascular defects	[94]
Endothelial cells	Defective revascularization of hind-limb ischemia	[95]
Cortical neurons	Defective axon specification	[96]
Whole body adult only	Hematopoietic stem cell depletion and pancytopenia	[97-99]
Cardiac myocytes	Cardiac dysfunction exacerbated by ischemia, atrial fibrillation and reduced capillaries	[100, 101]
Skeletal myocytes	Impaired contraction-induced glucose uptake, enhanced glucose tolerance, lower fasting glucose and insulin	[102, 103]
Thoracic nerves	Axon degeneration, hind-limb paralysis	[104]
Hepatocytes	Hyperglycemia, increased gluconeogenesis and lipogenesis	[73]

LKB1 is thought to be a constitutively active kinase and that regulation of its substrates phosphorylation is due to its subcellular localization. Ste20-like adapter protein (STRAD) is a pseudokinase that binds to LKB1 and allows its nuclear export [81]. Interestingly, an LKB1 mutation in PJS leads to loss of STRAD-binding and nuclear export, rather than loss of its kinase activity [81]. The scaffolding protein mouse protein 25 (Mo25) binds to STRAD to stabilize its interaction with LKB1 and keep it cytoplasmic [82-84]. Phosphorylation of LKB1 at serine431 by PKA and p90 was originally thought to be required for its kinase activity since it was required for LKB1-dependent cell cycle arrest [85]. The importance of LKB1 serine431 has since been contested as expression of a short isoform of LKB1, mostly expressed in testis, or mutation of this site to alanine does not affect LKB1-phosphorylation of AMPK [86].

LKB1 cellular functions.

As mentioned, LKB1 is an important kinase that activates all but MELK of the AMP kinases via direct phosphorylation of a conserved threonine residue (Figure 4) [72]. Through downstream activation of AMPK and MAP/microtubule affinity-regulating kinases (MARKs), LKB1 has been ascribed a role in cellular energy sensing and cell polarity, respectively.

The most well studied function of LKB1 is its tumor suppressing ability. LKB1 associates with p53 and is thought to mediate mitochondrial-directed apoptosis [105]. However, intestinal polyps from LKB1-/+ mice still express p53, instead it is thought that

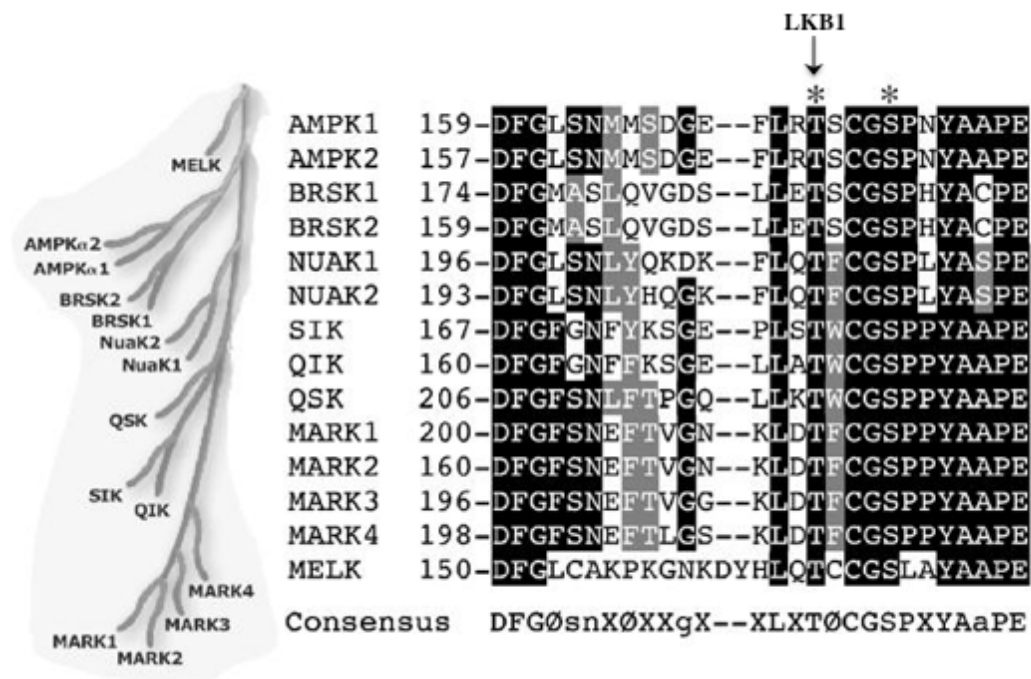


Figure 4. LKB1 activates 12 members of the AMP kinase family. Left, dendrogram from [57] depicting relatedness of all AMP kinases. Right, LKB1 consensus sequence alignment of all AMP kinases, arrow pointing to LKB1-phosphorylated threonine residue in all AMP kinases, identical residues in black and conserved residues in grey. Adapted from Figure 1 in [72].

these tumors arise from inappropriate Harvey rat sarcoma viral oncogene homolog (Ha-Ras) activity seen in LKB1^{-/-} MEFs and in polyps from PJS patients [106]. Furthermore, LKB1 is not a typical tumor suppressor, and the polyps seen in PJS are thought to be benign for this reason. In the absence of LKB1, AMPK is not active which renders cells more sensitive to energy stress and increased AMP/ATP resulting in increased apoptosis [107].

LKB1 may also play a role in chromatin remodeling and gene expression to coordinate cellular growth arrest. In the nucleus, LKB1 binds to Brahma-related gene 1 (Brg1) and stimulates its ATPase activity allowing for cell growth arrest [108]. LKB1 has also been shown to be critical for cellular polarity as deletion of its orthologs in *C. elegans* and *Drosophila* has severe defects during development [109, 110]. In mammalian cells, overexpression of LKB1 polarizes intestinal epithelial cells even in the absence of cell-cell contact that was previously thought to be required [111]. LKB1 is also thought to attenuate mammalian target of rapamycin (mTor)-dependent translation through AMPK-tuberous sclerosis 2 (TSC2) inhibition [112]. Furthermore, mTor is critical for beta cell survival and responds to nutrient and glucose stimulation [113]. Taken together, LKB1 is thought to mediate crucial cellular functions in order to convey appropriate metabolic and cell growth cues. **From the known cellular functions of LKB1, in particular growth suppression and energy sensing, I hypothesis that LKB1 inhibits beta cell growth and insulin secretion (subject of Chapter 4).**

As LKB1 is ubiquitously expressed, in order to look at LKB1 function specifically in beta cells, I will use the Cre/LoxP recombinase system with the pancreas specific Pdx1 promoter. The other consideration is that LKB1 is required for normal pancreas development (Table 1) and as such the Cre recombination must occur after pancreas development. To accomplish this time-controlled deletion I will use a modified Cre that takes advantage of estrogen receptor signalling. This Cre (Cre-ER^{Tam}) is fused to a fragment of the estrogen receptor which retains Cre in the cytoplasm unless the drug tamoxifen is present to bind, activate and translocate the receptor fused Cre into the nucleus. Once in the nucleus Cre can recombine LoxP sites inserted into a gene of interest, thus to investigate the role of LKB1 in beta cell biology, I will mate Pdx1-Cre-ERT2 mice with mice containing homozygous LKB1 alleles flanked with LoxP sites [114]. The resulting mice are LKB1 adult beta cell knockout (LABKO) mice and their control littermates which have LoxP-flanked LKB1 alleles but are Cre negative and are called L/L. Using the same system for investigating the role of SIK2 in beta cell function we will generate SIK2 adult beta cell knockout (SABKO) mice and their control littermates S/S.

LKB1-AMPK activation.

AMPK is the most well studied downstream kinase of LKB1 and was the first substrate described for LKB1. AMPK is a heterotrimeric complex comprised of catalytic alpha and regulatory beta and gamma subunits. In general, binding AMP or ADP at two sites on the gamma subunit leads to a conformational change that allows for LKB1-phosphorylation of threonine172 and stabilization of AMPK in the active state [115, 116].

Once active, AMPK has a vast number of cellular targets which promote catabolic processes such as glucose uptake, glycolysis, fatty acid oxidation, and mitochondrial biogenesis and inhibition of anabolic processes such as protein, sterol and fatty acid synthesis [117].

Glucose inactivates AMPK by increasing ATP/ADP, antagonizing binding of AMP and promoting dephosphorylation of threonine172 [118]. Many studies have shown the requirement of LKB1 for activation of AMPK; however, in LKB1 null contexts AMPK activity and phosphorylation of threonine172 has been observed. New evidence reveals that Ca^{2+} /Calmodulin-dependent Kinase Kinase 2 (CAMKK2) or Transforming growth factor beta-Activated Kinase (TAK1) can also phosphorylate and activate AMPK in HeLa, neuronal and yeast cells [119-121]. Interestingly LKB1 deficiency in the heart lead to a loss in AMPK α 2 activity only, whereas in skeletal muscle there was a loss of activation of both α isoforms [100, 103]. Evidence exists for LKB1-independent activation of AMPK although the importance of these alternate upstream regulators of AMPK in known AMPK biological functions has yet to be demonstrated.

AMPK biological functions.

Studying AMPK loss of function has been difficult due to the large number of AMPK subunit isoforms (α 1, α 2, β 1, β 2, β 3, γ 1, γ 2, γ 3), the potential for compensation and differences in isoform expression across different tissues. Thus careful consideration must be taken when drawing conclusions from the various

knockout models that have been produced. Like LKB1, double knockout of both alpha1 and alpha2 AMPK subunits is embryonic lethal [122].

Whole body knockout of AMPK alpha2 and not alpha1 abolishes the increase in glucose-uptake of skeletal muscle following treatment with 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) [123]. However, in response to muscle contraction, AMPK alpha1 or alpha2 was able to compensate for loss of the other with no effect on glucose uptake [123]. These data reveal the potential for overlapping and distinct functions between different AMPK isoforms. Similarly AMPK gamma3 isoform is more abundant in white skeletal muscle and knockout of this subunit leads to defects in glycogen synthesis following exercise [124]. Furthermore knockout of AMPK alpha2 in the liver leads to enhanced glucose production and hyperglycemia, effects that may have been mediated through increased CRTC-CREB activity although that was not measured in this study [125]. Loss of hypothalamic AMPK alpha2 in a specific subset of glucose-responsive neurons results in obesity, reduced energy expenditure and a loss of neuronal excitability in response to glucose [126].

Taken together from loss of function studies it is clear that AMPK is an important regulator of maintaining energy homeostasis in response to glucose. Even still there is a large degree of controversy over the role that AMPK plays in beta cell biology stemming largely from differences in experimental approaches. **From a review of the current literature and from findings in my own studies I will present support for AMPK having an important role in regulating beta cell function (please see Chapter 5).**

In summary, the goals of my thesis are to further investigate i) the beta cell specific regulation of CRTC-CREB pathway (Chapter 2 and 3), ii) the role of serine/threonine protein kinase LKB1 (Chapter 4) and iii) downstream kinases AMPK and SIK2 in beta cell biology (Chapter 5 and Appendix 1). I believe that a greater understanding of these processes will help to develop future therapeutic strategies for the treatment of diabetes.

Chapter 2. Glucose controls CREB activity in islet cells via regulated phosphorylation of TORC2 (now called CRTC2).

Article published:

Jansson, D., Ng, A. C-H, **Fu A**, Depatie, C., Al Azzabi, M, and Screaton, R.A. Glucose Controls CREB Activity in Islet Cells via Regulated Phosphorylation of TORC2. **PNAS** 105: 10161-10166. 2008.

Summary:

CREB was found to be activated by binding nuclear CRTC2 and first requires dephosphorylation at serine 171 and nuclear entry of CRTC2. In this study we identified a novel phospho-regulatory site serine 275 of CRTC2. Importantly, this novel regulatory site is regulated by glucose in beta cells and represents a molecular pathway that conveys an important beta cell functional stimulus to beta cell survival via CREB activation.

Contributions:

As co-author I contributed to this study by performing the experiments described below, interpreting results and editing of the manuscript.

I assisted Mufida Al Azzabi in conducting a kinome-wide overexpression kinase identification screen in 293Ts. I constructed and purified a GST-tagged peptide representing amino acids 268-283 of CRTC2 containing the newly identified phosphorylation site S275. We performed in vitro kinase assays on an overexpression library containing 280 of the known kinases in the human genome (Figure 4a). From this screen we identified Mark2 and Mark3 as kinases able to phosphorylate S275 of CRTC2. To confirm this finding and

investigate whether other AMP kinases might also phosphorylate Crct2 S275, I performed a series of secondary in vitro kinase assay screens on the entire AMP kinase family (Figure 4b and data not shown). Firstly, I observed that most of the AMP kinases can phosphorylate Crct2 S275 in vitro, secondly, I observed that Mark2 and Mark3 could phosphorylate Crct2 S275 to a greater extent than all the other AMP kinases (Appendix 2). This preferential phosphorylation by Mark2 suggested that it might have been the endogenous kinase regulating CRT2 at S275. In another study on CRT2 phosphorylation, I found that the mitogen-activated dual leucine zipper kinase (DLK) could also phosphorylate Ser275 in vitro revealing the possibility for multiple signal inputs into CRT2-CREB activity (Regulation of the CREB coactivator TORC by dual leucine zipper kinase at different levels. Phu do T, Wallbach M, Depatie C, **Fu A**, Screatton RA, Oetjen E. Cell Signal. 2011 [127]).

Title: Glucose Controls CREB Activity in Islet Cells via Regulated Phosphorylation of TORC2

Authors: Deidre Jansson¹, Andy Cheuk-Him Ng¹, Accalia Fu¹, Chantal Depatie¹, Mufida Al Azzabi¹, Robert Screatton^{1*}

Abstract: CREB is a cyclic AMP- and calcium-responsive transcriptional activator that is required for islet beta cell proliferation and survival. Glucose and incretin hormones elicit beta cell insulin secretion and also promote synergistic CREB activity by inducing the nuclear relocalization of TORC2 (also known as Crct2), a coactivator for CREB. In islet cells under basal conditions when CREB activity is low, TORC2 is phosphorylated and sequestered in the cytoplasm by 14-3-3 proteins. In response to feeding stimuli, TORC2 is dephosphorylated, enters the nucleus, and binds to CREB located at target gene promoters.

The dephosphorylation of TORC2 at Ser171 in response to cAMP is insufficient to account for the dynamics of TORC2 localization and CREB activity in islet cells. Here we identify Ser275 of TORC2 as a 14-3-3 binding site that is phosphorylated under low glucose conditions and which becomes dephosphorylated by calcineurin in response to glucose influx. Dephosphorylation of Ser275 is essential for both glucose and cAMP-mediated activation of CREB in beta cells and islets. Using a cell-based screen of 180 human protein kinases we identified MARK2, a member of the AMPK family of Ser/Thr kinases, as a Ser275 kinase which blocks TORC2:CREB activity. Taken together, these data provide the mechanistic underpinning for how cAMP and glucose cooperatively promote a transcriptional program critical for islet cell survival, and identifies MARK2 as a potential target for diabetes treatment.

Introduction

Loss of pancreatic beta cell function is the central feature of all forms of diabetes mellitus [128]. Glucose is a critical stimulus for beta cell proliferation, and the capacity of the islet cell mass to expand (islet hyperplasia) in response to chronically elevated blood glucose, together with functional adaptive responses, permits secretion of sufficient insulin to meet demand in prediabetic patients [129]. Failure of beta cells to proliferate and increase insulin output to meet increasing demand leads to type 2 diabetes. While growth factor signaling pathways have been implicated in the process of beta cell expansion, the molecular details of how glucose promotes islet cell proliferation are still unclear [130, 131].

Insulin secretion is triggered by numerous metabolites, principal among which are glucose and incretin hormones [132]. Beta cell glucose oxidation and consequent ATP production results in membrane depolarization, influx of extracellular calcium, and insulin release [133]. Incretins promote the insulin secretory response primarily through activation of cAMP-dependent protein kinase A (PKA), and also promote cellular proliferation, increased beta cell mass, and resistance to apoptosis [134-141]. In addition to insulin secretion, calcium and cAMP signaling events also synergistically elicit through cyclic AMP response element binding protein (CREB) a transcriptional program that is thought to restore the beta cell to a metabolically fit state in preparation for the next round of feeding [142]. CREB (and the related proteins CREM and ATF1) is the critical transcriptional activator that mediates cellular gene regulation in response to cAMP. CREB-dependent gene regulation is critical for governing islet cell proliferation and survival in vivo, as promoting CREB activity in islets via stabilization of the CREB:CBP complex increases beta cell mass, and, conversely,

disrupting CREB function in insulin-producing cells promotes apoptosis, glucose intolerance and eventual diabetes in the mouse [50, 70, 143].

A distinct set of CREB coactivators, Transducers of Regulated CREB Activity (TORCs), are also activated by PKA, yet by a distinct mechanism whereby cAMP-PKA signaling promotes TORC relocation from the cytoplasm to the nucleus where they bind to the DNA-binding/dimerization domain of CREB [65, 66]. An increasing body of evidence indicates that TORCs play a central role in signal integration to activate CREB in response to glucose and hormonal cues [53, 73, 144]. TORC2 is negatively regulated by phosphorylation at Ser171, which is a substrate for the salt-inducible kinases (SIKs) and AMP-activated protein kinase (AMPK) of the AMPK family [53, 144]. Phospho-Ser171 forms docking site for 14-3-3 proteins, phosphorylation-dependent allosteric ‘chaperones’ [145, 146] that, upon binding, mask an adjacent nuclear localization signal and thereby promote cytoplasmic accumulation of TORC2 [53]. Upon receipt of extracellular cues that increase intracellular cAMP, and in some contexts calcium, TORC2 is released from 14-3-3s and relocates to the nucleus [53, 144, 147]. While mutation of Ser171 to Ala is sufficient to reduce 14-3-3-mediated cytoplasmic retention of TORC2, lowering the threshold for TORC2 nuclear entry and for TORC-dependent CREB target gene activation, dephosphorylation of Ser171 alone in response to cAMP signals does not fully activate TORC2 in all cell types. In this study, we identify the remaining sites on TORC2 that mediate binding to 14-3-3s, identify MARK2 as a TORC2 kinase, and delineate how glucose and cAMP signals converge on two of these sites to control TORC2 activity in islet cells.

Results

Identification of a Second Regulatory Phosphorylation Site on TORC2 in Islet Cells.

CREB activity in islet cells is synergistically enhanced by costimulation with glucose and cAMP [53]. In agreement with these data, we observed that treatment of glucose starved MIN6 insulinoma cells with glucose and the incretin hormone exendin-4 (EX4, a cAMP agonist) provides an 11-fold and 7-fold increase in mRNA for the CREB target genes NR4A2 and IRS2, respectively, as compared with 4-fold for EX4 alone (Fig. 1A). Given that TORC2 nuclear relocalization underlies this synergy, and a phosphorylation defective Ser171Ala mutant of TORC2 isolated from HIT-T15 insulinoma cells retains 14-3-3 binding capacity Fig. 1B and [53], we sought to identify additional regulatory phosphorylation sites on TORC2 that bind to 14-3-3s. To identify these site(s), we used a far western approach using GST-tagged 14-3-3 protein to screen a series of N- and C-terminal deletion mutants of TORC2 purified from HIT-T15 cells for their capacity to bind directly to 14-3-3s. Whereas TORC2 wt and a C-terminal deletion (aa 1-389) of TORC2 both bound to 14-3-3, gross deletion of the N-terminus (deletion mutant aa 389-692) abrogated 14-3-3 binding (Fig. 1B, lane 4). Further truncation of TORC2 to aa 1-321 did not further reduce 14-3-3 binding. This residual binding required phosphorylation as treatment

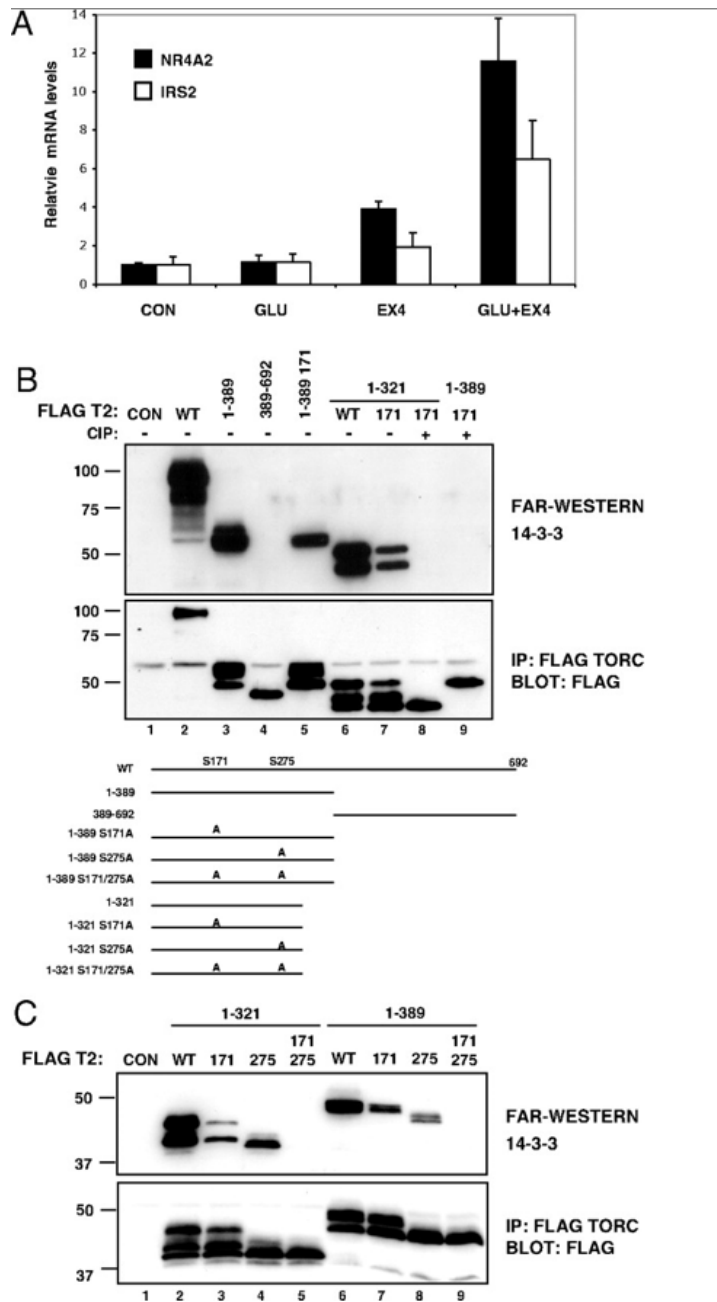


Figure 1. Serine 275 of TORC2 is a 14-3-3 binding site. (A) Treatment of glucose starved (in KRB buffer for 1h) MIN6 cells with 20 mM glucose (GLU) and 10 nM exendin-4 (EX4) elicits a synergistic increase in mRNA levels of CREB target genes NR4A2 and IRS2. (B) Top: Far western analysis with FLAG-TORC2 wt (1-692) and deletion constructs. Amino acid endpoint of the constructs indicated. TORC2 polypeptides isolated from HIT-T15 cells

were subjected to far western overlays with GST-14-3-3. 14-3-3:TORC2 complexes were detected with anti-GST antibody. 171 = Ser171Ala mutant. Treatment of the FLAG-TORC2 IPs with phosphatase is indicated (CIP). Bottom: schematic of truncation and phosphorylation mutants used in (B) and (C). (C) 14-3-3 Far western analysis with truncated FLAG-TORC2 polypeptides 1-321 and 1-389 containing point mutations at Sers 171 and 275 isolated from HIT-T15 cells. CON = vector control.

of the TORC2 immunoprecipitate with phosphatase (CIP) prior to SDS-PAGE abolished 14-3-3 binding (Fig. 1B, lanes 8 and 9). Importantly, whereas CIP treatment abolished 14-3-3:TORC2 complex formation, mutation on Ser171 to Ala reduced, but did not prevent, 14-3-3 interaction with the N-terminus of TORC2 (Fig. 1B, lane 7). Thus we conclude that an additional TORC2 phosphorylation site for 14-3-3 interaction lies within aa 1-321.

As Ser171 is highly conserved from human to zebrafish in TORCs1-3 [53, 65, 66], we assumed that the additional regulatory site that mediates 14-3-3 binding would also be well conserved. As TORC2 is exclusively phosphorylated on serine residues in HIT-T15 cells [53], we selected five additional serine residues in TORC2 (Ser70, Ser127, Ser238, Ser245, Ser275) to evaluate as possible 14-3-3 interaction sites (Fig. 1C), as they fit the following criteria: 1. reside in amino acids 1-321, 2. conform to a consensus or near-consensus 14-3-3 binding site (Mode 1 (R/KXXpSXP) and Mode 2 (R/KXXXpSXP), where pS is the phosphorylated serine and X is any amino acid [145]), and 3. highly conserved between human and mouse TORCs 1 and 2. Given that TORC2:14-3-3 binding requires TORC2 to be phosphorylated, we expected to see that loss of critical phosphorylation sites would lead to a

corresponding loss of 14-3-3 binding in the far-western screen. To test this, these five Ser residues were mutated to Ala in the 1-321 and 1-389 deletion constructs, and these new mutants were screened for 14-3-3 binding by far western analysis. Whereas mutation of either Ser 171 or Ser275 significantly reduced 14-3-3 binding (Fig. 1D, lanes 3, 4, 7, and 8) mutation of these residues together abolished binding in the context of both 1-321 and 1-389 (Fig. 1D, lanes 5 and 9). Individual substitution of Ser127, Ser238, and Ser245 for Ala did not appreciably reduce 14-3-3 binding (data not shown). In the context of the full-length TORC2 polypeptide, the mutation of Ser171 and Ser275 together were not sufficient to prevent 14-3-3 binding, indicating there was at least one more phosphorylation site on TORC2 that could serve to recruit 14-3-3s (Supp. Fig. 2). As we previously identified Ser369 in a tryptic peptide derived from TORC2 [53], we tested a triple Ser/Ala mutant of TORC2 in which Sers 171, 275 and 369 were all mutated to Ala in the far western assay it was unable to bind 14-3-3s. We conclude that 14-3-3 proteins interact with TORC2 at Sers 171, 275, and 369.

Ser171 and Ser275 control TORC2 nuclear localization and activity in islet cells. If the idea is correct that 14-3-3:TORC complex formation prevents TORC nuclear entry, then a TORC2 mutant that cannot bind 14-3-3s should relocate to the nucleus of insulinoma cells in the absence of cAMP and calcium stimuli. To test if such a correlation exists, we first determined the subcellular localization of full-length FLAG-TORC2 mutants harboring Ala mutations at Sers 171, 275 and 369 alone or in combination in unstimulated HIT-T15 cells. FLAG-TORC2 positive cells were scored as cytoplasmic (C), nuclear (N) or cytoplasmic + nuclear (C+N) (Fig. 2A). Whereas TORC2 wt and all single Ser/Ala point mutants remained

in the cytoplasm, we observed a pronounced nuclear relocalization of the Ser171/275Ala mutant (> 80% nuclear localization). Additional Ala substitutions at Ser127, Ser238, Ser245 or Ser369 in the FLAG-TORC2 Ser171/275Ala background did not significantly increase its nuclear localization (not shown). We conclude that the interaction of 14-3-3s with TORC2 via Ser171 and Ser275 regulate TORC2 subcellular distribution, and that the 14-3-3:Ser369 complex governs an unknown function of TORC2.

As calcium-dependent signaling events are initiated as a result of cell depolarization triggered by glucose influx [148], we tested whether glucose could promote nuclear entry of endogenous TORC2. In the absence of glucose, endogenous TORC2 was localized to the cytoplasm of HIT-T15 cells (CON, Fig. 2B). Consistent with results using TORC2 constructs, only upon stimulation with glucose and cAMP agonist did TORC2 relocalize to the nucleus, the same conditions that elicit a significant increase in CREB target gene levels in cells and in isolated islets (Figs. 1A, 2C). These data indicate that glucose cooperates with cAMP to induce TORC2 nuclear entry and activate CREB target genes. We predicted that a constitutively nuclear TORC2 should promote signal-independent CREB activity. To address this, we measured

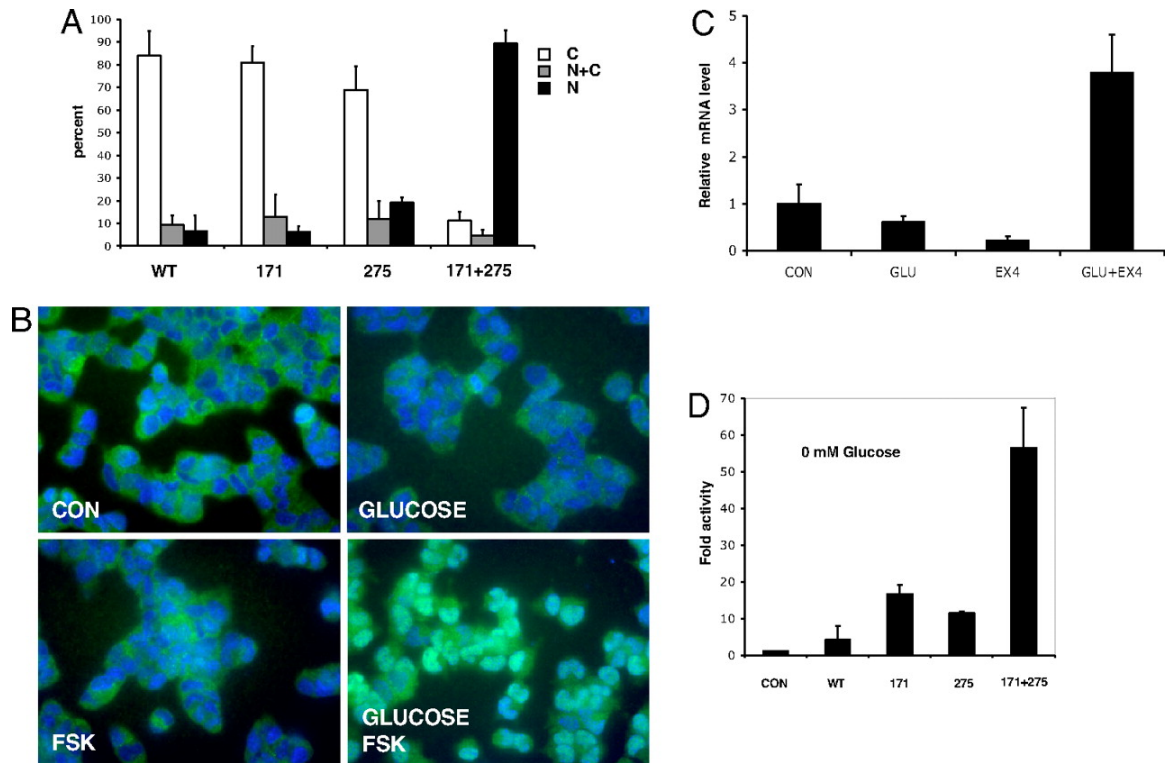


Figure 2. Ser171 and Ser275 control subcellular localization and activity of TORC2 in islet cells. (A, top) Histogram showing quantitation of subcellular localization of FLAG-TORC2 phosphorylation site mutants in HIT-T15 cells. Percentage of FLAG-TORC2-expressing cells with TORC2 in cytoplasmic (C), nuclear (N) or both (N+C) compartments indicated. (B) Immunofluorescence analysis of endogenous TORC2 (green) in HIT cells. Cells were starved for glucose for 1 hr and stimulated with 20 mM glucose, 10 μM forskolin (cAMP) or both for 60 min. Nuclei are revealed with DAPI (blue). (C) Quantitative PCR analysis of CREB target gene NR4A2 in isolated mouse islets. Treatment of glucose starved (CON) islets with 20 mM glucose (GLU), 10 nM EX4 indicated. (D) CREB reporter assay in HIT-T15 cells showing CREB activity induced by TORC2 phosphorylation site mutants cultured in the absence of glucose, shown $\pm$ standard deviation of two independent experiments performed in triplicate.

CREB activity induced by the TORC2 phosphorylation mutants in glucose starved HIT-T15 cells, conditions used for insulin secretion studies. In glucose starved (0 mM) conditions, we observed a greater than 50 fold increase in CREB reporter activity in HIT-T15 cells when Ser 171 and Ser275 were mutated together, compared to TORC2 wt (4 fold) or Ser 171 (16-fold) or Ser275 alone (11 fold) (Fig. 2D). Western blot analysis indicated that all mutant proteins accumulated to equal levels as FLAG-TORC2 wt (Supp. Fig. 1). Titration of the plasmids encoding TORC2 wt, Ser171Ala, or Ser171/275Ala double mutant confirmed these results (Fig. 2E). Taken together, these data indicate that glucose and cAMP promote TORC2 nuclear relocalization and that constitutively nuclear TORC2 can promote CREB activity in the absence of stimuli.

Glucose promotes dephosphorylation of TORC2 at Ser275 in islet cells.

As TORC2 nuclear entry is triggered by dephosphorylation of TORC2 and glucose is required for TORC2 nuclear relocalization, we pursued the possibility that glucose treatment would promote TORC2 dephosphorylation. Western blot analysis of endogenous TORC2 in the glucose-responsive cell line MIN6 demonstrated that glucose starvation for 1 hr led to rapid TORC2 phosphorylation (as measured by inhibition of mobility on SDS-PAGE) that could be reversed by 30 min stimulation with 20 mM glucose (Fig. 3A). Given the magnitude of the mobility shift that we observed here and in the FLAG-TORC2 mutants carrying a Ser275Ala mutation (Figs 1D, Fig. 1), as well as the involvement of Ser275 in TORC2 subcellular localization, we predicted that the level

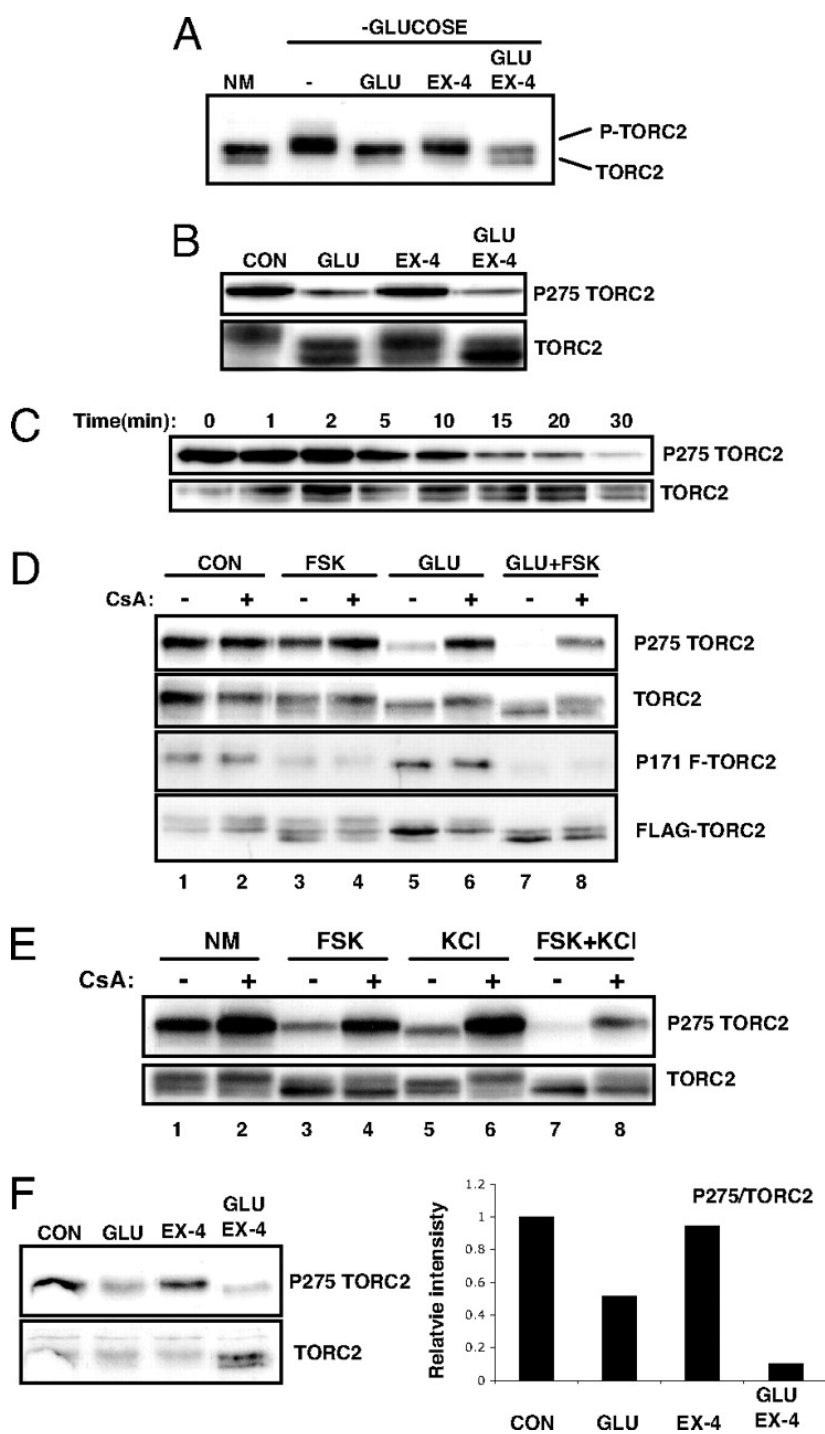


Figure 3. TORC2 Ser275 phosphorylation is regulated by glucose and calcineurin. (A)

Western blot analysis of endogenous TORC2 protein in MIN6 cells cultured in normal medium (NM) containing 20 mM glucose, or in glucose starved conditions for 1 hr (-GLUCOSE). 30 min treatment with 20 mM glucose (GLU), 10 uM exendin-4 (EX-4), or

both (GLU+EX-4) is indicated. (B) Western blot analysis of P-Ser275 levels in MIN6 cells. Total TORC2 protein blot shown as control. (C) Western blot of P275 levels on TORC2 protein in glucose starved MIN6 cells treated with 20 mM glucose for the indicated times. (D) Western blot of HIT-T15 cell extracts from glucose starved cells (CON, 1 hr) with P-Ser275 antibody. Treatments with glucose (GLU) and cAMP agonist (FSK) shown. Pretreatment with cyclosporin A (CsA, 1 uM) indicated by + signs. Blot with TORC2 antibody shown as control. Extracts from FLAG-TORC2 expressing HIT-T15 cells treated as shown were blotted for P-Ser171 and for FLAG as control. (E) Western blot analysis of P-Ser275 levels in HIT-T15 cells cultured in normal glucose-containing medium (NM). Treatment with cAMP agonist and depolarizing stimulus indicated. Pretreatment with cyclosporin A (CsA, 1 uM) indicated by + signs. Blot reprobed with non-discriminating TORC2 antibody shown as control. (F) Western blot of islet extracts with P-Ser275 antibody and TORC2 antibody. Glucose starved (CON) and islets treated with 20 mM glucose (GLU) and 10 nM exendin-4 (EX) is shown. Densitometric analysis of the ratio of P275:total TORC2 signal following treatment of glucose starved islets is shown at right. Signal in the glucose-starved lane (CON) is set to 1.

of phosphoSer275 (P-Ser275) would decrease upon glucose stimulation. To address this, we generated an antibody that recognizes P-Ser275 on TORC2 and performed western blot assays on protein extracts from glucose starved MIN6 and HIT-T16 cells. The level of phosphorylation at Ser275 decreased dramatically with 30 min treatment of MIN6 cells (Fig. 3B) and HIT-T15 cells (Fig. 3C) with 20 mM glucose, with a 50% reduction in P-Ser275 levels observed after 5-10 min of treatment of HIT-T15 cells. In contrast, P-Ser275 levels were unaffected by treatment with cAMP agonist, which specifically reduced the level

of P-Ser171. These data confirm that glucose alone can regulate P-Ser275 levels. Furthermore, addition of CsA to HIT cultures prior to glucose stimulation completely blocked Ser275 dephosphorylation (compare lanes 5 and 6, Fig. 3D), but did not block dephosphorylation of Ser171 (compare lane 3 and 4, Fig. 3D). Interestingly, we observed that the presence of glucose in the culture medium had a priming effect on cAMP-mediated dephosphorylation of Ser275. In the absence of glucose, the effects of cAMP agonists (EX-4 or FSK) on global TORC2 dephosphorylation (Supp. Fig. 3, compare lanes 6 and 10, and lanes 7 and 11) and on reducing P-Ser275 levels specifically (Figs. 3B-3D) were negligible, yet these were both enhanced in the presence of glucose. To confirm these effects in primary islet cells, we treated purified, glucose-starved islets with 20 mM glucose with or without 10 nM EX-4, and observed that TORC2 Ser275 phosphorylation decreases after 1 hr glucose treatment, an effect that is enhanced by EX-4 (Fig. 3F). Taken together, these data indicate that in beta cells, in contrast to the phosphorylation status of Ser171 (which responds primarily to cAMP signals [53, 144], that of Ser275 is regulated by glucose and is mediated by calcineurin. Moreover, they demonstrate that dephosphorylation of Ser275 is a critical event required for cAMP-mediated TORC2 activation in islet cells.

TORC2 Ser275 is an AMPK family substrate. In an effort to identify the cellular machinery that governs Ser275 phosphorylation, we sought to identify protein kinases that phosphorylate this residue. To this end, we generated a cDNA expression library of 180 GST-tagged human protein kinases (for complete list, see Supp. Fig. 4) to permit expression and isolation of the kinases from mammalian cells, for their use in parallel arrayed in vitro kinase assays. This approach allows for post-translation modifications of the kinases

required for their activity and provides an opportunity to study human protein kinase:substrate relationships using extracellular triggers (growth factors, hormones, drugs, etc.) in their proper context. The screen permits simultaneous interrogation of kinase activities for their ability to phosphorylate a recombinant substrate harboring the phosphorylation site in question, and is outlined in Figure 4A. To identify a specific Ser275 kinase, we provided a peptide sequence (268-PPMNTGGSLPDLTNL-283) as substrate for the kinase assay, and the results from the screen are presented in Supp. Fig. 5. Of the 180 library kinases, only MAP/microtubule affinity-regulating kinases (MARKs) 2 and 3 (also known as Par1b/EMK1 and Par1a/C-TAK1, respectively [149]), members of the AMPK family, phosphorylated the Ser275-containing peptide. To confirm these results, we performed a secondary screen using GST-MARKs 2 and 3 using the TORC2 268-283 peptide (Fig. 4B) and confirmed that Ser275 is a MARK substrate. AMPK activity increases in cells under conditions of nutrient deprivation including in beta cells starved for glucose [150]. Given that MARKs and AMPK belong to the same protein kinase family, and that ambient glucose concentrations control P-Ser275 levels on TORC2, we next tested the possibility that AMPK represents the TORC2 Ser275 kinase. Consistent with a role for AMPK in phosphorylating TORC2 in beta cells, we detected an increase in AMPK P-Thr172 levels in glucose starved HIT-T15 cells (Supp. Fig 6). However, expression of a constitutively active AMPK polypeptide (AMPK-CA) was unable to block TORC2 induced CREB activity in a CREB reporter

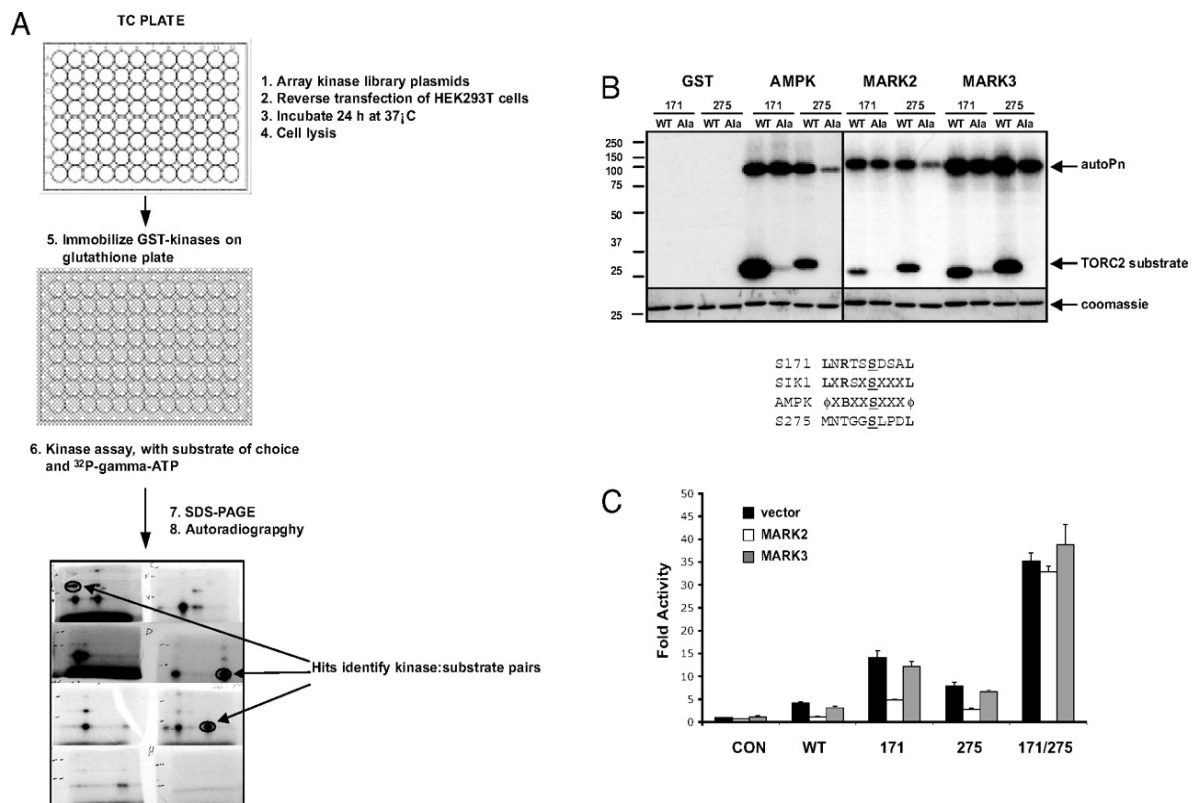


Figure 4. MARK2 is a TORC2 Ser275 kinase. (A) Flow chart of kinase library screening platform. Kinase assays performed in parallel with 180 human protein kinases is performed by reverse transfection of the arrayed library encoding GST-tagged human protein kinases, followed by cell lysis and isolation of active GST-kinases on glutathione coated plates. Following incubation with the desired substrate(s) and ^{32}P -gamma-ATP, phosphorylated substrates are resolved on SDS-PAGE and detected by autoradiography. Screen results are presented in Supplementary Information 5. (B) In vitro kinase assay with GST-tagged AMPK alpha 1, MARK2, and MARK3 purified from HEK293T extracts using TORC2 161-179 (171 WT) and TORC2 267-283 (275 WT) as substrates. Corresponding Ser to Ala mutant substrates (Ala) and kinase autophosphorylation are indicated. Coomassie staining of substrate is shown. Primary sequence context surrounding Ser171 and Ser275 in TORC2 and

alignment with consensus SIK1 [53] and AMPK [158] phosphorylation sites shown at bottom. (C) CREB reporter assay in HIT-T15 cells showing inhibitory effect MARK2 on TORC2:CREB activity. Transfection with vector control (CON), Ser171Ala (171), Ser275Ala (275), and the Ser171/275Ala mutant (171/275) are indicated. Assay was performed in the presence of glucose to maximize the potential inhibitory effects of the kinases.

assay in HIT-T15 cells (Supp. Fig 7). Consistent with this, treatment of MIN6 cells with the AMPK agonist AICAR had no effect on P-Ser275 levels, despite the observed increase in P-ACC levels, a known AMPK substrate (Supp. Fig 8). However, MARK2 but not MARK3 blocked TORC2-induced CREB activity, supporting a role for MARK2 as a specific TORC2 inhibitor in cells (Fig. 4C). The constitutively nuclear TORC2 Ser171/275Ala mutant was resistant to the inhibitory effects of MARK2. Taken together, our data indicate that MARK2 is a TORC2 Ser275 kinase, and that inhibition of TORC2:CREB activity by MARK2 involves phosphorylation of both Ser171 and Ser275.

Discussion

Induction of glucose-dependent insulin secretion by cAMP and calcium signals is the defining signaling event in islet beta cells. We show here that these signals also control CREB activity in islets by modulating the phosphorylation of status of two residues of TORC2: Ser171 (cAMP) and Ser275 (glucose). In particular, we show that Ser275 of TORC2 is a critical sensor of glucose/energy status in islet cells. We discuss here the roles of

these key signaling pathways in the regulation of TORC2 phosphorylation and function of beta cells.

Two signaling pathways: two phosphorylation sites. TORC2 nuclear entry is an essential event in signal-dependent activation of CREB target genes. Our data provide the mechanistic basis for the cooperativity of cAMP and glucose/calcium signaling for TORC nuclear relocalization that is required for CREB activity in beta cells. Ser171 of TORC2 is a critical site for negative regulation of TORC2 in beta cells and hepatocytes. In response to cAMP, PKA directly phosphorylates SIK2, leading to its dissociation from TORC2 and the dephosphorylation of TORC2 at Ser171. In the absence of cAMP, glucose/calcium signals play a minor role in Ser171 dephosphorylation [53]. By contrast, glucose governs the phosphorylation status of Ser275, which can be fine-tuned by cAMP signals. Ser275 (unlike Ser171) appears only to respond to cAMP in the presence of glucose, indicating that both the signals and protein complexes that regulate these two sites are different.

We developed the human protein kinase screening platform to permit unbiased identification of protein kinase:substrate pairs and facilitate the delineation of signal transduction pathways. Using this approach, we identified MARK2 as a Ser275 kinase. Given the key role of AMPK in responding to increased energy demand, we were surprised to find that neither Ser171 nor Ser275 is regulated by AMPK in beta cells, as evidenced by the relative inefficiency with which Ser275 is phosphorylated in vitro by AMPK, the absence of modulation of P-Ser275 levels with AICAR treatment, and lack of inhibition of TORC2:CREB activity in the presence of activated AMPK. Our data indicate that TORC2

represents the sole target of MARK2 required for CREB inhibition, and moreover that MARK2 inhibits TORC2 by phosphorylating both regulatory sites. MARK2 knockout animals display a metabolic phenotype [151] and learning and memory defects [152], areas in which CREB plays a central role [153]. Future work will determine the relative importance of MARK2 in regulating TORC2:CREB activity in beta cells.

We have used a biochemical screen to identify additional regulatory TORC2 phosphorylation sites. It is curious to note that constitutively nuclear TORC2 (Ser171/275Ala) is still able to bind to 14-3-3 proteins. This indicates that there is perhaps a function for TORC:14-3-3 complexes within the nucleus, perhaps in stabilizing the transcriptionally active form of TORC. However, we observed no functional consequence for TORC:CREB activity when Ser369 was mutated to Ala alone or in combination with Ser171/275Ala. Ser369 does not conform to a consensus 14-3-3 binding site, and the sequence context in which it is embedded bears no discernable homology with a site in TORC1 or TORC3.

TORC2 activity is regulated by glucose. We have shown that the phosphorylation status of Ser275 on TORC2 is modulated by extracellular glucose in islet cells. This indicates that the CREB pathway receives input from glucose via TORC2, providing a molecular link between glucose and a transcriptional program linked to beta cell proliferation and survival. The rapid kinetics of reduction in P-Ser275 levels in response to glucose are consistent with known responses to cellular energy status, such as the increase in AMPK phosphorylation at Thr172 [154]. The sequence context surrounding both Ser171 and Ser275 two sites is identical in all

three mammalian TORCs, zebrafish and fruitfly TORCs [65], indicating that both residues constitute critical regulatory sites. It will be of interest to examine the involvement of Ser275 in the control of TORC2:CREB activity in other tissues. We attribute the accumulation of phosphate at Ser275 in the presence of the immunosuppressant cyclosporin A to this site undergoing rounds of reversible phosphorylation catalyzed by calcineurin, even at steady state. CsA-induced hyperphosphorylation of Ser275 was only observed in cells cultured in high glucose, supporting a role for glucose signaling in phosphate turnover at this site. Chronic treatment with calcineurin inhibitor contributes to new onset diabetes in patients receiving immunosuppression therapy [155, 156]. As CsA treatment prevented Ser275 dephosphorylation, TORC2 nuclear entry, and CREB activation (effects that were obviated by constitutively nuclear TORC2), we expect that inhibitors of TORC2 Ser275 kinases such as MARK2 may be used as adjuvant therapies for these patients.

Materials and Methods

Chemicals: Forskolin, exendin-4 and anti-FLAG agarose were from Sigma. Cyclosporin A was from Calbiochem.

Constructs. Recombinant TORC constructs were prepared using standard PCR cloning techniques. Point mutations were generated with PfuTurbo and the QuickChange protocol (Stratagene). The presence of desired mutations was verified by sequencing.

Cell culture. HEK293T were cultured in DME +10% FCS (Hyclone). HIT-T15 cells were cultured in 1:1 DME+10%FCS:OPTIMEM (Invitrogen). MIN6 cells were cultured in HEK293T medium supplemented with 50 uM beta-ME. All media contained penicillin and streptomycin. For glucose starvation experiments, cells were incubated with Krebs-Ringer Buffer + 0.1%BSA lacking glucose (KRB) for 30 min, rinsed again with KRB and incubated a further 30 min (for a total of 1 hour) prior to stimulation with 20 mM glucose and/or 10 nM EX4 (final concentration). Identical results were obtained with cells cultured with 2.5 mM glucose prior to stimulation.

Antibodies and immunoblotting. TORC2 antibodies were generated by Covance custom antibody service (Covance, USA). Anti-FLAG-M2 was from Sigma. Antibodies were used at 1-2 ug/ml for western blotting and immunofluorescence as described [53]. 14-3-3 Far Western assays were performed as described [53].

Reporter assays. HEK293T and HIT-T15 cells were transfected overnight in 24 or 48 well plates using Lipofectamine 2000 (Invitrogen) and 150 ng (24 well) or 250 ng (48-well) total plasmid per well. 24h (HEK293T) and 40 h (HIT-T15 and MIN6) after transfection cells were treated with glucose and/or EX4 for 4-6 hours at 37°C prior to determination of luciferase and beta-galactosidase activity as described [53].

Immunofluorescence Microscopy: For endogenous TORC2 detection, HIT-T15 cells were seeded onto poly-L-lysine covered coverslips. For FLAG-TORC2 detection, this was done 16 hr post-transfection. Cells were stimulated for 30 min with forskolin (10 μ M), KCl (45 mM) prior to fixation with 4% PFA. In some cases, cells were starved with KRB as above prior to stimulation with glucose or cAMP agonist. Images were captured on a Nikon Axiovert inverted microscope with a 63X oil immersion lens with a CCD camera. For quantitation of subcellular localization, 200-300 cells per condition were scored for the presence of FLAG-TORC2 wt or phosphorylation mutant in the cytoplasm, nucleus, or in both compartments. TORC2 antibody for immunofluorescence has been described [157].

Kinase Library Screen. Full-length human protein kinase cDNA clones derived from the MGC/ORFeome (Open Biosystems, Invitrogen) were Gateway recombined with pDEST27 vector (Invitrogen) to generate in-frame GST-kinase ORFs. Integrity of the GST-proteins was verified by western blotting. Plasmids encoding GST-kinases were arrayed in 96-well plates and transfected into HEK293T cells with Lipofectamine 2000. 24 h later, GST-proteins in lysis buffer (25 mM Tris 7.5, 150 mM NaCl, 50 mM NaF, 5% glycerol, 5 mM beta glycerolphosphate, 1 mM EDTA, 1 mM NaVO₄, 1 mM DTT, 1 mM PMSF) were captured on glutathione-coated plates (Pierce). Wells were rinsed and equilibrated with kinase buffer (25 mM Tris 7.5, 5 mM beta glycerolphosphate, 1 mM NaVO₄) prior to addition of TORC2 substrate (1 μ g), MBP (1 μ g) as internal control, and 2 μ Ci gamma-32P-ATP. Reactions were carried out at 30°C for 30 min, stopped with 2X SDS sample buffer, and samples were boiled prior to separation on SDS-PAGE gels. Gels were dried and exposed to XAR film. Secondary screens were performed by transfecting 1 μ g plasmid encoding GST-AMPK alpha1, MARK2, and MARK3 into HEK293T cells seeded in 6-well

plates and isolating the GST-kinase on glutathione beads prior to kinase assay with indicated substrates.

Islet Isolation. Animal work was approved by the University of Ottawa Animal Care Committee. Pancreata from anaesthetized 6-12 week old male C57/Bl6 mice were isolated by perfusion of the pancreatic bile duct with 0.7 mg/ml Type XI collagenase (Sigma) in Hanks Buffered Salt Solution. Islets were purified from acinar tissue by two rounds of manual selection using a dissecting microscope, and cultured in RPMI medium containing 11 mM D-glucose, 10% FCS, 10 mM HEPES pH 7.4, and 100 units/ml penicillin, 100 ug/ml streptomycin (growth medium) on bacterial dishes for 24 hr prior to treatment.

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Author Contributions: D.J., A.N., A.F., C.D., M.A. and R.A.S. performed research, D.J., A.N., A.F., C.D., and R.A.S. analyzed data, R.A.S. wrote the paper.

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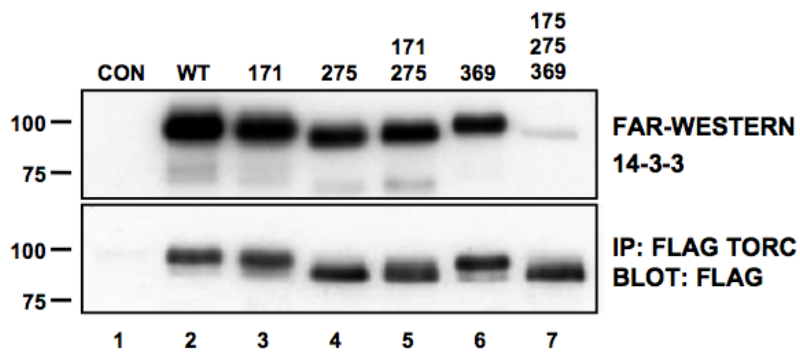
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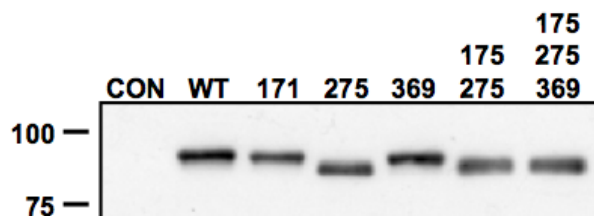
Mode 1 RXX-[pS/pT]XP
 Mode 2 RXXX[pS/pT]XP

		14-3-3 binding
S171	T1: WRTNSDSALH T2: LNRTSSDSALH T3: LNRTNSDSALH	+
S70	T1: QYYGGSLPNVN T2: SHYGGSLPNVN T3: QYHGGSLPNVS	-
S127	T1: GRQADSCPYGT T2: TRHIDSSPYSP T3: GRQFDGSAFGA	-
S238	T1: TKKTGSRPKSC T2: AKKLSSSSSRP T3: TKEIQSLSGRP	-
S245	T1: SSRPRSCVPG T2: SSRPRSCVPG T3: SGRPRSCDVGG	-
S275	T1: HNTGGSLPDLT T2: MNTGGSLPDLT T3: LNTGGSLPDLT	+

Supplemental Figure 1. Alignments of amino acid sequences from the three mammalian TORC proteins (T1, T2, T3) that flank candidate 14-3-3 binding sites. Consensus binding sites (Mode I, Mode II) for 14-3-3 cargo proteins are listed at the top. Plus signs indicate that the site mediates binding to 14-3-3s (i.e. a loss of binding for the Ser/Ala mutant at the indicated site was observed by far-Western analysis).



Supplemental Figure 2. 14-3-3 far-Western analysis with full-length FLAG-TORC2 harboring Ser/Ala substitutions at the indicated Ser residues.



Supplemental Figure 3. Western blot with anti-FLAG antibody showing protein levels of FLAG-TORC2 wt and phosphorylation site mutants. Vector only control (CON) and position of Ser/Ala mutants are indicated by numbers.

Chapter 3. Using Kinomics to Delineate Signaling Pathways: Control of CRTC2/TORC2 by the AMPK Family.

Article published:

Fu A, and Screatton, RA. Using Kinomics to Delineate Signaling Pathways: Control of CRTC2/TORC2 by the AMPK Family. Cell Cycle 7(24), 3823-8. 2008.

Summary:

Following our characterization of the new inhibitory phosphorylation site S275 on CRTC2 described in Chapter2, we were invited by Cell cycle to write a perspectives article. We had identified that Mark3 phosphorylated a S275 peptide of CRTC2 and went on to identify the entire AMP kinase family as potential kinases regulating Crc2 In beta cells. I found that all members of the AMP kinase family are able to phosphrylate CRTC2 in vitro but that Mark2 is likely main kinase responsible to phosphorylate CRTC2 *in vitro* which I present new data for in this review. In addition, I discuss the role of AMP kinases AMPK, Mark2 and Sik2 in the regulation of CRTC2 and the novel screening strategy developed in our lab to identify kinase:substrate pairs. I go on to describe other known and characterized functions of Mark2 and Sik2, and review current technologies able to delineate signaling pathways and described advantages to our mammalian cell kinase screening approach in comparison to yeast and bioinformatics approaches.

Contributions:

I wrote and conceived of topics for this review in equal contribution with my supervisor. I conducted a thorough literature review and wrote 2 of the 3 sections in the body

of this review. Dr. Screatton helped with organization of topics that I discussed in the review, wrote the introductory section and edited the manuscript for flow and clarity. In addition I provided further data to support the idea that Mark2 is a CRTTC2 kinase since it can form a complex with CRTTC2 and displays linear enzymatic activity on S275-peptide and not S171-peptide of CRTTC2 (Figure 3 and 6). I designed the model in figure 4 and performed the kinase assays in figure 6. Dr. Screatton designed the experimental model in Figure 1, performed the 96-well kinase assay in Figure 2 and the co-immunoprecipitation in Figure 3.

Title: Using Kinomics to Delineate Signaling Pathways: Control of CRTC2/TORC2 by the AMPK Family

Authors: Accalia Fu and Robert A. Screatton

Keywords: MARK2, beta cells, CREB, CRTC2, glucose, kinomics

Abbreviations: AMPK: AMP-activated protein kinase; **CAMKKbeta:**

calcium/calmodulin-dependent protein kinase kinase beta; **cAMP:** cyclic AMP; **CRE:** cyclic AMP response element; **CREB:** cAMP response element binding protein; **CsA:** cyclosporin A; **GLP1:** glucagon-like peptide-1; **IRS2:** Insulin-receptor substrate 2; **LKB1/STK11:** tumour suppressor gene encoding AMPK family master kinase; **MARK:** MAP/microtubule affinity-regulating kinase; **PKA:** cAMP-dependent protein kinase; **SIK:** salt-inducible kinase; **TORC2/CRTC2:** Transducer of Regulated CREB activity 2/CREB-Regulated Transcriptional Coactivator 2

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Abstract

The classical role of AMP-activated protein kinase (AMPK) as an energy status sensor is expanding to include other members of the AMPK family. Recent genetic and cell biological evidence points to a role for MAP/microtubule affinity-regulating kinase 2

(MARK2/EMK/Par1b) in the regulation of metabolic events¹ as well as in the control of CREB-dependent transcription activated by glucose in pancreatic islet beta cells². We have recently developed an in vitro kinase screening platform to identify novel kinase:substrate pairs, the building blocks of signal transduction pathways. Application of this technology led us to identify MARK2 as the kinase that targets a novel glucose-regulated phosphorylation site on Transducer of Regulated CREB Activity 2 (TORC2, referred to as CREB-Regulated Transcriptional Coactivator 2, or CRTC2), a transcriptional coactivator essential for CREB activity in beta cells². We discuss these recent developments and suggest a model whereby members of the AMPK family integrate numerous signals to coordinate energy metabolism and cellular polarity with gene expression to regulate cell function/proliferation.

Using Kinomics to Elucidate Signal Transduction Pathways. Assigning kinase:substrate pairs is critical to the establishment of a comprehensive map of how cells respond to environmental stimuli, and will allow us to establish the basic biochemical links that exist that form the basis of signal transduction pathways. Several approaches for identifying kinase:substrate pairs have been described, both in forward (kinase → substrate) and reverse (substrate → kinase) signaling³⁻⁵. One elegant method for substrate identification starting with a kinase of interest involves engineering a mutant form of the kinase to selectively ‘tag’ substrates with a modified ATP analog that is used to distinguish them from phosphoproteins targeted by endogenous kinases⁶. To identify kinases for a phosphorylated substrate of interest, three approaches have been taken: using bioinformatics to search for known kinase phosphorylation consensus sites, detecting complex formation with biochemical techniques, or using a trial-and-error approach to evaluate candidates based on their known biological

functions. We have developed an approach that permits rapid and systematic kinase:substrate pair identification, as well as dissection of signaling pathways using functional outcomes in cell based overexpression assays ⁷. The screen involves the interrogation of 180 human protein kinases, expressed as GST-fusion proteins in mammalian cells for purification, for their ability to phosphorylate a specific individual or pooled set of substrates.

There are two advantages to this ‘reverse signaling’ approach (Figure 1): First, the kinases are generated in and purified from mammalian cells. Human protein kinases can be purified in large quantities from bacteria, yet as bacteria lack serine, threonine, or tyrosine protein kinases, the purified enzymes lack modification required for activity. The yeast kinome is comprised of 122 protein kinases, reflecting neither the diversity of the mammalian kinome nor the complexity of regulatory processes that occur in multicellular organisms. Second, this approach permits experiments in which stimuli (such as chemotherapeutic drugs, growth factors, hormones, etc.) can be used to study the behaviour of the human kinome in the proper biological context. In order to exploit these advantages, we are currently expanding the GST-kinase library to achieve > 90% coverage of the kinome. As nearly 30% of all proteins are expected to be phosphoproteins ⁸, and many phosphorylation sites identified to date have no known functional consequence, we select substrates with sites known to be phosphorylated in cells that are of regulatory consequence, and lastly, are well-conserved. The screening platform provides for excellent substrate specificity (Figure 2) and can be applied to

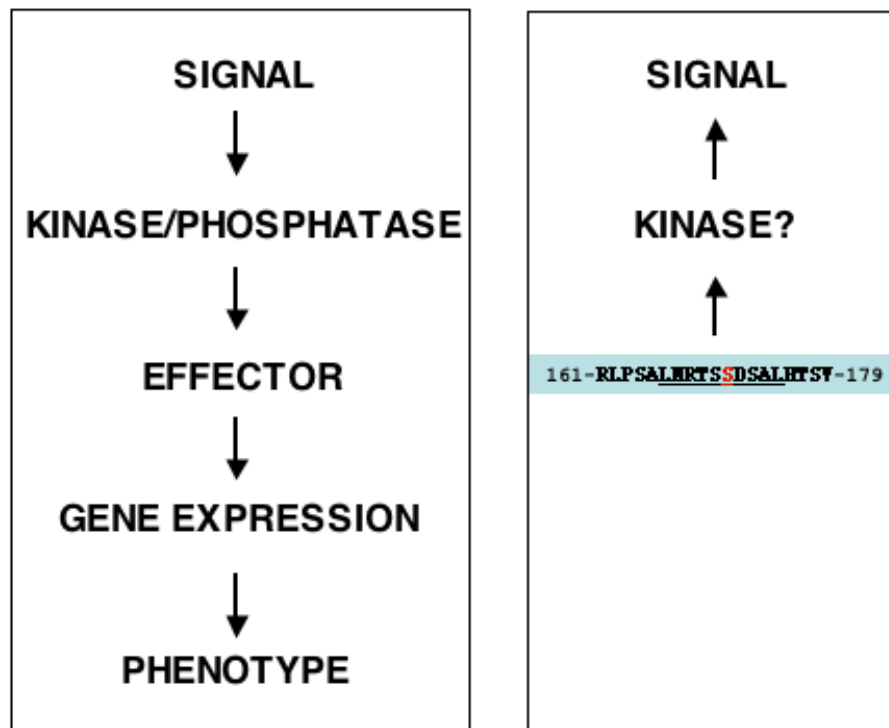


Figure 1. Reverse Signaling using In Vitro Kinomics. Interrogation of the human protein kinase library with protein substrates (peptides, domains, or intact proteins) permits identification of kinase substrate pairs, the building blocks of signal transduction pathways.

identify kinases that regulate target proteins involved in numerous medically relevant signaling pathways, among them mitochondrial vesicle dynamics, neuronal axon outgrowth, stem cell fate, cell survival, migration/phagocytosis, and cell polarity. We recently identified a novel glucose-regulated phosphorylation site, Ser 275 in mouse CRT2 and used a 16 amino acid peptide substrate corresponding to the sequence

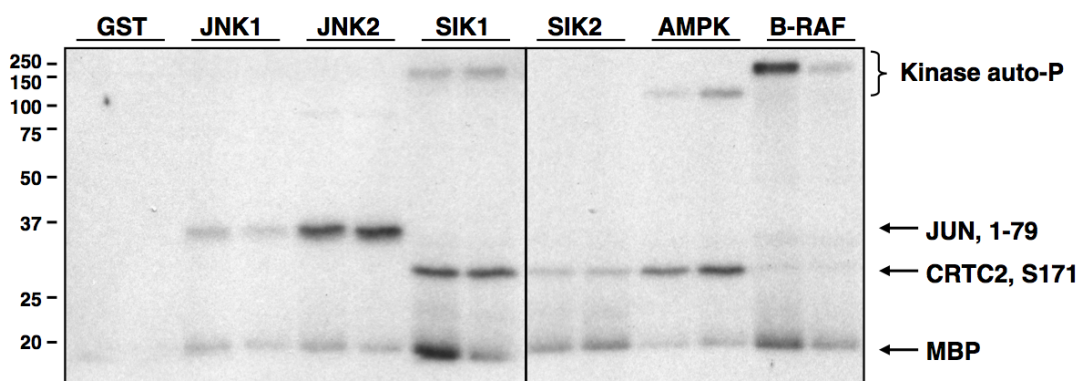


Figure 2. Substrate specificity in a 96 well kinase assay. A small scale screen was performed in duplicate using known kinase:substrate pairs as described in Jansson et al., 2008. Substrates are indicated at right: GST-JUN 1-79, CRTC2 GST-161-179 and MBP, myelin basic protein. The high degree of substrate selection by related kinases is a feature of the screening platform.

flanking Ser275 to screen the library and identify MARK2 and MARK3, members of the AMPK family, as candidate regulatory kinases ². We confirmed that MARK2, but not MARK3, could block CRTC2-induced CREB activity in cells, likely owing to the ability of MARK2, but not MARK3 to form a complex with CRTC2 in cells (Figure 3).

CRTCs: Signal-Dependent Coactivators. Originally identified as potent activators of CRE-dependent transcription by functional cell-based screen ^{9, 10}, CRTCs are now recognized as critical signal-dependent coactivators for CREB. Under basal conditions, CRTCs 1 and 2 are localized to the cytoplasm ^{11, 12}. In the absence of stimulus CRTC2 complexes with AMPK family members SIK2 and MARK2, as well as the calcium-

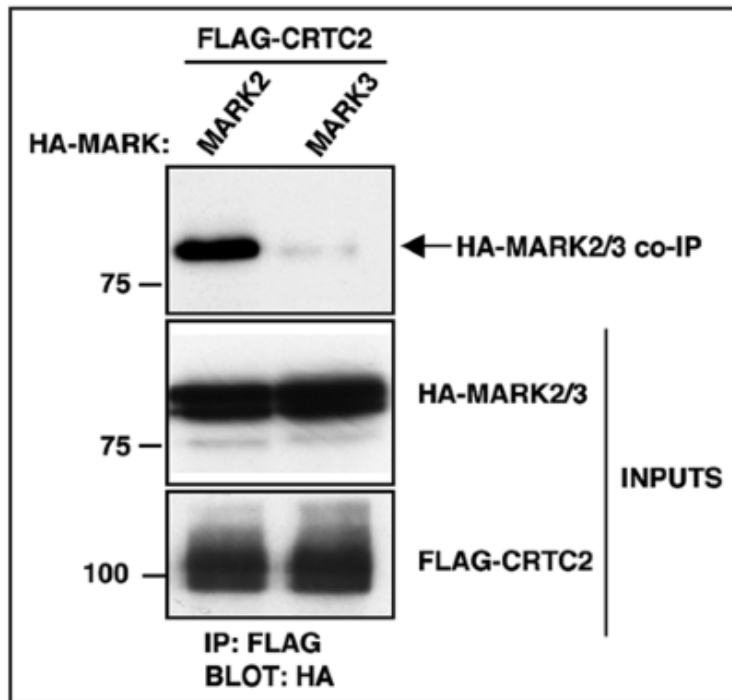


Figure 3. MARK2 and CRTC2 form a complex in cells. FLAG-CRTC2 was immunoprecipitated from extracts of HEK293T cells in the presence of HA-MARK2 or HA-MARK3. This association likely underlies the specificity of the inhibitory effect on CRTC2:CREB exerted by MARK2 and not MARK3.

activated Ser/Thr phosphatase calcineurin ^{2, 11}. Phosphorylated CRTC2 is subject to cytoplasmic retention mediated by 14-3-3 proteins, that prevent CRTC2 nuclear entry and its access to CREB ¹¹. In response to elevated cAMP concentrations, PKA phosphorylates SIK2, leading to dissociation of SIK2 from the CRTC2 complex, CRTC2 dephosphorylation, dissociation from 14-3-3s, and nuclear entry ¹¹. SIK2 was first identified as in diabetic mice where its activity and expression was increased in white adipose tissue ¹³,

and is activated by Akt2 in response to insulin ¹⁴. Elevated SIK2 activity is also associated with insulin resistance in db/db mice ¹⁵. In fibroblasts and hepatocytes, the critical inhibitory CRTC2 phosphorylation site is Ser171 ^{11, 16}. In beta cells, we recently found that Ser275 is also and is bound by 14-3-3s in resting cells; mutation of Ser171 and Ser275 together to Ala generates a constitutively active CRTC2 that confers constitutive, signal-independent CREB activity ². Interestingly, unlike Ser171, which is regulated by cAMP-PKA signals, Ser275 phosphorylation is regulated by extracellular glucose. Stimulation with glucose and consequent Ser275 dephosphorylation appears to be required to 'prime' cAMP-dependent dephosphorylation of Ser171 (Figure 4). The dephosphorylation of CRTC2 at two regulatory sites provoked by two distinct signals provides a mechanism to explain the synergy with which glucose and cAMP activate CREB in beta cells. This 'two pathways, two sites' model is reminiscent of the regulation of the guanine nucleotide exchange factor TSC2, which is targeted at distinct sites by the AMPK and Wnt pathways leading to mTOR inhibition ¹⁷.

The capacity of the islet cell mass to expand (hyperplasia) in response to chronically elevated blood glucose, together with functional adaptive responses, permits sufficient insulin secretion to meet demand in prediabetic patients ¹⁸. While the role of growth factor signaling pathways and cell cycle machinery in the process of b cell expansion is comparatively well studied ¹⁹⁻²⁵, the molecular details of how glucose - a critical stimulus for b cell proliferation ²⁶ - stimulates this process are largely unknown ²⁷⁻²⁹. One candidate target is IRS2, a glucose-regulated gene that is critical for glucose-mediated induction of b cell mass ^{30, 31}. IRS2 is also a CREB target gene ³², thus glucose-induced CRTC2

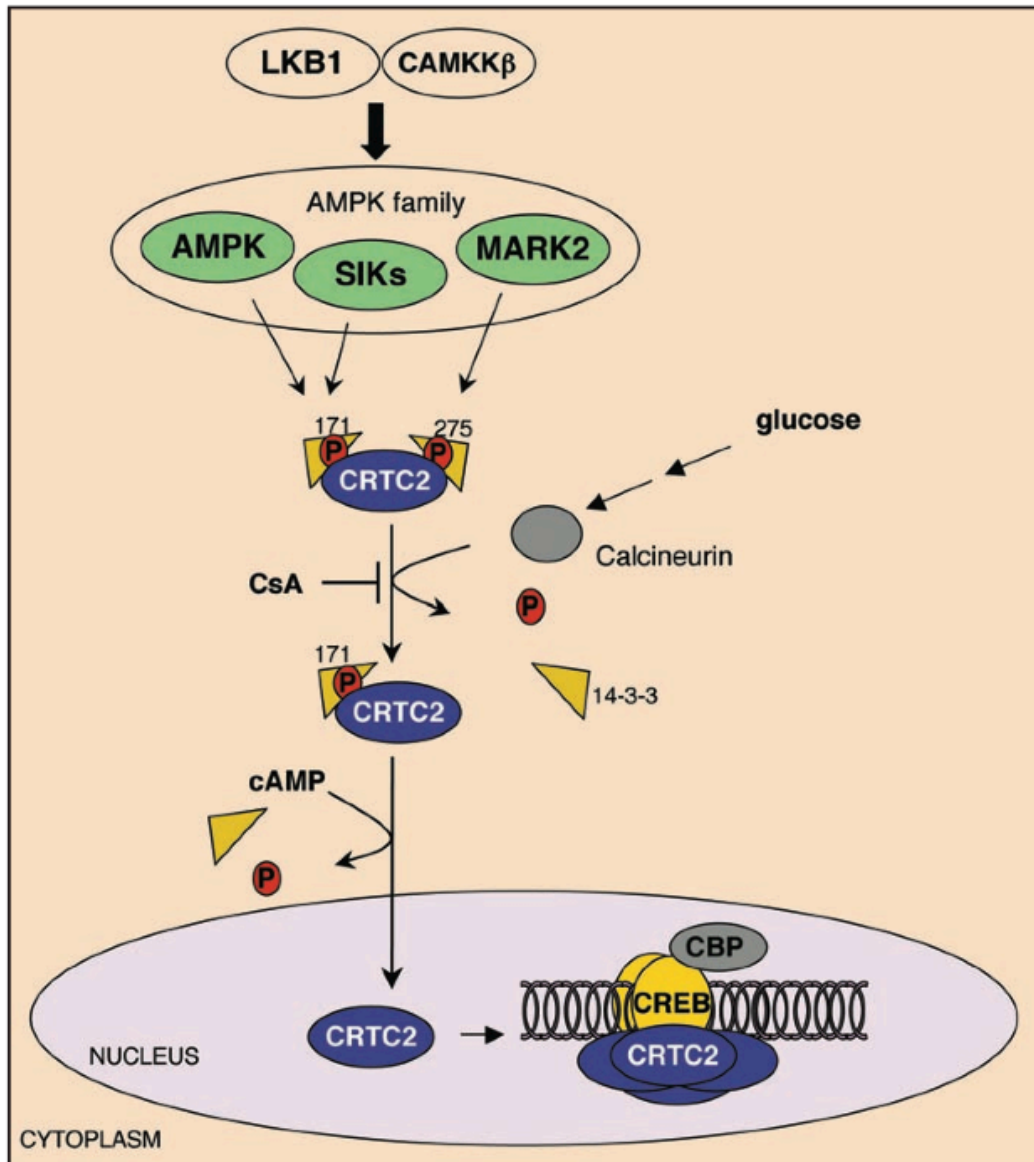


Figure 4. Model for activation of CRTC2 in beta cells. AMPK family kinases (that are known to target CRTC2) and their target sites are shown. Glucose stimulation leads to membrane depolarization, calcium influx, and activation of the Ser/Thr phosphatase calcineurin. Calcineurin dephosphorylates Ser275, the glucose-regulated phosphorylation site, which can be blocked by the immunosuppressant Cyclosporin A (CsA). Glucose stimulation ‘primes’ cAMP-dependent dephosphorylation of Ser171, leading to loss of 14-3-

3 binding and CRTC2 nuclear entry. The known AMPK kinases, LKB1 and CAMKK β activate AMPKs via activation loop site phosphorylation.

activity may underlie beta cell proliferation via CREB-dependent activation of IRS2 gene expression. We determined that glucose-stimulated dephosphorylation of Ser275 is catalyzed by the Ser/Thr phosphatase calcineurin, which can be blocked by cyclosporin A (CsA), a calcineurin inhibitor. As a consequence, CsA prevents CRTC2 nuclear entry and CREB activation. Cyclosporin and FK506 inhibit the Ser/Thr protein phosphatase calcineurin, required for T-cell

cytokine production, and thus suppress host immune rejection of foreign tissue³³. Chronic use of calcineurin inhibitors is associated with late-onset diabetes and, in the case of Type 1 diabetes treatment, loss of function of the transplanted islets and suppression of beta cell regeneration³⁴⁻³⁷. It will be of interest to determine to what degree inhibition of CRTC2:CREB activity underlies these unwanted side effects. Future work will clarify the role of Ser275 in glucose-dependent CRTC2 regulation in other excitable cells such as neurons, and the requirement for Ser275 phosphorylation for CRTC2 inhibition in non-excitable cells.

MARK2: diversifying biological roles. MARK2 was identified as a negative regulator of microtubule:MAP interactions and in a screen for regulators of *C. elegans* early embryonic polarization^{38,39}. In mammals there are 4 highly related genes (MARKs1-4) which encode a protein Ser/Thr kinase with > 50% homology in the kinase domain to that of AMPK, the best-studied member of the 14 gene AMPK family. AMPK itself is activated in response to

nutrient stress, and must be phosphorylated for maximum activity on Thr172 in the T-loop region of the α -subunit by the master AMPK family kinase, LKB1^{40,41}, or the alternate AMPK kinase, CAMKK β ⁴²⁻⁴⁴. Once activated, AMPK promotes lipolysis and glycolysis to turn on fuel generating (catabolic) processes and inhibits the mTOR and Akt pathways to shut down biosynthetic (anabolic) processes to restore cellular energy status, subjects of excellent recent reviews⁴⁵⁻⁴⁷. A series of studies has established that MARK2 regulates cellular polarity fate⁴⁸⁻⁵⁸ and acts downstream of LKB1 in numerous tissues, including skeletal muscle, pancreas, and cardiac muscle⁵⁹⁻⁶¹. Knockout mouse models have revealed that MARK2 has numerous biological roles, ranging from immune and cognitive functions to the control of energy metabolism^{1,62,63}. MARK2's recently discovered role in metabolic regulation indicates that control over these processes is not solely under AMPK control⁶⁴.

Our data indicate that, in beta cells, the functional overlap in the AMPK family with respect to the negative regulation of the CRTC2:CREB pathway includes SIKs, AMPK and MARK2. Regulation of CRTC2 by kinases that receive input from nutrient status (AMPK), hormone receptors (SIKs) and atypical PKCs (MARK2)⁶⁵ suggests that CRTCs may integrate metabolic, systemic, and architectural inputs in their role as CREB-dependent activators. The high degree of conservation of the AMPK, SIK and MARK catalytic domains suggests that they may have evolved from a single ancestral kinase and may share some substrates in common (Figure 5). In the case of CRTC2, SIKs and AMPK all phosphorylate Ser171 in vivo¹⁶. In the liver, AMPK shuts down gluconeogenesis via direct phosphorylation of CRTC2, leading to CREB inhibition and

CRTC2 Ser171:	L	N	R	T	S	<u>S</u>	D	S	A	L
consensus AMPK:	^b / _Φ	X	p	p	X	<u>S</u>	X	X	X	L
consensus SIK:	Φ	X	p	^S / _T	X	<u>S</u>	X	X	X	L
consensus MARK2:	Φ	X	^K / _R	X	X	<u>S</u>	X	X	p	L
CRTC2 Ser275:	M	N	T	G	G	<u>S</u>	L	P	D	L

Figure 5. Minor changes in consensus phosphorylation sites for AMPK, SIK1, and

MARK2. The consensus sites are derived from Hurov and Piwnicka-Worms, 2007 and

Screaton et al., 2004. Sequences are shown using single amino acid code; p, polar residue (S,

T, D, E, K, R); F, hydrophobic residue (G, A, V, L, I or M); b, basic residue (H, K or R).

Ser171 and Ser275 of CRTC differ only in the +3 position, which may explain the selectivity of MARK2 for Ser275.

represents the underlying mechanism whereby metformin exerts its therapeutic effect for

hyperglycemia⁶⁶. It is likely that SIKs and AMPK act in parallel, acting in response to

cellular energy demand (AMPK) and to systemic fasting cues (SIKs). The relative

contribution made by AMPK and SIKs to CRTC2 inhibition via phosphorylation of Ser171

remains to be clarified, both in hepatocytes and other cell types. Our data indicate that

AMPK, SIK2 and MARK2 can phosphorylate both Ser171 and Ser275 of CRTC2 in vitro

with AMPK and SIKs targeting Ser171² and MARK2 targeting Ser275 preferentially (Figure

6). This raises the possibility that in different cell types, distinct mechanisms may exist for

targeting Ser 171 and Ser275 by members of the AMPK family.

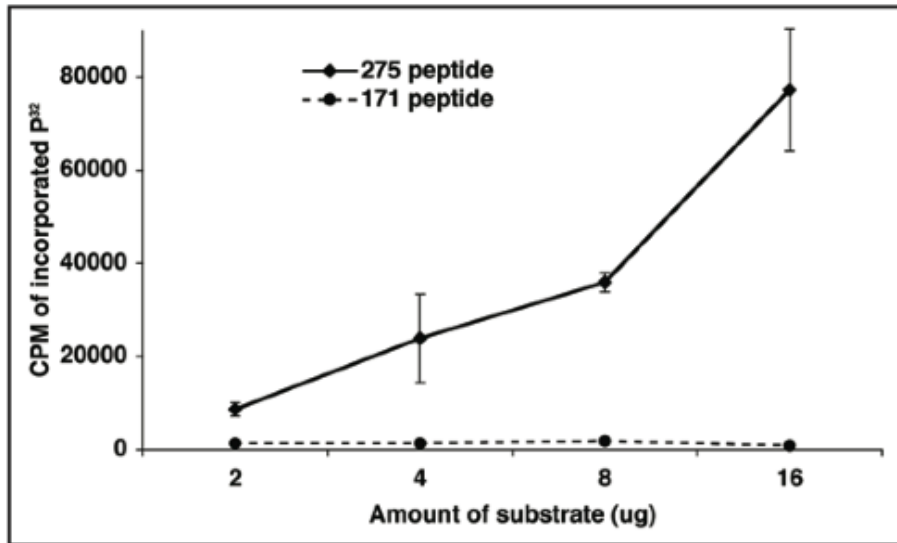


Figure 6. MARK2 phosphorylates Ser275. In vitro kinase assay with MARK2 and increasing amounts of GST-161-179 (Ser171) and GST-268-283 (Ser275) substrate peptides for 30 min at 30°C reveals that MARK2 selectively phosphorylates Ser275 in vitro.

Little is known about possible functions of the AMPK family in beta cells, and the role of AMPK itself in this context is controversial. Whereas AMPK activation has been correlated with reduction in insulin gene transcription, insulin secretion, and apoptosis⁶⁷⁻⁶⁹, recent data suggests that while AMPK is involved in nutrient sensing, it plays no role in the control of insulin secretion⁷⁰. The use of kinase-dead alleles, which may not act as dominant negatives under all experimental conditions, may underlie some of these conflicting data; studies using islets from genetic models lacking AMPK catalytic subunits should clarify these questions.

Concluding Remarks:

By inhibiting CRTC2 via phosphorylation, MARK2 inhibits CREB and may play a role in the glucose-activated gene transcription pathway². Given the well-established role of MARK2 in regulating cell polarity, future work will determine if CRTC and CREB play a

role in establishing a gene expression program that responds to polarity cues, and moreover if there is a role for MARK2 in regulating beta cell function in vivo. It will also be of interest to determine if AMPK regulates CRTC2 in other cell types besides hepatocytes and to clarify the roles of the two AMPKKs LKB1 and CAMKK beta in CRTC2 regulation in these settings.

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Chapter 4. Loss of LKB1 in adult b cells increases b cell mass and enhances glucose tolerance in mice.

Article published:

Fu A, Ng A, Depatie C, Wijesekara N, He Y, Wang G-S, Bardeesy N, Scott, FW, Touyz RM, Wheeler MB, and Sreter RA. Loss of LKB1 in adult b cells increases b cell mass and enhances glucose tolerance in mice. Cell Metabolism. **2009**.

Summary:

Following my discovery that many of the AMP kinases could phosphorylate CRTC2 and that all members were expressed in beta cells (please see Appendix 3) I sought out to look at the effects of reducing activation of the entire family of kinases. Since the threonine kinase LKB1 directly phosphorylates and activates all members of the AMP kinase family, I decided to investigate the effects of deletion of LKB1 in beta cells. Thus shutting down all of the members of the AMP kinase family and the CRTC2:CREB pathway, my prediction was that depletion of LKB1 in beta cells would lead to increase beta cell survival and function. Indeed I observed that LKB1 knockout beta cells had increased proliferation and enhanced insulin secretion which provided protection from high fat diet-induced hyperglycemia.

Contributions:

As first author I contributed to the majority of the work in this manuscript by executing experiments for over 75% of the panels in the paper. In addition I thought of experimental hypotheses to test and interpreted results for model-building along with Dr.

Screaton, Dr. Wheeler and Dr. Scott. Dr. Screaton and I wrote the manuscript and organized data for logical flow.

I provided all panels of Figure 1, 2, 5, for other figures I provided all panels except the work I describe in detail of co-authors listed below:

Figure 3a-c and Supplementary Figures 2 and 3: N. Wijesekara processed pancreas samples that I harvested and fixed from L/L and LABKO mice, at a facility at University of Toronto which were stained for insulin and glucagon using immunohistochemistry.

Figure 4a-c: Initial conditions for siRNA-mediated LKB1 knockdown in MIN6 cells were performed by A. Ng, using these conditions I and C. Depatie performed GSIS experiments in LKB1 knockdown MIN6 cells. A. Ng also helped to optimize conditions for using HTRF insulin assays to measure insulin secreted by MIN6 cells. I used these conditions to perform MIN6 GSIS experiments with knockdown of AMPK, Sik2 and Mark2 (Figure 4d-f).

Figure 4g: C. Depatie and Y. He performed the calcium influx experiments.

Figure 5a: Dr. Wang and Dr. Scott provided a detailed protocol and helped me with troubleshooting immunofluorescent detection of BrdU incorporation.

In addition, I contributed experimental samples from this work and co-authored a manuscript entitled “Depletion of intranuclear rodlets in mouse models of diabetes.” (please see Appendix 4). In this study we correlated increased insulin secretion and islet function with the frequency of intranuclear rodlet staining, I prepared all of the treatments on the mice and samples, P. Milman performed all the immunofluorescence staining and quantitation.

Title: Loss of Lkb1 in adult b cells increases b cell mass and enhances glucose tolerance in mice

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Running Title: Lkb1 controls glucose homeostasis and b cell mass

Summary

The Lkb1 tumor suppressor exerts its biological effects through phosphorylation and consequent activation of the AMP kinase (AMPK) family. Extensive genetic and biochemical evidence supports a role for Lkb1 in cell cycle arrest, establishment of cell polarity, and in cellular energy metabolism. However, the role of Lkb1 and the AMPK family in β cell function in vivo has not been established. We generated conditional knockout mice with a deletion of the *Lkb1* gene in the β cell compartment of pancreatic islets; these mice display improved glucose tolerance and protection from diet-induced hyperglycemia. *Lkb1*^{-/-} β cells are hypertrophic due to elevated mTOR activity; they also proliferate more and secrete more insulin in response to glucose. These data indicate that inhibiting Lkb1 activity in β cells may facilitate β cell expansion and glucose tolerance in vivo.

Introduction

Regulation of insulin secretion by the pancreatic β cell is critical for the proper maintenance of glucose homeostasis. During feeding, insulin secretion is triggered by elevations in blood glucose and is modulated by incretin hormones (Drucker, 2006; Jia et al., 1995). Glucose induced insulin secretion involves glucose sensing (e.g. glucose uptake and retention mediated by GLUT2 and GK), and events that lie at or downstream of mitochondria (e.g. oxidation of glucose and other nutrients to generate ATP, K-ATP channel closure, β cell membrane depolarization, and extracellular calcium influx) (MacDonald et al., 2005; Newgard and McGarry, 1995; Newsholme et al., 2007). Influx of calcium ions into the cytoplasm from extracellular and intracellular stores is a critical event that couples glucose stimulation to insulin secretion. Impaired insulin response resulting from dysfunction or loss of β cells underlies all forms of diabetes (Mathis et al., 2001; Muoio and Newgard, 2008).

The *Lkb1* gene (also known as *Stk11*) encodes a serine/threonine protein kinase that was first described as a tumour suppressor gene mutated in Peutz-Jeghers syndrome (PJS), a familial form of cancer (Giardiello and Trimbath, 2006; Hemminki et al., 1998; Katajisto et al., 2007; Miyaki et al., 2000). Consistent with a role for *Lkb1* as a tumour suppressor, expression of *Lkb1* induces G1 cell cycle arrest (Tiainen et al., 1999) and *Lkb1*^{-/+} mice are predisposed to developing hamartomatous intestinal polyps associated with activation of mTOR kinase (Bardeesy et al., 2002; Shaw et al., 2004b). *Lkb1* functions in a complex with the scaffold protein MO25 and the pseudokinase STRAD (Baas et al., 2003; Boudeau et al., 2003; Boudeau et al., 2004; Hawley et al., 2003; Milburn et al., 2004), exerting its known

biological effects via phosphorylation and consequent activation of all members of the AMPK family (Lizcano et al., 2004).

Given the diversity of biological roles attributed to the AMPK family of kinases, Lkb1 has been implicated in a number of distinct biological phenomena: tumor suppression, energy metabolism via AMPK-TSC2-mediated mTOR inhibition and activation of eEF2 kinase (Browne et al., 2004; Inoki et al., 2003), and the establishment of cell polarity via AMPK1/2, MARKs1-4 and Brsk1/SAD-B/Brsk2/SAD-A kinases (Baas et al., 2004; Barnes et al., 2007; Kishi et al., 2005; Martin and St Johnston, 2003; Watts et al., 2000) (Lee et al., 2007; Mirouse et al., 2007; Zhang et al., 2006; Zheng and Cantley, 2007). While Lkb1 null mice die at E8.5-11, indicating an essential role for Lkb1 in development (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002; Ylikorkala et al., 2001), targeted loss of Lkb1 in different settings, including skeletal muscle, liver, and pancreas has provided further evidence for Lkb1 as a tumor suppressor and a critical regulator of cell polarity and energy metabolism (Hezel et al., 2008; Katajisto et al., 2008; Koh et al., 2006; Sakamoto et al., 2005; Sakamoto et al., 2006; Shaw et al., 2004a).

AMPK is a central cell energy sensor that is activated via an increase in the intracellular AMP:ATP ratio under low nutrient conditions (Hardie, 2007b). Given the involvement of AMPK in exercise-dependent glucose uptake in muscle (Mu et al., 2001), inhibition of gluconeogenesis in the liver (Hundal et al., 2000; Iglesias et al., 2002), regulation by hormones leptin, resistin and adiponectin (Banerjee et al., 2004; Minokoshi et al., 2002; Yamauchi et al., 2002), and activation in response to treatment with the diabetes therapeutic

metformin (Zhou et al., 2001), it has been extensively studied as a potential target for treatment of hyperglycemia (Zhang et al., 2009). With respect to energy metabolism, Lkb1-Ampk function is required in the liver to inhibit the gluconeogenic program driven by the CREB coactivator Crtc2/Torc2; as such, Lkb1 agonists have been proposed to be potential therapeutics for hyperglycemia associated with Type II diabetes (Shaw et al., 2005).

However, activation of AMPK has been reported to impair glucose-induced insulin secretion and survival of pancreatic b cells and islets (da Silva Xavier et al., 2000; Leclerc et al., 2004; Richards et al., 2005), observations that suggest that inappropriate Lkb1 activity may be deleterious for b cell function. Given the interdependence of hepatic and pancreatic contributions to glucose metabolism, in this study we sought to determine the role of Lkb1 in adult b cells via conditional ablation of the *Lkb1* gene.

Results

Ablation of Lkb1 in adult b cells

Lkb1 is expressed in all epithelial cell lineages in both endocrine and exocrine compartments from embryonic day E16.5 (Hezel et al., 2008). In order to study Lkb1 function in adult animals and also avoid potential developmental complications due to absence of Lkb1, we employed a conditional gene ablation approach in mice to inactivate the *Lkb1* gene in mature b cells (Figure 1A). In adult mice, Pdx1 expression is restricted within the islet to the b cell compartment. We therefore bred *Lkb1*^{loxP/loxP} mice to Pdx1-CreERT2 transgenic mice that express the tamoxifen-inducible Cre recombinase via the

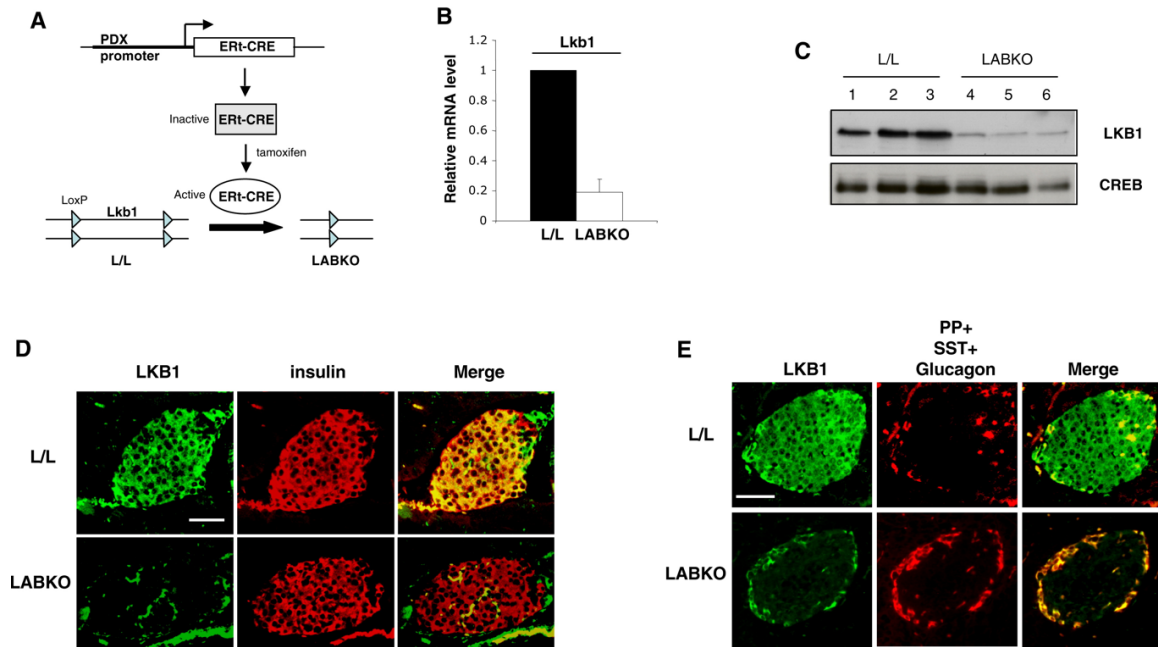


Figure 1. Deletion of Lkb1 in adult b cells. (A) Adult $Lkb1^{loxP/loxP}$ mice carrying the Pdx1-CreERT2 transgene were injected with tamoxifen to induce b cell specific deletion of Lkb1. (B) QPCR analysis showing reduction of Lkb1 mRNA levels in total RNA from islets isolated from $Lkb1^{loxP/loxP}$ (L/L) and $Lkb1^{loxP/loxP}$, Pdx1-CreERT2 (LABKO) mice. RNA is pooled from 3 mice per genotype. Lkb1 mRNA levels are normalized to levels of 36B4 mRNA as internal control and are representative of two experiments. All data are represented as mean $\pm$ SEM. (C) Western blot analysis showing loss or reduction of Lkb1 protein from extracts of isolated islets from LABKO mice. CREB levels are shown as loading control. (D) Pancreatic sections stained with insulin and Lkb1 antibodies reveal loss of Lkb1 in insulin-positive cells in LABKO mice, whereas all control (L/L) insulin-positive cells expressed Lkb1. Bar = 25 μ m. (E) Immunostaining of pancreatic sections of LABKO mice with a cocktail of antibodies to non-b cells (somatostatin/delta, glucagon/alpha, pancreatic polypeptide/PP) reveals intact Lkb1 expression in these compartments. Merge of

Lkb1 (green) and non-b cell (red) staining is shown at right. Bar = 25 μ m.

Pdx1 promoter (Gu et al., 2002). Progeny with genotype $Lkb1^{loxP/+}$, Pdx1-CreERT2 were mated to $Lkb1^{loxP/loxP}$ mice to obtain $Lkb1^{loxP/loxP}$, Pdx1CreERT2 mice and control $Lkb1^{loxP/loxP}$ littermates. The Cre system does not elicit phenotypic changes from wt Fvb control mice (Hezel et al., 2008), Supp Fig 1).

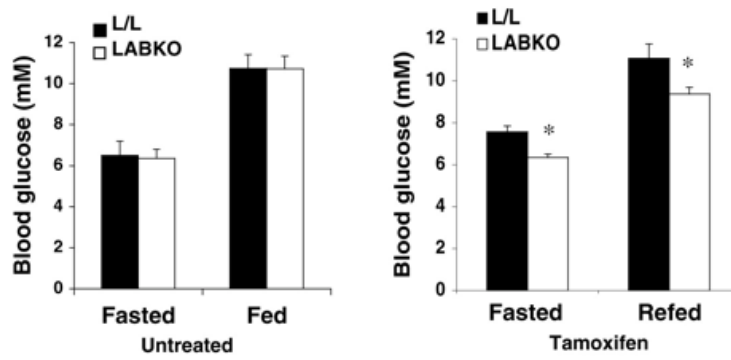
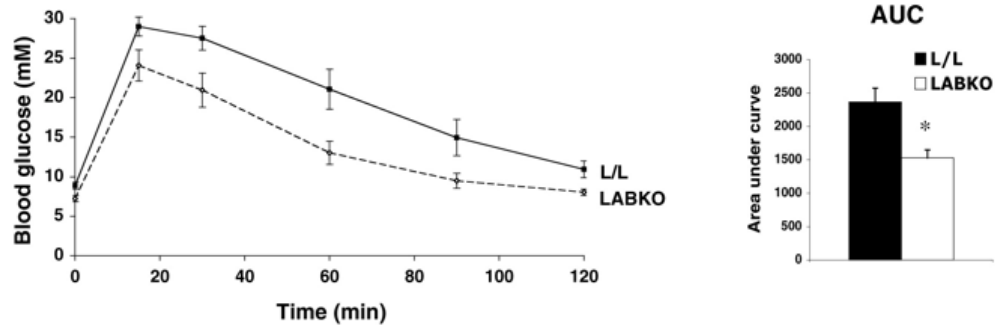
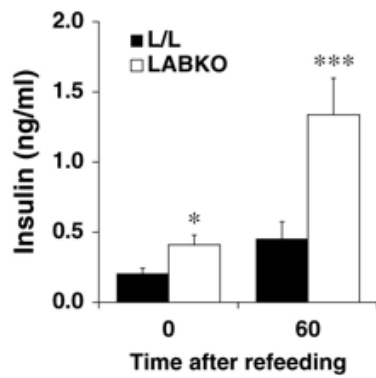
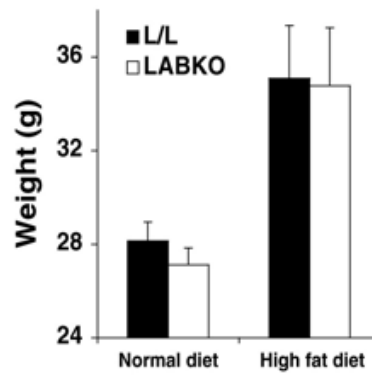
Cre activity was induced in 8- to 10-week old $Lkb1^{loxP/loxP}$, Pdx1-CreERT2 male mice by IP injection of tamoxifen. After 1 week of tamoxifen treatment, we observed that Lkb1 mRNA expression was reduced by 80% in islets from $Lkb1^{loxP/loxP}$, Pdx1-CreERT2 mice (hereafter referred to as Lkb1 Adult Beta cell Knockout, or LABKO), as compared to $Lkb1^{loxP/loxP}$ control ($Lkb1$ L/L, or L/L) mice as measured by quantitative RT-PCR of total RNA purified from isolated islets (Figure 1B). It is likely that unperturbed expression of Lkb1 in non-b islet cells (alpha, delta, epsilon and PP cells) explained the residual mRNA detected in total islet RNA by QPCR as we investigate below. Western blotting of protein extracts prepared from isolated islets indicated nearly complete loss of Lkb1 protein in islets from LABKO animals, consistent with the observed reduction in Lkb1 mRNA (Figure 1C).

Immunofluorescence staining of pancreatic sections from adult control mice demonstrated that Lkb1 was expressed primarily in the islets (Figure 1D). There was nearly a complete loss of staining in LABKO pancreata, demonstrating the efficiency of CreERT2-mediated Lkb1 gene recombination. Costaining with anti-Lkb1 and anti-insulin antibodies demonstrated uniform absence of Lkb1 protein in b cells in islets from LABKO mice. Acinar cells in sections from both wt and LABKO animals were either negative or weakly positive

for Lkb1 staining, consistent with the observed absence of Lkb1 protein in acinar tissue in adult mice (Hezel et al., 2008). To confirm that Lkb1 had been deleted exclusively from the b cell compartment, we costained sections for Lkb1 along with a cocktail of antibodies to glucagon (alpha cells), somatostatin (delta cells), and pancreatic polypeptide (PP cells; Figure 1E), and observed that these cell types still expressed Lkb1. These data confirm that the Lkb1 deletion was specific to the b cell compartment and we conclude that any phenotypic alteration observed in LABKO mice is due to loss of Lkb1 in b cells.

Improved glucose tolerance in LABKO animals

We next performed an analysis of the glucoregulatory system in the LABKO animals. Prior to tamoxifen treatment, mice carrying the Pdx1-CreERt transgene showed no difference in fasting or random fed blood glucose levels (Figure 2A, left panel). After tamoxifen treatment LABKO animals displayed marginally lower blood glucose levels than controls when fasted or refed for 2 hrs (Figure 2A, right panel). We next performed glucose tolerance tests to assess the dynamics of the response to glucose in LABKO mice. Loss of Lkb1 significantly accelerated glucose clearance by glucose tolerance test compared to control L/L mice (IPGTT; Figure 2B); Pdx-CreERt+ mice carrying one wt lkb1 allele demonstrated a normal IPGTT profile indicating that loss of Lkb1 underlies the improvement in glucose tolerance (Supp Fig 1). To determine the underlying cause of this enhanced glycemic control, we measured plasma insulin levels in LABKO animals after an overnight fast and 60 min refeeding. Plasma insulin levels of LABKO mice were

A**Blood glucose levels****B****C****Plasma insulin levels****D**

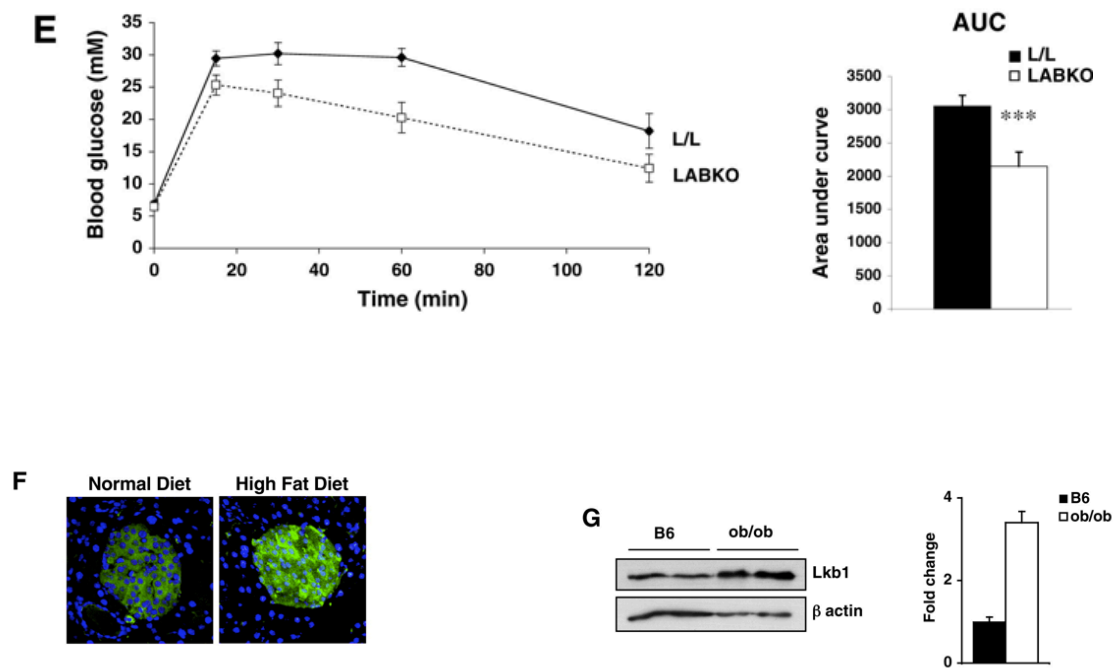


Figure 2. Enhanced glucose tolerance in LABKO animals. (A) Blood glucose levels of fasted and random fed control (L/L) and LABKO mice were measured prior to tamoxifen treatment (left panel; n=5) or from fasted or refed (2 hrs) animals 1 week after tamoxifen treatment (right panel; * $p < 0.05$ for fasted mice; n=9 L/L, n = 17 LABKO). All data are represented as mean $\pm$ SEM. (B) Glucose tolerance test (2 mg glucose/g, IP injection) for tamoxifen treated L/L and LABKO mice, n=9 for both groups. Blood glucose concentrations were determined at indicated times. Area under the curves shown at right, * $p < 0.02$. (C) Plasma insulin levels after 60 min of refeeding in control (L/L, n=6) and LABKO mice (LABKO; n=12). * $p < 0.03$ for fasted, *** $p < 0.001$ for refed. (D) Weights of control (L/L) and LABKO animals on high fat diet for 16 weeks. (E) Glucose tolerance test of control (L/L) and LABKO animals on high fat diet for 16 weeks. Area under the curve shown at right, ***, $p < 0.003$. (F) Immunostaining of pancreatic sections on control mice

with anti-LKB1 antibody after 18 weeks on normal or high fat diet. (G) Western blot analysis of Lkb1 protein levels in extracts of isolated islets from control C57BL/6J and B6.V-Lep^{ob}/J (“ob/ob”) mice compared to B6 control mice. Quantitation of Lkb1 levels normalized to actin loading control shown at right.

more than 2-fold higher than controls during both fasting and refeeding (Figure 2C), indicating that enhanced basal and nutrient stimulated insulin secretion likely underlies the improved glucose tolerance.

To provoke an increased functional response from the β cell mass, LABKO and control mice were fed a high fat diet for 16 weeks, a regimen that induces features of human metabolic syndrome including obesity, hyperglycemia, and insulin resistance. After 16 weeks on a high fat diet, control and LABKO animals had indistinguishable body weights (Figure 2D), but control mice displayed a progressive deficiency in glucose clearance in IPGTT assays (Figure 2E). Remarkably, glucose tolerance in the LABKO mice was similar to animals fed normal diet (compare Figure 2E on high fat diet to Figure 2B on normal diet), indicating that loss of Lkb1 improves the capacity of the β cell mass to facilitate restoration of euglycemia in this setting. Glucose readings for L/L animals on high fat diet were frequently above the limit of glucometer detection (33.3 mM), thus these data would underestimate the effect of Lkb1 on lowering blood glucose in this context. In contrast, immunohistochemical analysis suggested that Lkb1 protein levels were increased in islets of L/L control mice fed a high fat diet-fed for 16 weeks compared to age matched controls (Figure 2F). Furthermore, Lkb1

protein was significantly increased in extracts of islets isolated from ob/ob mice compared to control B6 mouse islets (Figure 2G), indicating that Lkb1 accumulation correlates with glucose intolerance in two distinct models of Type 2 diabetes.

Loss of LKB1 in adult islets increases b cell mass and insulin secretion

To determine if a defect in insulin storage contributed to the hyperinsulinemia observed in fed Lkb1 mutant mice, we measured insulin content in islets isolated from LABKO mice.

Insulin content of LABKO islets (Figure 3A) and the b cell mass doubled in LABKO animals (Figure 3B) while alpha cell mass was unaffected (Figure 3C).

Quantitation of insulin staining from pancreatic sections (shown in Figure 3B) confirmed the increase in insulin staining in LABKO islets (Supp Fig 2) and revealed a 20% increase in islet size (Supp Fig 3). Taken together, these data indicate that increased insulin accumulation in Lkb1-deficient b cells and expansion of b cell mass may underlie the improved glucose clearance. To determine if the LABKO islets displayed a corresponding increase in glucose-stimulated insulin secretion and investigate the cell autonomy of the phenotype, we performed static incubation GSIS assays using isolated islets from LABKO and control mice one week after tamoxifen injection. Basal insulin secretion increased 3-fold in LABKO islets and after 1 hr of stimulation with 20 mM glucose, LABKO islets secreted 40% more insulin than controls (Figure 3D). We next performed islet perfusion studies to evaluate the dynamics of insulin secretion in response to glucose. Consistent with the static incubation data, we observed an increase in total secreted insulin from LABKO islets following 20 mM glucose treatment (Figure

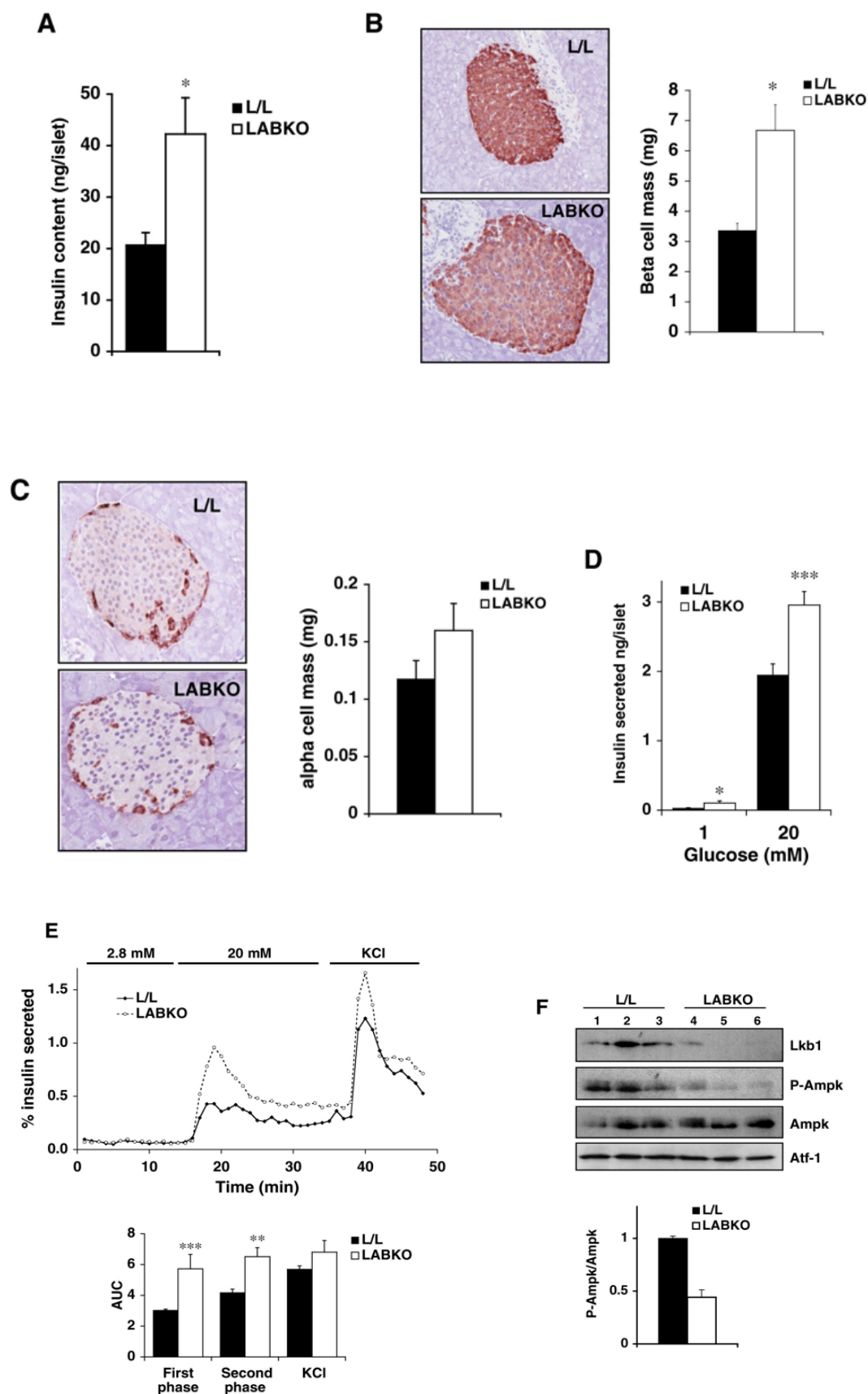


Figure 3. Loss of Lkb1 in islets enhances insulin secretion. (A) Insulin content in islets isolated from LABKO and control mice (n=5, * p < 0.03). All data are represented as mean $\pm$ SEM. (B) Left, insulin staining of pancreatic sections from control L/L and LABKO mice. Right, histogram showing b cell mass (mg per pancreas) in control L/L and LABKO islets. n = 6-7, * p < 0.02. (C) Left, glucagon staining of pancreatic sections from L/L and LABKO mice. Right, histogram showing alpha cell mass in control L/L and LABKO mice (n = 5-6, p < 0.7). (D) Increased insulin secretion in LABKO islets. 20 islets per genotype were incubated in low glucose (1 mM) and stimulated with 20 mM glucose for 1 hour at 37°C. Supernatants were analyzed for insulin content by HTRF assay. Data shown are representative of 4 independent experiments with islets from 4-8 mice per genotype are shown. Low glucose: *, p < 0.04, high glucose: ***, p < 0.002. (E) Top: Glucose-stimulated insulin secretion assay by islet perfusion from L/L and LABKO mice. Islets incubated in 2.8 mM glucose were stimulated with 20 mM glucose and then 45 mM KCl at as indicated. Insulin content in collected fractions was determined by HTRF assay and presented as secreted insulin normalized to islet insulin content (% insulin secreted). n=3 animals per genotype, data presented are from one of three independent experiments. Bottom: Area under the curves. *** p < 0.0005; ** p < 0.004. For KCl treatment, p = 0.3. (F) Western blot analysis of P-Ampk and total Ampk levels in isolated islets from control (L/L) and LABKO animals. Blots for Lkb1 and ATF1 loading control are shown.

3E). In particular, we noted a significant increase in first and second phase insulin secretion from LAKBO islets that could not alone be accounted for by the increase in insulin content, suggestive of enhanced glucose coupling efficiency in islets lacking Lkb1.

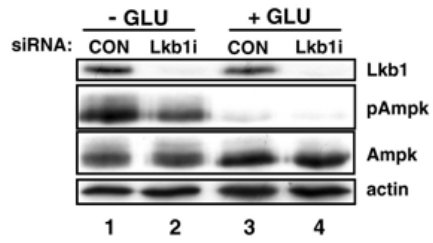
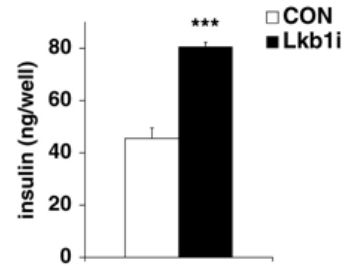
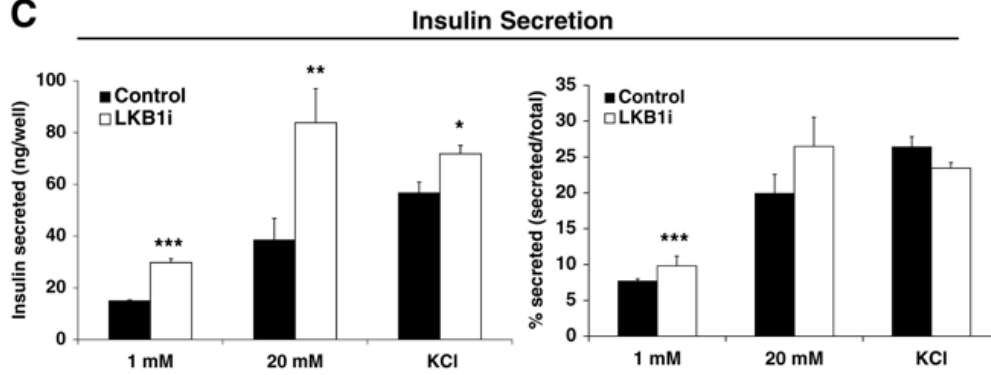
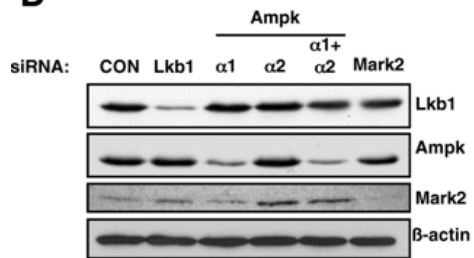
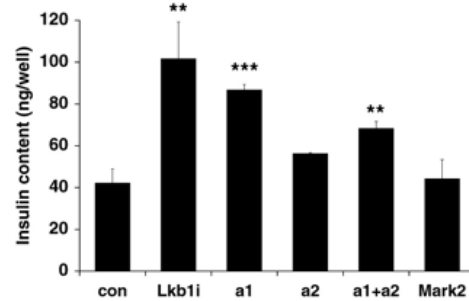
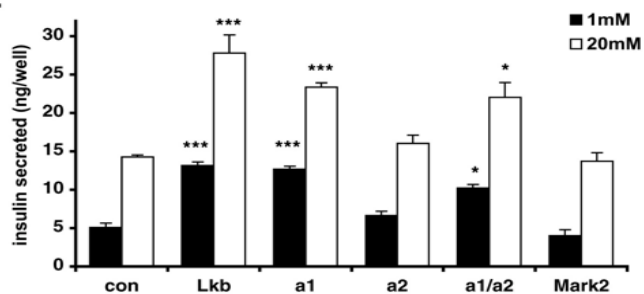
AMPK is activated under conditions of high AMP:ATP observed during conditions of nutrient exhaustion, hypoxia, and ischemia among others (Hardie, 2003). AMP binding to the AMPK γ subunit and Thr172 phosphorylation of the α subunit by Lkb1 or other AMPKK is required to achieve full AMPK activity (Lizcano et al., 2004). As a consequence, AMPK activity is attenuated in settings where Lkb1 protein is reduced. To determine whether or not P-Ampk activity in islets may be controlled in accordance with fluctuations in glucose across the fasted-fed transition (6-11 mM), we stimulated islets cultured in low glucose (2.8 mM) with increasing glucose concentrations up to 15 mM and monitored changes in the level of P-Ampk. Comparison of the levels of P-Ampk at 7.5 mM glucose and higher (fed range) to those found at 5 mM (fasted range) revealed a 60-80% reduction in levels of P-Ampk (Supp Fig 4). Examination of P-Ampk levels in glucose starved islets from LABKO mice revealed a similar reduction in P-Ampk levels (Figure 3F). Importantly, the absence of Lkb1 completely prevented pharmacological activation of Ampk in LABKO islets treated with the Ampk activator, 5-aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR; Supp Fig 5), indicating that Lkb1 is the principal AMPK kinase in islet β cells. Taken together, we conclude that AMPK activity is regulated by physiological changes in glucose concentration observed during feeding and fasting, and that Lkb1 is the principal upstream Ampk kinase in islet β cells.

Loss of Ampk $\alpha 1$ promotes insulin accumulation and basal secretion

To facilitate examination of the improved insulin secretion phenotype, we attempted to model LABKO islets using siRNA-mediated knockdown of Lkb1 in the glucose-responsive

cell line, MIN6. Western blot analysis confirmed reduction of Lkb1 protein by 90% in cells transfected with siRNA targeting Lkb1 relative to nonspecific control (Figure 4A, compare lanes 1 and 2), as well as a 50% reduction in P-Ampk levels in low glucose that was enhanced in the presence of glucose (Figure 4A, compare lanes 2 and 4; quantitation is shown in Supp Fig 6). Thus the effect of Lkb1 loss on Ampk activity in islets can be recapitulated in Lkb1 knockdown MIN6 cells. Consistent with the results from LABKO islets, Lkb1 knockdown resulted in a 2-fold increase in insulin content (Figure 4B) and a 2-fold increase in insulin secretion in low glucose (Figure 4C, left panel). When stimulated with 20 mM glucose, Lkb1 knockdown cells similarly secreted nearly 2 times more insulin (80 ng insulin/well, compared to 45 ng/well for control cells). When the amount of secreted insulin was normalized to content, the increase in secretion appeared to result largely although not entirely from an increase in insulin stores (Figure 4C, right panel). Consistent with this, treatment of control and Lkb1 knockdown cells with the depolarizing agent KCl resulted in unchanged insulin secretion when normalized to content.

Phenotypic alterations due to loss of Lkb1 are expected to result from the net loss of the activities of all members of the AMPK family. To investigate the enhanced basal insulin secretion phenotype seen in Lkb1 knockdown cells, we transfected siRNA targeting those Ampk family members that have to date been implicated in metabolic control: Ampk α 1, Ampk α 2, and Mark2. Western blot analysis using an antibody that recognizes both

A**B****C****D****E****F**

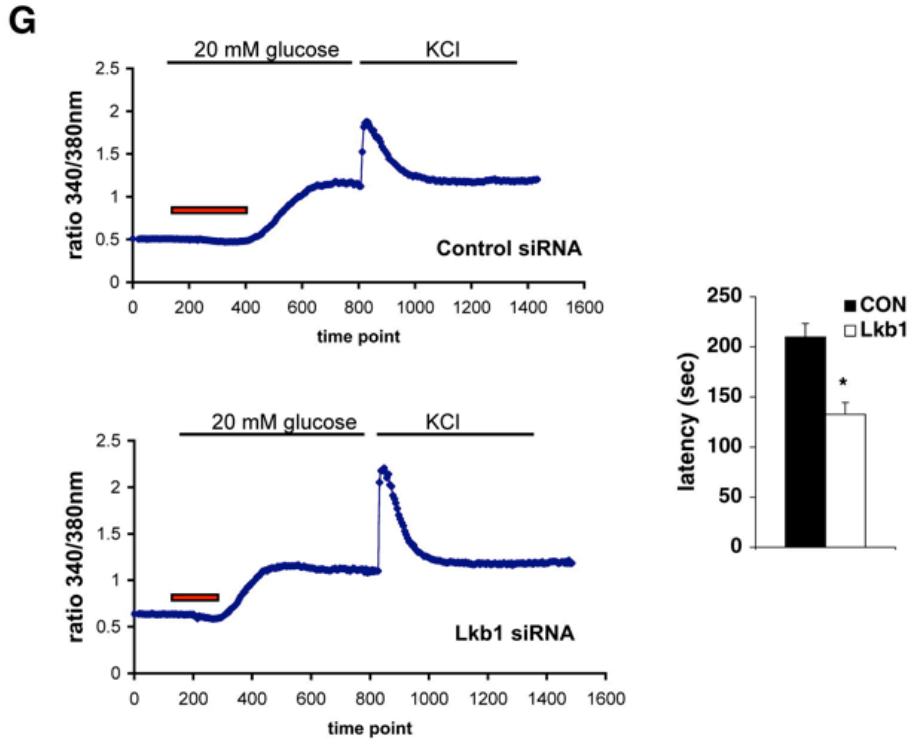


Figure 4. AMPK $\alpha 1$ knockdown promotes insulin accumulation and secretion. (A)

Western blot analysis of P-Ampk, total Ampk and Lkb1 levels in MIN6 cultures treated with control or Lkb1 siRNA in the presence of low (1 mM) or high (20 mM) glucose. b actin loading control is shown. (B) Insulin content in Lkb1 knockdown and control siRNA treated cells cultured in 1 mM glucose, *** $p < 0.001$. All data are represented as mean $\pm$ SEM. (C) Left, insulin secretion in Lkb1 knockdown and control siRNA-treated MIN6 cells cultured in low (1 mM; *** $p < 0.00001$) or high (20 mM; ** $p < 0.02$) glucose, or stimulated with 45 mM KCl (* $p < 0.05$) for 1 hour. Right, histogram showing insulin secretion values normalized to insulin content (*** $p < 0.001$). Data in (B) and (C) are representative of at least 3 experiments. (D) Western blot analysis of Lkb1, Ampka1/a2, and Mark2 levels in MIN6 cultures treated with the indicated siRNA duplexes. b actin loading control shown. (E) Insulin content in MIN6 cells transfected with the indicated siRNA duplexes and cultured in

1 mM glucose. P-values for insulin readings compared to control siRNA were as follows: Lkb1, ** $p < 0.02$; Ampka1, *** $p < 0.004$, Ampka1+a2, ** $p < 0.01$. (F) Insulin secretion in Ampk a1 knockdown and control siRNA-treated MIN6 cells cultured in low 1 mM glucose or stimulated with 20 mM glucose for 1 hour. Lkb1: ***, $p < 0.00005$; Ampk a1: ***, $p < 0.00008$; Ampk a1+ a2: **, $p < 0.02$. Data in (E) and (F) are representative of at least 3 experiments. (G) Lkb1 knockdown cells display an accelerated response time in the rise of intracellular calcium concentration of control (top left) and Lkb1 knockdown cells (bottom left) cultured in 1 mM or 20 mM glucose. Treatment with 45 mM KCl depolarization stimulus is indicated. The latency is marked with a red bar for clarity. (Right) Histogram showing reduction in Ca^{2+} influx latency in LABKO cells. Data are $\pm$ SEM of three independent experiments, * $p < 0.03$.

Ampk a1 and a2 demonstrated efficient knockdown of Ampk a1 but not Ampk a2, indicating that Ampk a2 is likely expressed at lower amounts compared to Ampk a1 in MIN6 cells (Figure 4D). Interestingly, knockdown of Ampk a1, but not Ampk a2 or

Mark2, recapitulated the effect of Lkb1 knockdown on both insulin content (Figure 4E) and insulin secretion (Figure 4F), indicating that loss of Ampk a1 promotes insulin accumulation and secretion. Similarly, the observed increase in insulin secretion in Lkb1 and Ampka1 knockdown cells appeared to result largely from increased insulin content. The small enhancement in insulin secretion in Lkb1 knockdown cells stimulated with 20 mM glucose and the enhanced first phase secretion seen in perfusion studies (Figure 4C, right panel) raised the possibility that loss of Lkb1 may also improve the coupling of glucose stimulation

to insulin secretion. To address this, we performed calcium imaging experiments in MIN6 cells. Analysis of cytosolic calcium revealed that basal levels of intracellular Ca^{2+} were comparable in control and Lkb1 knockdown MIN6 cells, indicating that control mechanisms governing Ca^{2+} content are preserved under low glucose conditions (not shown). However, glucose induced a more rapid response in the Lkb1 cells versus controls (Figure 4G), consistent with an improvement in glucose sensing.

Enhanced b cell proliferation, hypertrophy and mTOR pathway activation in the absence of Lkb1.

To determine if enhanced b cell proliferation in the LABKO mice also contributed to the increase in islet size (Supp Fig 2), we performed BrdU incorporation studies (Figure 5A). Quantitation of BrdU+ cells in islets of LABKO animals revealed a 2.7 fold increase in the number of BrdU+ insulin+ cells per islet (Figure 5B), indicating that loss of Lkb1 activity also enhances recruitment of b cells into the cell cycle. Examination of pancreatic sections stained with hematoxylin and eosin also revealed larger b cells and an altered distribution of nuclei within the b cell compartment (Figure 5C), alterations consistent with a role for Lkb1 in homeostatic maintenance of b cell size and architecture (Dhawan et al., 2007; Hezel et al., 2008). Staining of pancreatic sections from Mark2^{-/-} mice revealed a similar alteration in nuclear positioning as seen in LABKO islets, indicating that loss of Mark2 activity in the LABKO islets underlies, at least in part, the observed morphological change (Figure 5D). We also observed what appeared to be larger b cells

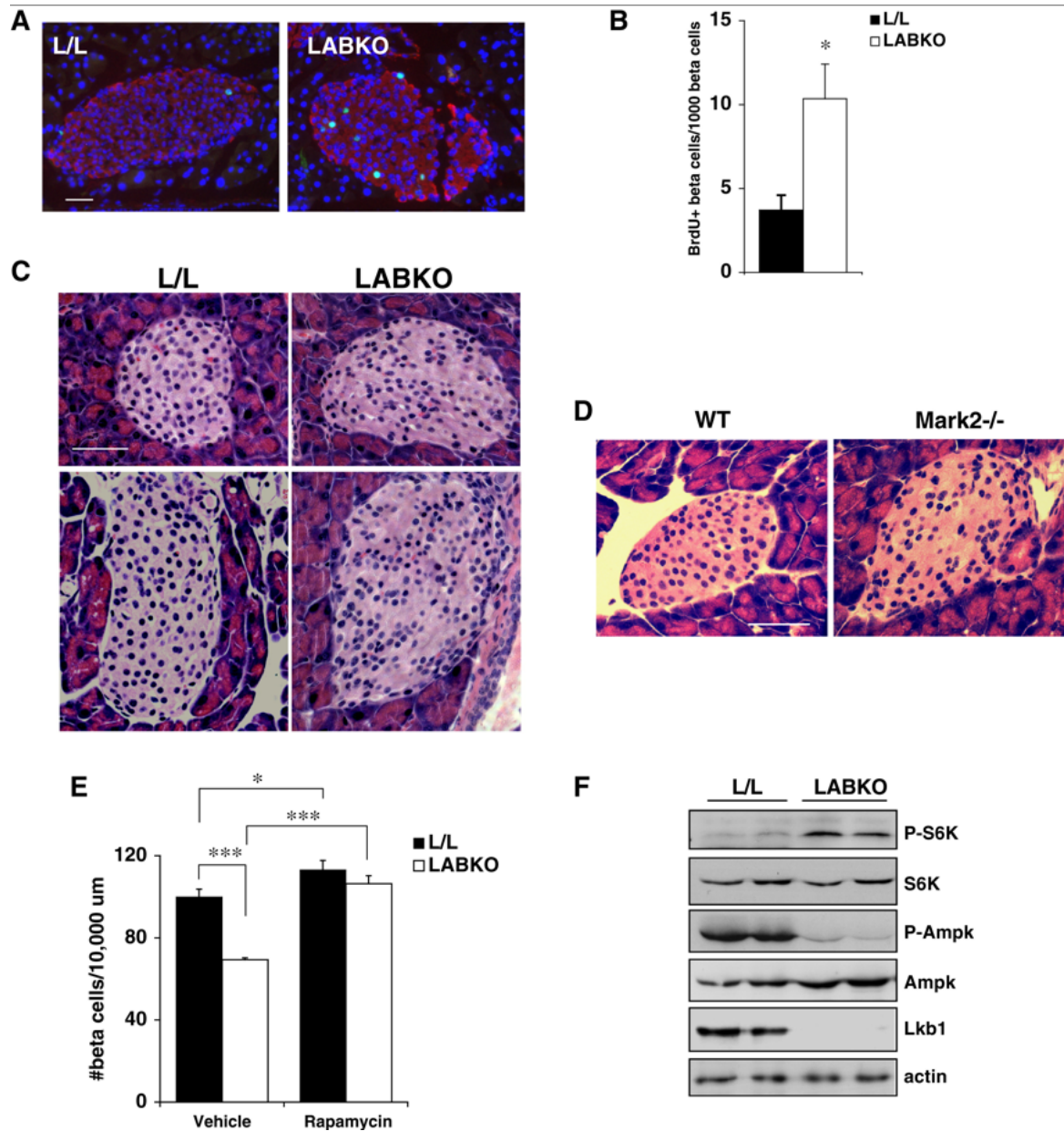


Figure 5. Lkb1 regulates b cell proliferation, size and architecture and activates mTor signaling. (A) Representative immunofluorescence staining for BrdU (green), insulin (red) and DAPI (blue) in pancreatic sections from control (L/L) and LABKO mice injected with BrdU. These were used to evaluate the b cell proliferation index shown in (B). (B) Histogram showing number of double BrdU+, insulin+ cells in L/L and LABKO mice (n=5-6, * p < 0.03). All data are represented as mean $\pm$ SEM. (C) H+E staining of islets in

pancreatic sections from L/L and LABKO mice. Two islets per genotype are shown. Bar = 50 μm . (D) H+E staining of islets in pancreatic sections from WT and Mark2^{-/-} mice. Bar = 50 μm . (E) Histogram showing number of b cells per 10,000 μm^2 (area of a medium sized islet) in pancreatic sections from L/L or LABKO mice. Effect of low dose rapamycin treatment on b cell size is shown (n=3-4; * $p < 0.04$; *** $p < 0.000004$). (F) Western blot analysis showing elevation in S6K phosphorylation, indicative of mTor pathway activation, in extracts of islets isolated from LABKO mice 18 weeks after tamoxifen injection. Western blots were reprobed with antibodies to total S6K, P-Ampk, Ampk, Lkb1, and b actin.

in LABKO islets. When this was quantitated, we found a 25% reduction in the number of b cells per unit area in LABKO islets, indicating that b cells lacking Lkb1 are larger than controls (Figure 5E).

Ampk activation promotes catabolic events and inhibits anabolic activities such as protein translation, which is directly linked to the growth state of the cell and controlled by mTOR kinase. To determine if increased mTor activity is required for b cell hypertrophy in islets of LABKO mice, we treated animals with a low dose of rapamycin to inhibit mTOR kinase activity in vivo. We observed that 7 days of rapamycin treatment increased cell number per unit area in control L/L mice and was also sufficient to revert the b cell hypertrophy in LABKO animals (Figure 5E). Consistent with this, in LABKO islets we observed increased phosphorylation of the mTor target S6 kinase, which serves as a readout for the activity of the protein translation machinery and of cell growth (Figure 5F). Taken together, these data

are consistent with the idea that loss of Ampk activity in cells lacking Lkb1 underlies the β cell hypertrophy in the LABKO islets.

Discussion

It is becoming increasingly clear that the diverse biological consequences of loss of Lkb1 activity, including tumor susceptibility and loss of polarity, result from dysfunction of members of the AMPK family, including AMPK itself. Our data provide further evidence for the emerging concept that there is an intimate connection between glucose metabolism/energy balance and polarity under control of Lkb1, here in pancreatic islets.

b cell mass is governed by at least four parameters, including proliferation, apoptosis, cell size and neogenesis (Ackermann and Gannon, 2007). We have found that within 7-10 days after acute Lkb1 deletion, b cell mass is increased, insulin accumulates in islet b cells and its secretion is enhanced, indicating that these are highly dynamic processes subject to fine control by Lkb1. Importantly, the improvement in glucose clearance in vivo is preserved under high fat diet challenge and sustained for at least five months after loss of Lkb1.

Moreover, the accumulation of Lkb1 protein in islets of mice rendered glucose intolerant due either to leptin mutation (ob/ob mice) or by excessive caloric intake supports the idea that Lkb1 may serve to antagonize b cell function in these settings. Future work will address the potential for regulation of apoptosis and neogenesis by Lkb1.

While the role of Ampk in b cell function in vivo has not been established, and defining a precise role for Ampk in insulin secretion has been controversial (da Silva Xavier et al., 2000; Gleason et al., 2007; Leclerc et al., 2004; Richards et al., 2005), our data are consistent with Ampk $\alpha 1$ having an inhibitory effect on insulin accumulation and secretion in the basal

state. While Ampk $\alpha 1$ knockout mice display normal glucose tolerance (Viollet et al., 2003), this may be due to the combined effects of loss of Ampk $\alpha 1$ from other tissues that influence glucose metabolism. That Lkb1 is the critical upstream regulator of Ampk activity in β cells is reinforced by the activation of mTOR pathway in LABKO islets and the Lkb1 phenocopy seen in Ampk $\alpha 1$ knockdown cells. The increase in islet insulin content is reflected in the hypertrophy of β cells lacking Lkb1, which together with the increased proliferative index results in a doubling of β cell mass in LABKO mice, observations reminiscent of mice with a deletion of Tsc2 in the β cell (Rachdi et al., 2008). As loss of Lkb1 removes a break on translation via AMPK-mTOR that would normally exist under nutrient deprivation (Shaw et al., 2004a), we suggest that the Lkb1:Ampk signaling cassette in mature β cells serves to restrain insulin secretion by limiting insulin stores. Indeed, Ampk activity in cultured β cell lines is activated by pioglitazone and metformin, both of which attenuate insulin secretion (Lamontagne et al., 2009; Leclerc et al., 2004). While the increased insulin secretion with loss of Lkb1 in vivo appears to result predominantly due to increased β cell mass, and in vitro from increased insulin content, there appears to be some contribution due to improved coupling of secretion to glucose as revealed by enhanced first phase secretion in perfused islets and the accelerated calcium influx following glucose stimulation in MIN6 cells. Notably, impaired first phase secretion is a hallmark of Type 2 Diabetes (Mitrakou et al., 1992); our data suggest that inhibiting Lkb1 in β cells may help to overcome such a deficiency.

A recent study reported a whole pancreas knockout of Lkb1 in mice, characterized by acinar cell degeneration and pancreatic insufficiency, development of a PJS-associated tumor type

(serous cystadenomas), smaller islets with a normal morphology and balance of cell types and improved glucose tolerance at 6 weeks of age (Hezel et al., 2008). In carcinogenesis, loss of Lkb1 promotes cell proliferation due to failure of the maintenance of cytoskeletal integrity (Hezel et al., 2008). β cells lacking Lkb1 showed alterations in nuclear distribution indicating the Lkb1 signaling contributes to steady-state determination of nuclear positioning in the islet. Of the candidates in the AMPK family that may mediate the positioning defect, AMPK1/2, MARK and SAD kinases, which have all been implicated in establishment of cell polarity (reviewed in (Hurov and Piwnicka-Worms, 2007)), H+E staining of pancreatic sections from Mark2^{-/-} mice indicate that the Lkb1-Mark2 pathway, at least in part governs this phenotype. While the precise reason for this effect is unknown, one possibility is that mature, non-dividing β cells are functionally polarized and this information is lost in the absence of Lkb1. Given the concomitant increase in the proliferative index in β cell populations lacking Lkb1, this suggests that the loss of polarity in the absence of Lkb1 may help to promote proliferation. It will be of interest to determine if proliferating cells undergo structural alterations that are governed by these polarity genes, which would indicate that cell polarization may serve to restrain proliferation in contexts where β cell expansion is compromised.

The absence of Lkb1 from the liver during feeding permits unrestrained gluconeogenesis owing to a failure to inhibit CRTC2:CREB activity (Shaw et al., 2005). We conclude that the same pathway in liver and endocrine pancreas acts with respect to glucose metabolism in a diametrically opposite fashion at the level of the whole organism. AMPK has attracted attention as a potential target for treatment of Type II diabetes (Hardie, 2007a), largely due

to its effects on liver and muscle glucose and lipid metabolism. The role of AMPK in stimulus-coupled insulin secretion is controversial (da Silva Xavier et al., 2003; Gleason et al., 2007; Richards et al., 2005), but given the complete inactivation of Ampk in glucose-stimulated b cells, it is unlikely to play a role in acute regulation of insulin secretion. Our data indicate that loss of Lkb1 and AMPK/mTOR activity in b cells promotes responses from the b cell mass, including b cell proliferation, cell growth, and generating a fertile soil for enhanced insulin secretion, all of which are consistent with the goals of b cell focused therapies for type I and type II diabetes. Furthermore, our data indicate that inhibition of pancreatic Lkb1 with small molecule antagonists may increase b cell mass thereby providing therapeutic benefit for hyperglycemia.

Experimental Procedures

Mice

Lkb1^{loxP/loxP} (FVB/n background, (Hezel et al., 2008) and Pdx-CreERT2 mice (ICR background; kind gift of D. Melton) were mated to generate Lkb1^{loxP/loxP}, Pdx-CreERT2⁺ mice. The Pdx-CreERT2 mice were backcrossed three times onto FVB/n background and then intercrossed. To induce Lkb1 gene ablation, 5 mg of tamoxifen (Sigma) per 40 g body weight was injected IP every second day for a total of 3 injections. Unless otherwise stated, analyses were performed 5-10 days after the last tamoxifen injection. For high fat diet studies, mice were fed normal lab diet or high fat diet (45 kcal% fat; Open Source Diets) for 16 weeks. Mark2/EMK +/- mice were the kind gift of H. Piwnicka-Worms. All procedures involving mice were approved by the Animal Care Committee of the University of Ottawa.

Glucose tolerance and insulin tests

Animals were fasted overnight and their fasting level of blood glucose was evaluated with an Ultra OneTouch glucometer (Lifescan). Mice were then injected IP with 2 mg/g body weight (normal diet) and 1.5 mg/g body weight of glucose, and blood glucose and insulin levels were measured at the indicated times. Plasma insulin levels were measured by ELISA using an ultra-sensitive mouse insulin kit (Crystal Chem). Statistical significance for all data was determined using Student's *t*-test; *p*-values < 0.05 were considered significant. Significance is indicated in all figures as follows: * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.005.

Immunostaining

Pancreata from 4 $Lkb1^{loxP/loxP}$ and 4 $Lkb1^{loxP/loxP}$, Pdx-CreERT2 mice were isolated after perfusion with fixative (4% PFA in PBS), weighed and stored in fixative overnight at RT. Sections were treated in an Antigen Unmasker (Pickcell) and stained with anti-LKB1 antiserum raised against the C-terminus of mouse $Lkb1$ (1:100), anti-insulin (1:200, DAKO), somatostatin/glucagon/PP antibody cocktail (all at 1:200, DAKO), and revealed using HRP- or Alexa dye-conjugated secondary antibodies for multiple labeling (Invitrogen). Sections were mounted in Vectashield with DAPI (Vector Labs) and images captured using a Zeiss AxioSkop2 microscope fitted with a CCD camera. For b cell and α cell mass measurements, three serial sections of 4 μ m were prepared from each pancreas at 100 μ m apart. One section from each level was stained with insulin (Biomed Diagnostics, 1:200) or glucagon and HRP-conjugated secondary antibodies using DAB as chromogen. Hormone-positive areas in three sections were quantified using Aperio Imagescope software by dividing the total insulin positive area by the total area and multiplying this value by the pancreas weight.

b cell size and proliferation

To calculate the mean size of individual b cells, the b cell area (marked by staining with insulin antibodies) was divided by the number of nuclei in insulin+ cells, counting 3 islets in 6 sections per mouse and 4-5 mice per genotype per treatment. Data are presented as the number of b cells per 10,000 μ m², the size of an average-sized islet. Rapamycin (0.6 mg/kg; LC Laboratories) was injected every second day for a total of 3 injections starting 1 day after the last tamoxifen injection. For BrdU incorporation studies, animals were injected at 8 weeks with tamoxifen and 10 days later with 0.1 mg/g body weight BrdU in PBS for 6 hr, perfused with 4% PFA and processed for staining with anti-insulin and anti-BrdU (1:150,

Sigma) antibodies. The number of insulin+, BrdU+ cells in a minimum of 1500 total insulin+ cells per pancreas was determined and presented as the number of BrdU+ b cells/1000 b cells.

RNA interference

MIN6 cells (p. 21-30) were cultured in DME + 10% FCS + antibiotics containing 100 μ M β -mercaptoethanol. siRNAs were transfected into cells using 1.1 μ l Dharmafect 1 (Dharmacon) or 1 μ l RNAiMAX (Invitrogen) per well. On day 0, 1.8×10^5 cells (for Dharmafect) or 1.0×10^5 cells (for RNAiMAX) were seeded in 48-well plates and transfected with 40 nM of the indicated siRNA (Smartpool, Dharmacon) or control siRNA at the time of seeding. Medium was changed on days 1 and 2 prior to GSIS analysis on day 3.

Glucose Stimulated Insulin Secretion (GSIS)

24 h after isolation, 20 size-matched small and medium islets were cultured in RPMI were equilibrated with Krebs 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, 0.1 % BSA] + 1 mM glucose for 30 min, prior to incubation in 500 μ l KRB + 1 mM glucose for 1 hr. The SN was removed and stored, and islets were stimulated with 500 μ l of KRB + 20 mM glucose for 1 hr. All steps were performed at 37°C in a tissue culture incubator. For islet perfusion, 50 small islets per genotype were preincubated in KRB + 2.8 mM glucose for 45 min, following which 1 ml fractions were collected from islets in KRB + 2.8 mM or 20 mM glucose. Islets were depolarized with 45 mM KCl. For MIN6 experiments, cells in 48 well plates were first equilibrated with 250 μ l KRB + 1 mM glucose for 1 hr, then incubated in

KRB + 1 mM glucose for 1 hr (basal). Cells were stimulated with KRB + 20 mM glucose or KRB + 45 mM KCl for 1 hr. Three independent wells were used per condition. For all experiments, total insulin was harvested by acid-ethanol extraction overnight. Insulin measurements were performed by HTRF assay (Cisbio) for GSIS experiments or by ELISA (Crystal Chem) for serum insulin.

Western Blotting

Western blots were performed as described (Screaton et al., 2004). 80-100 isolated islets were starved with 2.8 mM glucose for 2 hr then treated with the indicated concentrations of glucose for 1 hr prior to lysis directly in Laemmli sample buffer. Antibodies used were Lkb1: Santa-Cruz, 1:500; GAPDH: Advanced Immunochemical Inc. 1:20,000; β -actin: Abcam, 1:10,000; ATF-1: Cell Signaling Technology, 1:1,000, pThr172-AMPK 1:500; AMPK 1:1,000; pT hr389-S6K, 1:1000; s6K, 1:1000; p-T56 eEF2 1:1000; eEF2 1:1000.

Measurement of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

$[\text{Ca}^{2+}]_i$ was measured using the fluorescent probe fura-2AM (Molecular Probes, OR) as described (Tabet et al., 2004) with the following modifications: on the day of the study, the culture medium was replaced with prewarmed KRB containing 1 mM glucose. Cells were loaded with fura-2AM dissolved in DMSO containing 0.02% pluronic F-127 (Molecular Probes) at a final concentration of 4 μM for 30 minutes at 37°C in a humidified incubator. Under these conditions, ratiometric fluorescence cell images are homogeneous, indicating no significant dye compartmentalization. Loaded cells were washed at RT and used after a 5- to 10-min stabilization period. Coverslips were placed in a stainless steel chamber of an

inverted microscope (X10 objective) with a Stallion High Speed Digital Microscopy Workstation imaging system (Slidebook, Zeiss). Fura-2AM loaded cells were excited at 340 and 380 nm and their emission at 510 nm was monitored. $[Ca^{2+}]_i$ responses were measured in cells exposed to KRB + 20 mM glucose for 10 min. Latency data are derived from calcium traces of >50 MIN6 cells per field.

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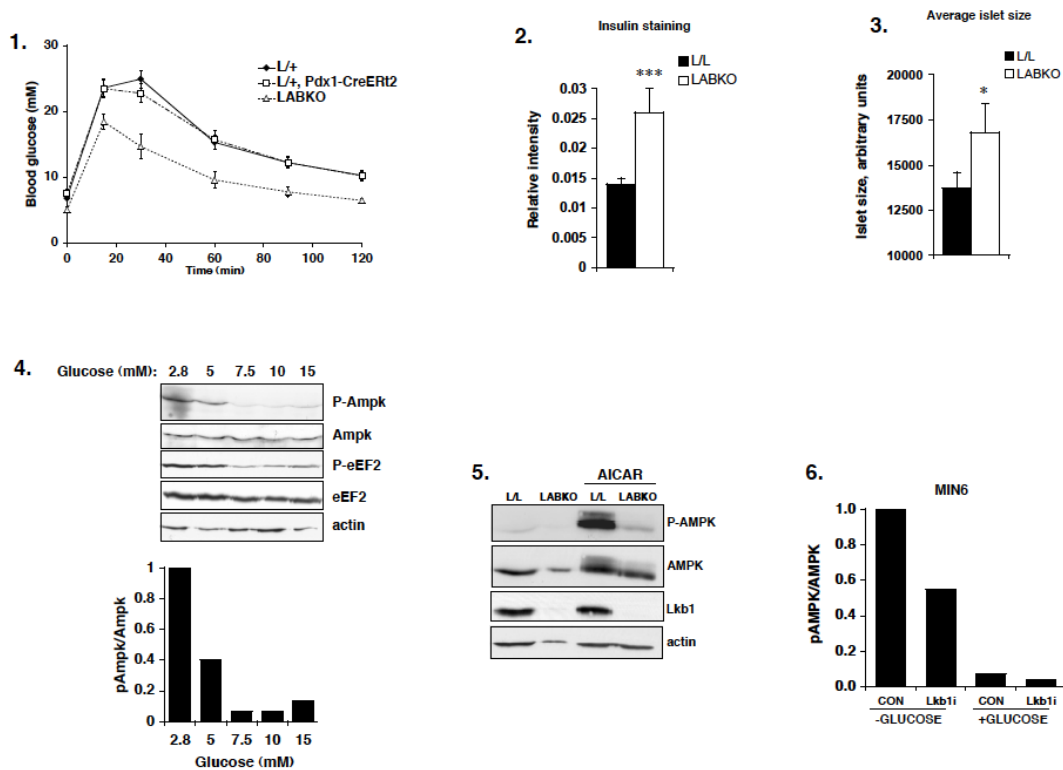
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Supplemental Data



(1) Glucose tolerance test (2 mg glucose/g, IP injection) for tamoxifen treated LABKO mice and L/+ strains with or without the Pdx-CreERT2 transgene. n=7-9 for all groups. Blood glucose concentrations were determined at indicated times. All data are represented as mean $\pm$ SEM.

(2) Histogram showing densitometric analysis of insulin staining in control and LABKO islets (n=5-6, *** p < 0.005). All data are represented as mean $\pm$ SEM.

(3) Histogram showing islet size in LABKO and control L/L mice derived from staining of sections stained with insulin antibody shown in Figure 3B. Size is expressed in arbitrary units (n=10, * p < 0.03). All data are represented as mean $\pm$ SEM.

(4) Top, Western blot analysis showing effect of 1 hr treatment of isolated islets from wild type FVB mice with KRB containing increasing concentrations of glucose on levels of P-

Ampk and the Ampk target eEF2. Islets were incubated for 2 hr in 2.8 mM glucose prior to stimulation as indicated. Bottom, histogram showing ratio of P-AMPK/AMPK signals normalized to P-AMPK/AMPK ratio in low glucose (2.8 mM) quantitated from Western blots shown in (A).

(5) Western blot analysis showing effect of 1 hr treatment of islets from LABKO and control L/L mice cultured in 16.7 mM glucose with or without 400 uM AICAR treatment for 1 hr. Lkb1 and actin loading control blots are shown.

(6) Histogram of densitometric analysis of Western blots in control or Lkb1 knockdown MIN6 cells (from Figure 4A) showing P-Ampk levels normalized to total Ampk levels in low (1 mM) or high (20 mM) glucose.

Chapter 5. Role of AMPK in pancreatic beta cell function.

Review published:

Fu A, Eberhard CE, Sreaton RA. Role of AMPK in pancreatic beta cell function. Mol Cell Endocrinol. **2013** Feb 25;366(2):127-34.

Summary and contributions:

We were invited to write a review in a special issue of the Molecular and Cellular Endocrinology journal focused on AMPK. In this review I described the insight gained on the role of AMPK in beta cell functions from the use of mouse models.

My overall contribution to writing the review was over 50% of the total content in the review, and I as a result I am first author on the review. Dr. Sreaton and I contributed the overall flow, outline and ideas for what would be discussed in this review. The review was written, edited and discussed by Chandra Eberhard and I along with Dr. Sreaton.

The review consists of 11 sections, I wrote 7 of these sections contributing to more than half of the literature review content, where equal contributions were made to 3 other sections, including introduction, conclusions and clinical considerations. In these 7 sections I discuss the regulation of AMPK activity in beta cells, the effects of AMPK agonists on beta cell dysfunction and cell death, the phenotypes of AMPK $\alpha 1$ and AMPK $\alpha 2$ knockout mice, the role of LKB1 in beta cells, the effects of downstream AMPK targets in beta cells and the role of all other AMP kinases on beta cell function (Mark2, 3, Sik2, SNARK).

Chandra Eberhard wrote 4 sections on her own discussing the acute versus long term effects of AMPK activity on GSIS, and the differences of outcomes from using pharmacological versus genetic approaches.

I thought of and designed the model slide in figure 1 of the role of AMPK in the insulin secretion pathway. Chandra Eberhard compiled the all of the information in Table 1 and generated the panels in Figure 2.

Title: Role of AMPK in pancreatic beta cell function.

Abstract: Pharmacological activation of AMP activated kinase (AMPK)¹ by metformin has proven to be a beneficial therapeutic approach for the treatment of type II diabetes. Despite

¹ **Abbreviations:**

ACC1: acetyl-CoA carboxylase 1, AICAR: 5-amino-4-imidazole carboamide, AMPK: AMP activated kinase, BAX: Bcl-2 associated X protein, BCL-2: B-cell lymphoma 2, BID: BH3 interacting domain death agonist, BIM: Bcl-2-interacting mediator, BRSK: Brain selective kinase, CA-AMPK: constitutively active AMPK, CAMKK2: Calmodulin-dependent protein kinase kinase 2, CREB: cAMP response element binding protein, CRTC: Creb regulated transcription coactivator, DN-AMPK: dominant negative AMPK, GLUT: Glucose transporter, GSIS: glucose stimulated insulin secretion, HIT: hamster insulinoma beta cell line, HMG: 3-hydroxy3-methylglutaryl, INS-1: rat insulinoma1 cell line, IBMX: Isobutylmethylxanthine, JNK: Jun kinase, MARK: MAP/microtubule affinity-regulating kinase, MELK: maternal embryonic leucine zipper kinase, MIN6: mouse insulinoma beta cell line, MIP: Mouse insulin promoter, mTOR: Mammalian target of rapamycin, NIAK: SNF-1 like kinase, POMC: proopiomelanocortin, PP1: Protein phosphatase 1, PPAR: Peroxisome proliferator-activated receptor, QIK: salt inducible kinase 2, QSK: salt inducible kinase 3, RIP2: Rat insulin promoter 2, ROS: reactive oxygen species, SIK: salt inducible kinase, SREBP: Sterol regulatory element-binding protein, STZ: streptozotocin, T2D: type II diabetes, TAK1: transforming growth factor beta-activated kinase, TSC2: tuberous sclerosis complex 2, TZDs: thiazolidinediones¹

improved glucose regulation achieved by administration of small molecule activators of AMPK, the potential negative impact of enhanced AMPK activity on insulin secretion by the pancreatic beta cell is an important consideration. In this review, we discuss our current understanding of the role of AMPK in central functions of the pancreatic beta cell, including its expression, regulation, and effect on central beta cell functions including glucose-stimulated insulin secretion (GSIS), proliferation, and survival. In addition we discuss the controversy surrounding the role of AMPK in insulin secretion, underscoring the merits and caveats of methods used to date.

Introduction: Pancreatic beta cell function deteriorates in T2D resulting in insufficient insulin secretion to meet higher demand. Activation of AMP activated kinase (AMPK) has been pursued as a potential strategy for the treatment of T2D, in large part due to AMPK-mediated inhibition of gluconeogenesis in the liver during feeding [1]. Consideration of the impact of targeting AMPK on other tissues, including pancreatic beta cells, will be crucial for evaluating the therapeutic potential of this approach. For a detailed discussion of AMPK as a potential drug target for treating T2D and its biological role in other tissues, we refer the reader to excellent recent reviews and articles in this issue [2, 3]. In this review, we will discuss our current understanding of the role of AMPK in central functions of the pancreatic beta cell, such as glucose-stimulated insulin secretion (GSIS), proliferation, and survival.

AMPK expression in the beta cell. AMPK is a heterotrimeric enzyme consisting of α (catalytic) and β/γ (regulatory) polypeptides, of which there are multiple isoforms for each. AMPK α 1 and α 2 proteins are detected in the rodent insulinoma cell lines MIN6 and INS1

[4, 5]. While the $\alpha 1$ subunit is predominately a cytoplasmic protein, $\alpha 2$ is present throughout the cell. Studies using immunodepletion and knockout animals have indicated that AMPK $\alpha 1$ is the major catalytic subunit found in both MIN6 cells and islets, and accounts for the vast majority of AMPK catalytic activity [5, 6]. Neither expression nor subcellular localization studies have been performed for AMPK beta and gamma subunits in beta cells.

Regulation of AMPK activity in beta cells. AMPK activity is regulated by nutrients (glucose and amino acids) [7], hormones, calcium, and cellular stress, and coordinates these signals via phosphorylation of numerous targets involved in glucose metabolism, ATP consumption and protein synthesis [8]. Interestingly, the cAMP agonists forskolin and IBMX do not impact AMPK activity, indicating that hormone-derived feeding signals are unlikely to modulate the enzyme [7]. In general, AMPK activity is regulated at the level of ***allosteric activation*** by adenine nucleotides and by direct ***phosphorylation by upstream activating kinases***, including LKB1, CAMKK2 and TAK1 [9-11]. Binding of AMP and ADP at two sites on the gamma subunit leads to phosphorylation of AMPK and a conformational change that prevents dephosphorylation, thereby maintaining AMPK activity [12, 13]. In contrast, binding of ATP promotes dephosphorylation. AMPK activation is modulated by the ATP, AMP and ADP levels as well as phosphorylation by an upstream AMPK kinase, LKB1 at Thr-172 of the α subunit [14]. Loss of LKB1 in beta cells abolishes AMPK phosphorylation at Thr172 and phosphorylation of AMPK target proteins, suggesting that LKB1 is a key regulator of AMPK in this setting (see below) [15-17]. Evidence for AMPK activation by Ca^{2+} /Calmodulin-dependent Kinase Kinase 2 (CAMKK2) or Transforming growth factor beta-Activated Kinase (TAK1) has been observed in HeLa, neuronal and yeast cells, but not

in beta cells to date [18-20]. As LKB1 is thought to be constitutively active, regulation of stimulus-dependent alterations in the phosphorylation status of AMPK occurs as a result of AMP/ADP binding, or through phosphatase-mediated dephosphorylation. Knockdown of the R6 regulatory subunit for protein phosphatase 1 (PP1) reduced PP1-R6 activity, preserved P-AMPK levels in high glucose, and inhibited insulin secretion in MIN6 cells, all of which required glucokinase activity [21]. These data indicate that AMPK activity is controlled following a glucose challenge by a regulated dephosphorylation step [21]. As beta cells have distinct glucose metabolism processes and rely heavily on glucose and energy sensing for function and survival, it will be important to validate the general mechanisms of AMPK activation in the beta cell which have been delineated to date in liver and skeletal muscle.

Role of AMPK in Insulin Secretion. Insulin secretion is triggered by glucose (and other nutrients including fatty acids and amino acids), which is metabolized to generate ATP in a stepwise fashion by glycolysis and the Krebs cycle in mitochondria (Figure 1). Elevated intracellular ATP triggers the stepwise closure of ATP-sensitive K^+ ion channels, plasma membrane depolarization, and calcium influx via voltage sensitive Ca^{2+} ion channels, which ultimately leads to insulin granule mobilization and insulin release [22-25]. The role of AMPK in glucose stimulated insulin secretion (GSIS) still requires resolution, despite over 20 published works devoted directly to the topic, as contradictory evidence for a role of AMPK as both a positive and negative regulator of insulin secretion has been reported.

Early evidence that AMPK regulation may be important for the maintenance of beta cell function, including insulin secretion, came from the observation that high passage hamster

insulinoma (HIT) cells, unlike their low passage counterparts, have lost their ‘glucose responsiveness’, i.e. the capacity to secrete insulin and reduce AMPK activity in response to glucose [26]. In addition, an increase in K/ATP channel trafficking to the cell

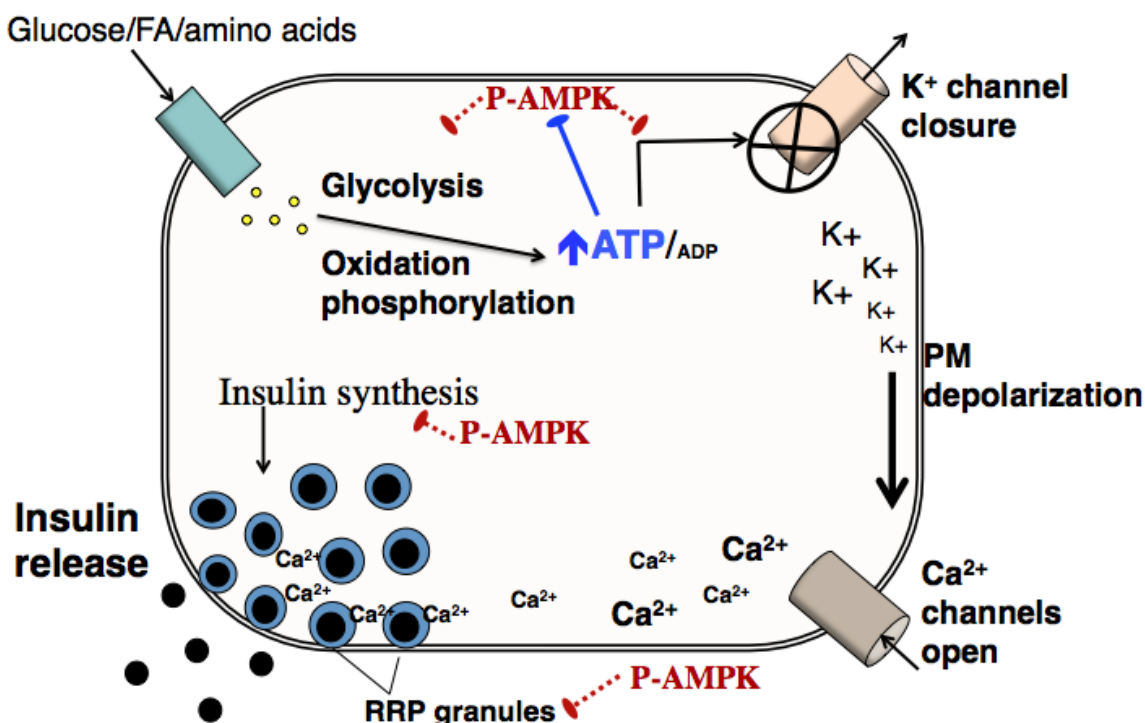


Figure 1. Role of AMPK in insulin secretion. Model of the events required for insulin secretion in pancreatic beta cells highlighting the role of AMPK. Nutrient uptake and metabolism increases the ratio of ATP/ADP leading to closure of the K⁺/ATP channel. Accumulation of K⁺ inside the cell leads to membrane depolarization that in turn opens voltage-sensitive calcium channels and calcium ion influx. Calcium triggers exocytosis of the RRP (readily releasable pool) of insulin granules docked at the plasma membrane resulting in the ‘first phase’ of insulin secretion. Secondary recruitment of distal granules also requires calcium and results in a lower magnitude ‘second phase’ of insulin release. AMPK may control several of these events to inhibit insulin secretion, including

glucose/nutrient metabolism, K⁺/ATP channel trafficking to the plasma membrane, insulin granule docking, and insulin gene transcription. The sum of AMPK's actions supports the concept that AMPK is a negative regulator of insulin secretion.

surface in cells treated with AICAR, an AMP analog that acts as an AMPK agonist, under high glucose conditions suggested a possible link between AICAR, AMPK and inhibition of insulin secretion via the K/ATP channel [27]. However, K/ATP independent effects of AICAR have also been reported in the beta cell [28]. Several studies over the past decade have challenged the notion that AMPK is required for insulin secretion. Without question a number of variables, including differences in cell type, glucose concentration, dosage of small molecule activators, and length of treatments have clouded the issue. For the reader's convenience, we have summarized these data in Table I. Below, we present the arguments for a role for AMPK in the process of insulin secretion in the form of a debate, 'The Government' vs. 'The Opposition'. Specifically, we discuss the evidence that AMPK regulates insulin secretion in 1) an acute treatment setting (hours) in which pharmacological agents are used to alter AMPK activity throughout the duration of insulin secretion assays, and 2) a chronic treatment setting (days), using both pharmacological and genetic approaches.

'The Government' - AMPK promotes GSIS.

A significant amount of *pharmacological* data generated using biguanides such as metformin and phenformin or AICAR have provided insight into the effects of *acute AMPK activation* on insulin secretion. Prompted by the observation that AICAR inhibits gluconeogenesis in hepatocytes, early work on AMPK in beta cells aimed to evaluate the impact of maintaining

AMPK activity with chronic AICAR treatment, i.e. throughout the duration of the insulin secretion assay [29]. Perfusion of pancreas at an intermediate concentration of glucose (6 mM) together with increasing doses of AICAR enhanced

Table 1
Overview of the impact of acute pharmacological activation of AMPK on GSIS.

Drug	GSIS (% of control)	Drug treatment (mM)	Glucose treatment (mM)	Time (min)	Cells	AMPK activity	Reference
AICAR	100–400	Co-treat 0.1–1	6	20	Perfused pancreas	Not shown	Akkan and Malaisse (1994)
	200	Pre-incubation only: 0.1–1	8.3	60	Rat islets	Not shown	Malaisse et al. (1994)
	25	Co-treat: 1	20	60	MIN6	ACC proxy	Zhang and Kim (1995)
	10–20	Co-treat: 0.2	30	20	MIN6	P-AMPK at 1hr in	da Silva Xavier et al. (2003)
		Co-treat: 0.4	16	20	Rat islets	MIN6.	
	150–200	Pre/Co-treat: 0.3	Pre-starve: 2 Treat: 5, 8, 14	30	Mouse islets	AMPK activity	Wang et al. (2005)
	100	Co-treat: 1	0 and 15	60	MIN6	P-AMPK and P-ACC	Targonsky et al. (2006)
	125	Co-treat: 0.2, 1	Pre-starve: 0 Treat: 30	90	MIN6	P-AMPK	Gleason et al. (2007)
	130 +/- DN AMPK	Pre/Co-treat 1	Pre-starve: 3 Treat: 12	120	INS-1 832/13 cells	AMPK activity	
	150–200	Pre/Co-treat: 1	Pre-starve: 3 Treat: 5, 8.3	120	Rat islets	Not shown	
Metformin	100	Co-treat: 1	0 and 15	90	MIN6	P-AMPK and P-ACC	Targonsky et al. (2006)
Troglitazone	50	Co-treat: 0.01	16.7	60	Rat islets	Not shown	Wang et al. (2007)
			25		MIN6	P-AMPK and P-ACC	
Berberine	50	Co-treat: 0.0025	16.7	60	Rat islets	Not shown	Zhou et al. (2008)
			25		MIN6	P-AMPK and P-ACC	
Pioglitazone	50	Pre/co-treat: 0.05	Pre-starve: 1 Treat: 25	45	INS-1	P-AMPK P-ACC	Lamontagne et al. (2009)

Table 1. Summary of published experimental conditions used to date for pharmacological analysis of AMPK activity and GSIS in the pancreatic beta cell.

insulin secretion, suggesting that AMPK activation promotes insulin secretion [30].

However, subsequent removal of AICAR resulted in a further increase in insulin secretion, raising the contradictory possibility that AMPK *inactivation* promotes insulin secretion [30].

Follow-up studies reported that AICAR treatment increased insulin secretion in cell lines and rat islets across a large range of glucose concentrations [4, 26, 28, 31, 32]. The effect of AICAR on the enzymatic activity of AMPK was reported in some but not all of these studies, precluding correlation of AICAR treatment with the anticipated increase in AMPK

biochemical activity. For instance, in high glucose, conditions in which AICAR elicits the greatest increase in AMPK activity, only mild potentiation of insulin secretion has been observed [4, 28, 32]. In our hands AICAR treatment increased both P-AMPK levels and GSIS in mouse insulinoma cells (MIN6) across a range of doses used in published work, which saturated at 0.4 mM AICAR (Figure 2). Perhaps most importantly, it remains unclear if the observed effects on GSIS result from AMPK-independent effects of AICAR. Thus an overreliance on AICAR as a pharmacological tool makes drawing conclusions regarding AMPK as an activator of GSIS a challenge.

'The Opposition' - AMPK inhibits GSIS

Acute pharmacological activation of AMPK inhibits insulin secretion

Substantial evidence suggests that AMPK negatively regulates insulin secretion. The first evidence for a negative role of AMPK in insulin secretion was reported in a study investigating glucose activation of Acetyl-CoA carboxylase 1 (ACC1), an AMPK substrate, in INS1 cells [33], in which AICAR both inactivated ACC1 and blocked GSIS. Consistent with this idea, AMPK activity was observed to be inversely related to insulin secretion across a wide range of glucose concentrations in the rodent insulinoma INS1, MIN6 and HIT cell lines [7, 26]. Similar observations were made when amino acids (Gln, Leu, Arg) were used as secretagogues [7]. Subsequent studies confirmed that acute/short term treatment with AICAR reduced insulin secretion triggered by glucose in MIN6 cells and isolated rat islets [26, 34, 35]. However, when documented, AMPK activity was not determined under the same conditions used for GSIS making it difficult to correlate AMPK activity with secretion [34]. The use of the anti-diabetic drugs thiazolidinediones (TZDs) has provided additional

support for an inhibitory effect of AMPK on GSIS. Interestingly, TZDs were originally

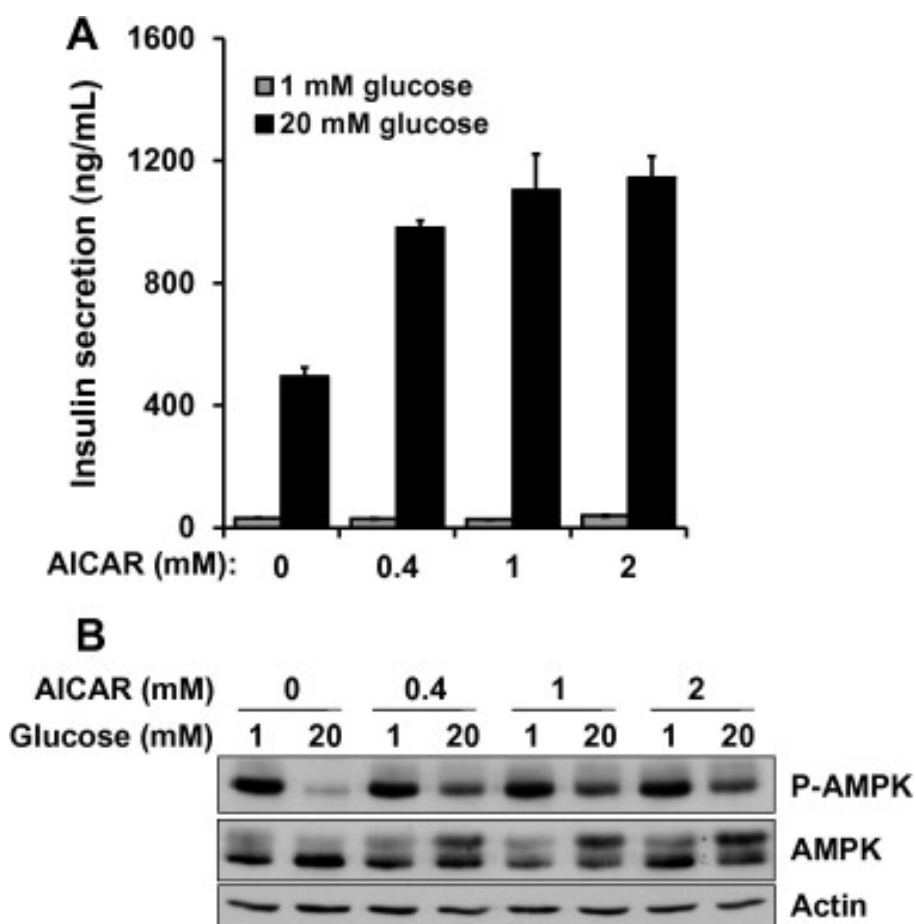


Figure 2. AICAR maintains AMPK phosphorylation and potentiates insulin secretion

in MIN6 cells. A) AICAR potentiates glucose-stimulated insulin secretion in MIN6 cell.

MIN6 cells were pre-starved for 30 min in 1 mM glucose in KRB with the indicated

concentration of AICAR. Insulin secretion was measured from MIN6 cells after 1 hr

incubation with 1 mM glucose in KRB and again after a 1 hr incubation in 20 mM glucose

KRB. When present, AICAR was used at the indicated concentrations throughout

treatments. **B)** Phosphorylation of AMPK at Thr-172 is elevated under high glucose

conditions in MIN6 cells treated with a range of AICAR concentrations. Cells harvested for

P-AMPK determination by western blot were treated in parallel and harvested at the end of the GSIS experiment.

thought to act through PPAR gamma, yet they have recently been shown to require AMPK for their biological effects first described in muscle cells [35]. Treatment with TZDs, including troglitazone, berberine and pioglitazone, can reduce insulin secretion and activate AMPK [36-38].

Long term AMPK activation by pharmaceutical agents inhibits insulin secretion

While results with acute activation of AMPK on GSIS have varied, long-term (i.e. several days) activation of AMPK has consistently been shown to inhibit insulin secretion in beta cell lines and islets [39-41]. Furthermore, AICAR inhibition of GSIS is restored by co-treatment with the AMPK inhibitor Compound C, indicating that impaired GSIS by AICAR occurs through activation of AMPK [42].

Long term modulation of AMPK activity using cDNA overexpression

Additional support for AMPK inhibition of GSIS has come from the use of ***dominant negative (DN-AMPK)*** and ***constitutively active AMPK (CA-AMPK)*** constructs. Under high glucose, AMPK kinase activity falls by ~ 50% [4, 5, 26, 28]. Similarly, overexpression of DN-AMPK in low glucose lowers AMPK activity by 50%, mimicking high glucose treatment. In contrast, expression of CA-AMPK maintains AMPK activity in high glucose. When the effects of these constructs were evaluated in MIN6 cells and human islets, DN-AMPK increased insulin secretion under low glucose conditions, whereas CA-AMPK

activity completely blocked GSIS [34, 41]. In contrast, DN-AMPK overexpression in INS1 cells elicited a modest reduction in AMPK activity and had no effect on GSIS [32]. In high glucose when AMPK activity is already reduced, DN-AMPK reduced AMPK activity a further 20% and moderately increased GSIS in human islets, but not cell lines or rat islets [41]. Perhaps most importantly, transplant of CA-AMPK islets into STZ-induced diabetic mice blocked normal restoration of glucose tolerance, whereas DN-AMPK islets improved glucose tolerance with greater efficacy than wt islets [43]. Of note, an increase in apoptosis in CA-AMPK infected cells may have contributed to impaired glucose clearance in the CA-AMPK setting, raising the possibility that CA-AMPK also affects beta cell survival.

From this work, two conclusions emerge: 1) long-term AMPK activation impairs insulin secretion and 2) restriction of AMPK activity at low levels increases insulin secretion. Indeed, expression of CA-AMPK (but not of DN-AMPK) in MIN6 cells reduced the number of docked insulin granules, glucose-stimulated vesicle movement, and actin reorganization required for exocytosis [44]. CA-AMPK expression blocked a rise of ATP and Ca^{2+} in response to glucose and inhibited insulin secretion stimulated by a low concentration (10 μM) of the sulfonylurea drug tolbutamide, suggesting that the impaired depolarization results from a reduced capacity to generate malonyl-CoA and, in turn, ATP [34, 45]. However, while the use of CA-AMPK has provided a potential mechanism for beta cell dysfunction caused by constitutive AMPK activity, thereby mimicking conditions of chronic pharmacological activation of AMPK, it does not permit evaluation of acute activation of AMPK on GSIS. Furthermore, a clear understanding of the relationship between AMPK activity and GSIS is complicated by the lack of consistent documentation of AMPK activity

with these constructs using the same conditions in the insulin secretion experiments.

So, why is there a debate at all? A cogent argument against a role for AMPK in the regulation of acute insulin secretion is that the catalytic activity of the enzyme is at its lowest under glucose-treated ('fed') conditions. That is, as AMPK is most active in low glucose, what role, if any, could AMPK play in insulin secretion in high glucose, i.e. 'fed' conditions. As expected, lowering extracellular glucose from 11 mM to 0 mM in INS1 culture inhibited insulin secretion, yet pre-treatment with the AMPK inhibitor Compound C sustained insulin secretion in low glucose [27], indicating that AMPK blocks insulin secretion under low glucose conditions. As such AMPK would provide a mechanism by which the beta cell can prevent insulin secretion when it is active in low glucose to prevent inappropriate hypoglycemia. Taken together, the preponderance of evidence suggests that AMPK inhibits GSIS, as there is strong correlation between nutrient and pharmaceutical modulation of AMPK activity. Alternatively, it is also possible that inhibition of AMPK in high glucose relieves inhibition of an as-yet-unidentified AMPK target required for insulin secretion. In fact the debate centres largely around the question of what role acute pharmacological activation of AMPK has on short term (1-2 h) GSIS, and in particular, whether or not the biological effects of small molecules are mediated solely by modulation of AMPK. The range of AICAR concentrations we tested resulted in similar induction of P-AMPK and GSIS (Figure 2), suggesting conflicting results in the literature are not the result of a biphasic response of AMPK to AICAR. Moreover, we would argue that higher concentrations of AICAR are perhaps eliciting AMPK-independent effects on the GSIS. Indeed, AMPK-independent effects of AICAR have been documented in other tissues by showing that

AICAR-induced apoptosis in B lymphocytes and breast tumor cell line occurs in the absence of AMPK α 1 [46, 47]. These findings demonstrate the necessity of the genetic approach in order to attribute the impact of a pharmaceutical agent to the expected target. Fortunately, limitations in studying AMPK function using small molecules can now be resolved through genetic approaches. Studies with acute administration of pharmacological agents in the absence of AMPK using RNAi should clarify their effects on AMPK activity.

AMPK agonists, beta cell dysfunction and death. The impact of long term AMPK activation has also been investigated in the context of beta cell survival. Exposure of MIN6 cells cultured in high glucose to AICAR for 24 hours blocks glucose oxidation and insulin secretion, and increases reactive oxygen species (ROS) and apoptotic cell death [48]. This correlated with modest accumulation of pro-apoptotic BH3- domain-containing members of the BCL2 family, BIM, BID, BAX [40], effects that were also achieved by extended culture in low glucose. Survival could be rescued by overexpression of anti-apoptotic BCL2 protein. A similar AICAR treatment protocol increased apoptosis in islets cultured in high glucose but had no impact in low glucose [49]. In addition, AICAR stimulated apoptosis to a greater extent in wt islets than α 2 KO islets, indicating a contribution of AMPK activation in apoptosis. Studies in MIN6 cells reported that low glucose treatment results in apoptosis mediated through the activation of Jun Kinase (JNK) and c-myc. These effects were reproduced by activation of AMPK with CA-AMPK or AICAR, suggesting that prolonged activation of AMPK may play a role starvation mediated apoptosis in beta cells [50, 51]. Conversely, in high glucose (27 mM), treatment with AICAR restored GSIS and reduced

apoptosis in INS1 cells suggesting that inappropriate AMPK inhibition may underlie impaired beta cell function in a glucotoxic environment [52].

An important consideration for the pharmacological approach is that ***AMPK agonists inhibit mitochondrial function***. Mitochondria are central regulators of the insulin secretion process as they use the products of glucose metabolism, amino acids, and fatty acids and convert these via oxidative phosphorylation into ATP thus transmitting the insulin secretion signal. The observation that the biguanide compound metformin inhibits complex I of the mitochondrial respiratory chain in hepatocytes raised the possibility that metformin activated AMPK due to a secondary effect of dysfunctional oxidative phosphorylation [53]. Indeed, complex I inhibition by metformin occurs in MIN6 cells, where it modestly increases lactate production via lactate dehydrogenase (LDH), which is diagnostic of a shift away from reliance on mitochondrial respiration [54]. Interestingly, metformin does not inhibit complex I activity in isolated mitochondria, indicating that the relevant target is a non-mitochondrial cellular component [55]. Unlike metformin, AICAR does not inhibit complex I, suggesting that these agents must work via AMPK-independent means to achieve a subset of their biological effects. Again, the use of genetic means (i.e. siRNA-mediated knockdown) to evaluate a requirement for AMPK for complex I inhibition should clarify these questions. Notwithstanding the extra-mitochondrial effects of these compounds, and given the central role that AMPK serves in cellular energy sensing, it would be reasonable to propose that during starvation AMPK directly affects mitochondrial function, perhaps by promoting the use of distinct fuels (i.e. fatty acids) that are more available under these conditions. It would

be interesting to investigate the potential involvement of AMPK in the beta cell in this setting.

Recent Approaches

AMPK $\alpha 1$ and AMPK $\alpha 2$ knockout mice: Whole animal knockout of the AMPK $\alpha 1$ or $\alpha 2$ catalytic subunit genes revealed a role for AMPK $\alpha 2$ in modulating glucose homeostasis [56, 57]. AMPK $\alpha 2$ knockout mice had high blood glucose levels and low serum insulin and were insulin resistant. Islets from AMPK $\alpha 2$ $-/-$ mice showed a normal insulin secretion profile, consistent with the observations that $\alpha 1$ is the major isoform in beta cells. The lack of phenotype in AMPK $\alpha 1$ knockout mice raises the possibility of compensation from $\alpha 2$. In contrast, the inability of $\alpha 1$ to compensate for loss of $\alpha 2$ in all tissues reveals the potential for $\alpha 1$ tissue-specific roles given that it may not be ubiquitously expressed or active in all tissues. This supports the idea that AMPK $\alpha 1$ can compensate functionally for loss of $\alpha 2$ in beta cells, although secretion from isolated islets from $\alpha 1$ knockout mice has not been evaluated.

Two groups independently generated mice lacking AMPK $\alpha 2$ in beta cells and the brain using RIP2-cre in the background of whole body knockout of AMPK $\alpha 1$ [6, 58]. Both studies reported that these AMPK $\alpha 1$ $-/-$; beta cell $\alpha 2$ $-/-$ mice have impaired glucose tolerance along with reduced plasma insulin as well as a slight but not statistically significant increase in beta cell mass. Sun et al. also observed a significant increase in beta cell proliferation (>2 fold) and reduction in cell size. While both studies reported an increase in insulin secretion in isolated $\alpha 1\alpha 2$ $-/-$ islets under low glucose, Sun et al. observed that these islets secrete more

insulin in response to glucose, whereas Beall et al. observed that they have a defect in GSIS. Age differences in the mice at the time of analysis may have contributed to these phenotypic differences. A complication in these studies is that global loss of $\alpha 1$ or loss of both $\alpha 1$ and $\alpha 2$ in the brain triggered by Cre recombinase expressed from the rat insulin II promoter (RIP2-cre) may mask potential beta cell-dependent defects in glucoregulation [59]. Indeed, AMPK $\alpha 2$ in POMC neurons of the hypothalamus (areas in which RIP2 expression has been observed) leads to an increase in body weight and fat together with a loss of glucose responsiveness, thus confounding any of the phenotypes observed in these models [60]. Furthermore, the AMPK $\alpha 1^{-/-}$; $\alpha 2^{-/-}$ beta cell mice are more insulin sensitive, have lower circulating insulin and higher blood glucose levels suggesting that loss of these enzymes in peripheral tissues are masking beta cell specific functions of these enzymes. Definitive conclusions regarding the impact of AMPK catalytic subunit deletion from the beta cell compartment on whole animal physiology cannot yet be drawn. This controversy will likely be clarified in the future via conditional deletion of AMPK $\alpha 1$ and $\alpha 2$ floxed alleles using the tamoxifen-inducible variant of the mouse insulin promoter cre transgene (MIP-CreERT2), expression of which appears to be restricted to beta cells [59].

Upstream of AMPK: LKB1 and the beta cell. The AMPK beta cell knockout phenotypes discussed above are discordant with the role of LKB1 in beta cells using Pdx1-creERT2 to delete *lkb1* in adult mice. We and others have found that loss of LKB1 leads to improved glucose homeostasis and increased insulin secretion *in vivo* and in isolated islets [15, 16]. In this setting, AMPK $\alpha 1$ appears to be the relevant LKB1 target, as knockdown of $\alpha 1$ in MIN6 cells mimics the effect of loss of LKB1 on insulin content and secretion, whereas AMPK $\alpha 2$,

SIK2 or MARK2 have no effect on either [15]. Interestingly, while beta cells lacking LKB1 are larger as a consequence of enhanced mTOR activation, presumably resulting from AMPK deficiency, hypertrophy is not observed in AMPK $\alpha 1\alpha 2$ double knockout beta cells. This may reflect an extra-pancreatic contribution of gene deletion with RIP-cre and Pdx1 transgenes or a compensatory action of another member of the AMPK family that also regulates cell size.

Targets of AMPK in the beta cell. Direct targets of AMPK include ACC1, glucose transporters (GLUTs), glycogen synthase, fatty acid synthase, HMG CoA reductase, tuberous sclerosis protein 2 (TSC2) and SREBP [61-65]. It is natural to assume that phosphorylation and modification of the activity of each of these targets would underlie AMPK's effects, but this is an understudied area in beta cell research. Indeed, of those listed here, ACC1, GLUT2, SREBP, and TSC2 have been studied in the beta cell [33, 61, 66, 67]. Monitoring an increase in phosphorylation status of ACC1 is the most commonly used proxy for AMPK activation. ACC1 catalyzes the rate-limiting step in fatty acid biosynthesis and is inactivated by direct phosphorylation by AMPK at three Ser residues, S79, S1200 and S1215. As phosphorylation of ACC1 attenuates lipid biosynthesis, targeting AMPK and ACC1 has been proposed as a strategy for treating obesity and insulin resistance. Chronic chemical inactivation of ACC1 inhibits GSIS, and similarly knockdown of ACC1 inhibits GSIS as well as glucose oxidation, ATP production and the generation of mitochondrial metabolites [66]. In addition, AMPK has been shown to control programs of gene expression via the transcriptional regulators SREBP, Foxo1a and HNF4 α , that modulate synthesis of fatty acids, cholesterol, and insulin, as well as apoptosis [67-69]. AMPK activity

also activates TSC2 to inhibit mTOR and protein synthesis, in beta cells this pathway is regulated by amino acids and glucose [32]. Furthermore, TSC2 knockout mice display improved glucose tolerance, increased insulin secretion and increased beta cell mass, consistent with the notion that AMPK and its targets normally attenuate insulin secretion and proliferation [70, 71].

Role of AMPK family members in the beta cell. The 14 members of the AMP kinase family, AMPK α 1, AMPK α 2, BRSK1, BRSK2, NUA1, NUA2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4 and MELK, feature a conserved phosphorylation site in their kinase domain that is a substrate for phosphorylation by LKB1 [9]. While largely understudied as a group, some overlapping biological functions among the members have been identified, including cellular energy sensing, cell polarity and survival mediated by cAMP response element binding protein (CREB) [72-75]. For their part, SIK2, MARK2 and AMPK phosphorylate the CREB coactivator CRTC2 and block activation of CREB target genes [1, 76]. CRTC2 is activated by glucose in beta cells [76] and we speculate that AMPK effects in beta cells are in part mediated by the CRTC2:CREB pathway, including its effects on insulin secretion. SIK2 knockout mice display increased neuronal survival and resistance to ischemia via increased CREB-regulated transcription coactivator 1 (CRTC1)-CREB activity [77]. The pleiotropic beta cell phenotypes observed in LKB1 knockout beta cells (hypertrophy, enhanced proliferation, increase in insulin content and secretion, polarity modifications) raises the possibility that additional AMPK family members also play important roles in beta cell biology. Of the 14 members, whole animal knockout mice have been generated for MARK2, MARK3 and SIK2 [77-79]. Among a constellation of

phenotypes, MARK2 $-/-$ animals also display numerous alterations in the glucoregulatory system, including improved insulin sensitivity and glucose clearance [78]. In contrast, MARK3 knockout mice are normoglycemic and maintain normoglycemia when challenged with a high fat diet [79]. In these models it is not possible to discern the contribution to whole animal glucose metabolism made by MARK2, MARK3 in the beta cell compartment. In addition, a function for SIK2 in the islet yet to be described. We anticipate that adult-onset beta cell knockouts will shed light in this area and that distinct roles will emerge as additional members of the AMPK kinase family come under close scrutiny. Interestingly, SNARK activity is attenuated by metformin in INS1 cells, and activated by energy stress stimuli, suggesting potential non-redundancy between some AMP Kinases and cross-regulation within the family [80].

Clinical Considerations. Despite the majority of the evidence indicating that AMPK activation is detrimental to beta cell function and survival, systemic treatment with AMPK activators result in overall improvement in symptoms associated with T2D. For example, AICAR administration prevents diabetic progression in Zucker fatty rats and improves glucose homeostasis in ob/ob mice [81, 82]. Furthermore, analysis of numerous clinical studies indicated that TZDs and metformin prevent the progression of diabetes in glucose intolerant individuals (reviewed in [83]). Thus, these collective benefits appear to outweigh negative effects of AMPK activation in the beta cell. It is likely that the maintenance of beta cell mass and/or function from these drugs is due to secondary effects of preventing hyperglycemia and hyperinsulinemia rather than from a direct effect on the beta cell. We anticipate that a greater appreciation for the role of AMPK in the beta cell will be

particularly informative for long-term effects of AMPK modulation on beta cell function and survival in humans.

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Chapter 6. Discussion

CRTC-CREB pathway in beta cells.

The second regulatory site of CRTC2 serine275 represents a beta cell specific mode of regulation of the CRTC-CREB pathway. This site becomes dephosphorylated in the presence of glucose, the major stimulus of insulin secretion. Importantly, we have recently shown that CRTC2 is required for insulin secretion and beta cell survival [159]. CsA is an immunosuppressant used in transplants and contributes to new onset diabetes after transplant (NODAT) by inhibiting beta cell function. Interestingly, overexpression of CRTC2 rendered constitutively active by mutation of both serine171 and serine275 to alanine, is able to restore insulin secretion in CsA treated beta cells [159]. These data suggest that CRTC2 activation promotes beta cell function and survival. However, prolonged overexpression (5-7 days) of constitutively active CRTC2 reduces insulin content in beta cells. This may be due to negative feedback and repression of insulin synthesis pathways following initial elevation of insulin secretion seen with active CRTC2.

Constitutive CRTC2-CREB activity impairs beta cell function.

In an effort to assess the role of CRTC2 in beta cell function, I attempted to characterize the phenotype of transgenic mice that overexpressed constitutively active CRTC2 in beta cells. The founders for this transgenic line were generated by Yves De Repentigny in collaboration with Dr. Rashmi Kothary. I found that the transgenic pups

expressing either wild-type or constitutively active (DM) CRTTC2 had a severe reduction in insulin staining and as a result had extremely high fed glucose levels >33.3 mM (Appendix 5). These mice were end-pointed at 7 weeks of age, and developed hyperglycemia at 3 weeks of age corresponding to time of weaning off maternal nourishment. During the weaning period juvenile mice become fully dependent on their own beta cells for insulin secretion. These data were only obtained for 2 transgenic mice so the findings are inconclusive and the study was discontinued. Interestingly, mice expressing a constitutively active CBP have enhanced CREB gene transcription, a doubling of beta cell mass and yet insulin secretion is severely impaired in these islets potentially due to enhanced levels of Pgc1a which is known to attenuate insulin release [160].

Constitutive loss of CRTTC-CREB activity impairs beta cell function.

On the other hand, consistent with earlier findings beta cell-specific deletion of CN, which presumably decreases CREB activation, impaired beta cell function. CN beta cell knockout mice had severely reduced insulin secretion and lethal diabetes around postnatal day 20 [161]. The authors attribute CN knockout effects to loss of Nuclear Factor of Activated T cells (NFAT) gene transcription, however they did not consider the potential contribution of CREB inactivation [161]. Thus it would be interesting to assess whether reconstitution of either NFAT or CREB activity could restore beta cell function in CN beta cell knockout mice.

Transient CRTTC-CREB activity is critical for beta cell function.

Nonetheless, inhibition of CREB target gene transcription via overexpression of dominant negative CREB leads to beta cell destruction and diabetes [50]. Thus inappropriate activation or inactivation of the CRTC-CREB pathway is detrimental to beta cell function and results in diabetes. Taken together, my work and the work of others suggest that transiently regulated CRTC-CREB activation is critical for beta cell function and survival. To confirm these studies, conditional knockouts of CRTC2 and CREB in beta cells should be generated.

Beta cells need rest.

The adverse effect seen with chronic CRTC-CREB activation is similar to and exemplifies the toxicity seen with chronic high glucose stimulation in beta cells. Thus transient activation of beta cell function is also critical for beta cell survival. It is possible that the toxic effect of chronic glucose stimulation is actually mediated in part through chronic CRTC-CREB activation. As such, it would be interesting to see if blocking inappropriate CRTC-CREB activation could relieve impairment of beta cell function in a glucotoxic environment.

In fact, for treatment of T2D new therapeutics are aimed at provoking periods of beta cell ‘rest’ wherein beta cell function is temporarily inhibited to promote improvement of beta cell function and insulin secretion [162]. Some therapies cause beta cell rest indirectly, such as Metformin, which acts by blocking glucose production and reducing blood glucose levels

and as a result reduces the demand for insulin secretion from beta cells [163]. Other therapies target beta cells directly using K^+_{ATP} channel openers, such as diazoxide, which are given temporarily to block insulin secretion and once the treatment is over and the drug is removed, native beta cell insulin secretion is improved [164].

CRTC2 inhibition by LKB1-AMP kinases implies functional redundancy.

I have observed that all the members of the AMP kinase family are capable of phosphorylating at serine171 or 275 CRTC2 in vitro. Interestingly some kinases preferentially phosphorylate serine171 or serine275 revealing the potential for specific regulation among the AMP kinases (Appendix 2). In particular, AMPK, SIK1 and SIK2 phosphorylated serine171 to the greatest extent, while MARK2 and MARK3 phosphorylated serine275 to the greatest extent. However, when AMPK, SIK2 or MARK2 is knocked down in MIN6 cells there is no obvious loss of CRTC2 phosphorylation (Appendix 6). Similarly in SIK2 knockout and MARK2 knockout islets CRTC2 phosphorylation is unchanged (Appendix 1 and Appendix 7). These data suggest that there is compensation for loss of one AMP kinase from another family member. In addition, I have found that all of the AMP kinases are expressed in beta cells, except Nuak2 and Brsk2 whose expression I did not assess (Appendix 3).

The presence of most AMP kinases in beta cells and their potential functional redundancy was initially discouraging for undertaking loss of function studies. Thus as an initial step, I sought out to investigate the effects of deletion of LKB1 and loss of activation

of the entire AMP kinase family in beta cells. I found that LKB1 functions as a way to limit insulin secretion, cell size, polarity and replication. My findings were confirmed in a complementary study by another group, which further described the altered polarity in LABKO beta cells [165]. Deletion of LKB1, therefore, enhanced beta cell mass and function and provided resistance to hyperglycemia induced by a high fat diet. In addition, I found reduced CRTC phosphorylation and elevated transcription of CREB target genes revealing that loss of activation of the entire AMP kinase family could result in increased CRTC-CREB activity (Appendix 7).

The increase in CRTC2-CREB activity in the context of improved beta cell function in LABKO islets, is in conflict with the idea that transient CRTC-CREB activity is required for healthy beta cells. This discrepancy may be explained by 2 possibilities: 1) the enhancement in CREB target genes is only modest (2-3 fold) in LABKO islets, whereas use of constitutively active CRTC2 leads to severe enhancement (50 fold) of CREB activity [166]. 2) The CRTC-CREB pathway is still transiently active, in that it is not fully activated in basal conditions but is further enhanced in response to glucose in contrast to constitutively active CRTC2 which increases CREB activity 50-fold in 0 mM glucose [166]. As such there is potential for enhanced CRTC-CREB activity playing a role in the phenotype of LABKO mice. Experiments aimed at reducing CRTC2 activity and identifying which phenotypes are reversed in LABKO mice can help to determine the contribution of CRTC2-CREB. Instead of pursuing this possibility, I switched focus onto identifying the role that the various kinases downstream of LKB1 play in beta cell biology.

The role of LKB1-AMPK in beta cell function.

Importantly, I have attributed the increase in cell growth and insulin secretion to loss of AMPK. This helped to clarify controversy over what role AMPK plays in insulin secretion, controversy that stemmed from technical experimental differences. As mentioned in Chapter 5, the lack of overlapping effects seen with AMPK alpha1 and alpha2 knockout beta cells is likely due to the use of a different non-beta cell specific model where AMPK is deleted in hypothalamus as well. This is especially important since AMPK knockout in the hypothalamus leads to obesity [126]. This study made use of the RIP-Cre system which was also used by the same group to generate an LKB1 knockout model which again showed non-overlapping phenotypes with those that I and others observed in LABKO mice [104, 167]. These differences underscore the importance of drawing precise conclusions with the model systems used. The MIP-Cre system is now thought to be the most beta cell specific model with no Cre expression in the brain unlike RIP-Cre and to a much lesser extent Pdx1-Cre [168]. However, using the Pdx-CreER^{Tam} system, in our hands, we observe no loss of LKB1 or SIK2, in LABKO or SABKO brains (Appendix 1).

The role of LKB1-MARK2 in beta cell biology.

There are significant differences between LABKO and MARK2 knockout mice as well. MARK2 knockout mice have germ line deletions thus loss of MARK2 is ubiquitous making direct comparisons to LABKO mice equivocal. MARK2 mice display a hypermetabolic phenotype and as a result have reduced insulin secretion [151]. In this model

the effects of MARK2 loss in beta cell function are likely secondary and masked by the effects of MARK2 loss in the brain and peripheral tissues. Nonetheless, I did observe similar altered nuclear positioning in MARK2 beta cells as seen in LABKO beta cells. However, when I isolated islets from MARK2 knockout mice I found no difference in insulin secretion suggesting that loss of MARK2 does not underlie the increased insulin secretion in LABKO islets (Appendix 8). Interestingly, cell polarity in pancreatic progenitor cells during embryogenesis is required for the development of islets and beta cells [169]. This suggests that perhaps beta cell progenitors might be affected by loss of MARK2 and LKB1. In order to test this MARK2 beta cell specific knockout mice could be generated, to first confirm the morphological change is due to an intrinsic beta cell alteration. Then the presence and characteristics of potential beta cell progenitors could be investigated by measuring expression levels of markers for beta cell progenitors, such as NGN3 and Pdx-1. Beta cell polarity has been previously reported, however there is as yet no evidence of a functional effect from this obvious morphological reality [170]. Thus further study of the effect of MARK2 in beta cells could provide the first insight into the functional consequence of beta cell polarity.

The role of LKB1-SIK2 in beta biology.

SIK2 was the first kinase identified as a CRTC2-CREB regulator. It has since been found to be required for down-regulating glycogen and lipid synthesis during starvation in *Drosophila* [171]. In addition SIK2 deletion provides enhanced neuronal survival following oxygen-glucose deprivation due to enhanced CRTC1-CREB activity [172]. These findings

highlight a role for SIK2 in mediating central metabolic functions. Interestingly, in *Drosophila*, SIK2 expression is highest in the brain, we also find that in mice SIK2 protein is enriched in brain and islets ([171] and Appendix 1). We sought out to investigate the role of SIK2 in beta cell biology and in mediating the phenotypes of LABKO mice.

Surprisingly, we found that loss of SIK2 impairs insulin secretion and glucose tolerance. These effects are exacerbated on a high fat diet where SABKO mice become severely hyperglycemic. Furthermore, loss of SIK2 inhibits insulin secretion by inhibiting p35-Cdk5-mediated opening of Ca^{2+} channels. The phenotype of SIK2 mice is in discordance with that of LABKO mice. Interestingly, LKB1 is required for SIK2 stabilization, as I have found reduced SIK2 protein levels in LABKO islets (Appendix 9). However, LKB1 does not play any role in regulating p35-Cdk5 activity. Thus these differences could be due to opposing actions of other AMP kinases on SIK2, in which case loss of LKB1 would nullify the effects of loss of SIK2 since an opposing kinase would also be inactivated. Taken together, we have found that SIK2 plays an opposing role to LKB1 in beta cell biology, and may act independently of LKB1 or perhaps as a negative feedback to attenuate actions of LKB1. Once more, in contrast to LKB1 function, SIK2 is required for in G1-S phase cell cycle progression and is upregulated in ovarian cancers [173]. It would be interesting to see if overexpression of SIK2 in LABKO islets would synergize to enhance insulin secretion thus revealing that SIK2 is acting on insulin secretion independently of LKB1. In contrast, if SIK2 overexpression had no effect, it could be a potential negative feedback mechanism on LKB1. Identifying the role of other kinases downstream of LKB1 in beta cell biology will help to clarify these questions.

Long-term loss of LKB1 in beta cells.

Considering that LKB1 is a tumor suppressor and that I observed an increase in beta cell replication in LABKO beta cells, one might predict that eventually LABKO mice would develop insulinomas. I, therefore aged LABKO mice to 2 years in order to assess the potential for tumorigenicity. For the LABKO mice that still had complete LKB1 depletion I did not observe any insulinomas by histological analyses, furthermore the improvement in glucose clearance persisted over time (Appendix 10). This is different from effects seen with long-term loss of TSC2 and increased mTor activation, where there's an initial burst in beta cell mass followed by negative feedback and eventual destruction of beta cells and hyperglycemia [174]. This suggests the underlying mechanism of enhanced glucose tolerance in LABKO mice is not due to enhanced mTor activation. The lack of development of insulinomas in LABKO mice is fitting with the fact that LKB1 is not a classical tumor suppressor. In the absence of LKB1, although there is increased cell growth, growth is limited due loss of AMPK activity and the inability to survive increased energy stress that tumors are typically subjected to [175].

From an in vitro screen to an in vivo model system.

I have identified that several AMP kinases phosphorylate and inhibit CRTC2 in an in vitro kinase screen. The identification of this family of kinases as regulators of CRTC-CREB provided the rationale to look at the effects of their upstream regulator LKB1 in beta cell

biology. I found that LKB1 is a critical regulator of beta cell mass and insulin secretion in mice. **Thus, I have illustrated the usefulness of conducting high throughput screens and discovery-based research to identify kinase-substrate pairs that regulate important biological functions.**

Targeting LKB1-AMP kinases for diabetes treatment.

Ubiquitous knockout of LKB1 in adult mice led to a complete loss of hematopoietic stem cells, revealing the potential dangers of targeting LKB1 for treatment of diabetes [97, 98]. This underscores the need to identify the role of the AMP kinases in contributing to the LABKO phenotypes. Surprisingly, SIK2 knockout has the opposite effect of loss of LKB1. However, considering that loss of SIK2 inhibits insulin secretion, blocking SIK2 for treatment of T2D may be beneficial to beta cell function in providing a beta cell rest period. SIK2 is especially attractive since it only mildly inhibits insulin secretion and is not likely to pose the risk of causing overt hyperglycemia. Targeting AMPK, on the other hand would enhance insulin secretion in beta cells, however in the liver targeting AMPK would increase glucose output and has the potential for causing hyperglycemia. Thus identification of other AMP kinases that may attenuate insulin secretion downstream of LKB1 and might be expressed exclusively in beta cell holds the most therapeutic promise.

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Appendix 1. Role of the Sik2-p35-Pja2 complex in pancreatic beta cell functional compensation.

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Summary

There were several phenotypes that I observed following Lkb1 depletion in mouse beta cells and for some I identified the Lkb1 target responsible. For example, loss of Ampk activation in LABKO beta cells underlies the increase in insulin secretion, and loss of Mark2 underlies the altered nuclear organization of LABKO beta cells. This chapter seeks to identify the role of the kinase Sik2 downstream of Lkb1 in beta cell biology. As such we generated and characterized Sik2 adult beta cell knockout (SABKO) mice. Surprisingly, SABKO mice display reduced insulin secretion and elevated blood glucose levels in contrast to LABKO mice. In addition, we identified the mechanism underlying the reduction in insulin secretion as Sik2 negatively regulates PJa2-p35-Cdk5 and is required for opening of voltage-gated calcium channels (VDCC) to permit insulin secretion. Furthermore Sik2 is elevated in diabetic models and when knockdown can restore hypersecreting diabetic islets to normal insulin levels. Taken together these data describe an important role for Sik2 in beta cell insulin secretion and functional compensation during increased insulin demand, with cellular functions independent and/or opposing to those of other Lkb1 targets.

Contributions

J. Sakamaki is the first author of this manuscript and conducted all experiments in the manuscript except those mentioned below and Dr. Srean wrote the paper. As co-author on this manuscript I contributed to experiments, experimental design, interpretation of results and assisted with editing the manuscript. I performed initial experiments on SABKO mice to validate the mice had cre-dependent recombination of LoxP sites inserted into exon8 of the Sik2 allele and effective knockout of Sik2. I performed the initial islet isolations, glucose tolerance test, serum insulin secretion measurements, islet perfusion, insulin immunohistochemical staining, and islet calcium imaging experiments with J. Sakamaki to train him in various islet techniques. I generated and optimized the use of Sik2-targeting shRNA lentiviruses for their ability to knockdown Sik2 in MIN6 cells. I performed seahorse experiments revealing that oxygen consumption and glycolysis are unaffected in Sik2 knockdown MIN6 cells (Supplemental Figure 3f and g).

Title: Role of the Sik2-p35-Pja2 complex in pancreatic beta cell functional compensation

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Keywords: Sik2; Cdk5r1/p35, Cdk5; beta cell; islet; beta cell compensation; Ampk; Pja2; glucose signaling; insulin secretion; insulin; calcium; hyperglycemia; diabetes

Abstract:

Metabolic syndrome and Type 2 Diabetes are increasingly prevalent global health risks. The transition from glucose intolerance/prediabetes to a diagnosis of diabetes occurs when pancreatic beta cells fail to compensate for the increasing demand for insulin. While mechanisms for increasing beta cell mass as part of the compensatory response have been described, the molecular underpinnings of beta cell functional compensation remain largely

unknown. Here we delineate a novel pathway featuring the AMP kinase (AMPK) family member Sik2, the Cdk5 activator Cdk5r1/p35 and the E3 ubiquitin ligase Pja2 that underlies beta cell functional compensation. Sik2 triggers calcium influx and insulin secretion via phosphorylation of p35 at Ser91, which leads to its ubiquitination via Pja2. Knockout of Sik2 in beta cells leads to accumulation of p35, insulin secretion defects, and glucose intolerance. Unlike AMPK, Sik2 is stabilized in beta cells cultured in high glucose and in models of metabolic syndrome, and is required for compensatory insulin secretion in these settings. In summary, we demonstrate that the Sik2-Pja2-p35 signaling cassette is essential for the maintenance of glucose homeostasis by driving functional compensation of the beta cell and define a novel role for Sik2 and the AMPK family in metabolism.

A longstanding goal in pancreatic beta cell biology is to understand fundamental mechanisms of insulin secretion to improve cell function for the treatment of diabetes. We and others previously demonstrated a critical role for the tumour suppressor Lkb1 as a central regulator of critical aspects of beta cell biology, including cell size, proliferation, and insulin secretion ¹⁻³. Biochemical and genetic evidence indicates that Lkb1 phosphorylates and activates Ampk, a key regulator of cellular energy metabolism, as well as a large family of Ampk-related kinases ⁴. In this regard, while we showed that loss of Ampk activity directly underlies the beta cell size hypertrophy and increase in insulin content in Lkb1 beta cell knockout mice, precisely how Lkb1 regulates insulin secretion is still unclear. The Ampk-related kinase Sik2 was originally isolated due to its upregulation in the adrenal glands of rats fed a high salt diet ⁵ and since has been implicated in diverse biological scenarios ⁶⁻¹⁴. Sik2 is thought to exert its biological effects by direct phosphorylation of the

Crtc transcription coactivators and regulation of a Creb-dependent gene expression program essential for beta cell function, proliferation and survival¹⁵⁻¹⁷. Given this, we sought to gain insight into the role of Sik2 in the beta cell, and thus employed the Pdx1-CreERT2 transgene to generate Sik2 adult beta cell knockout (SABKO) mice (Figure 1a). SABKO mice displayed nearly complete loss of Sik2 mRNA compared to control Sik2^{loxP/loxP} littermates (S/S), while mRNA levels for the remaining Siks, Sik1 and Sik3, were unchanged (Supplementary Fig. S1a). Western blotting for Sik2 protein in isolated islets, cortex, and hypothalamus confirmed that knockout of Sik2 occurred only in the islet (Figure 1b). While islets from SABKO mice displayed normal morphology and insulin staining (Figure 1c), Ampk-mTOR signaling (Supplementary Fig. S1b-d) and Crtc2-Creb activity (Supplementary Fig. S1e-g), SABKO mice displayed elevated blood glucose (Figure 1d) due to a 30% reduction in plasma insulin after refeeding (Figure 1e). These changes were seen in both male and female SABKO animals only after tamoxifen treatment (Supplementary Fig. S2a-e), without a change in body weight (Supplementary Fig. S2f) or insulin sensitivity (Supplementary Fig. S2g).

Dynamic analysis of glucose clearance in SABKO mice compared to controls was similarly impaired (Figure 1f, S2h and i), which we attributed to a 30-40% reduction in plasma insulin levels (Figure 1g, S2j and k). Insulin secretion triggered directly by

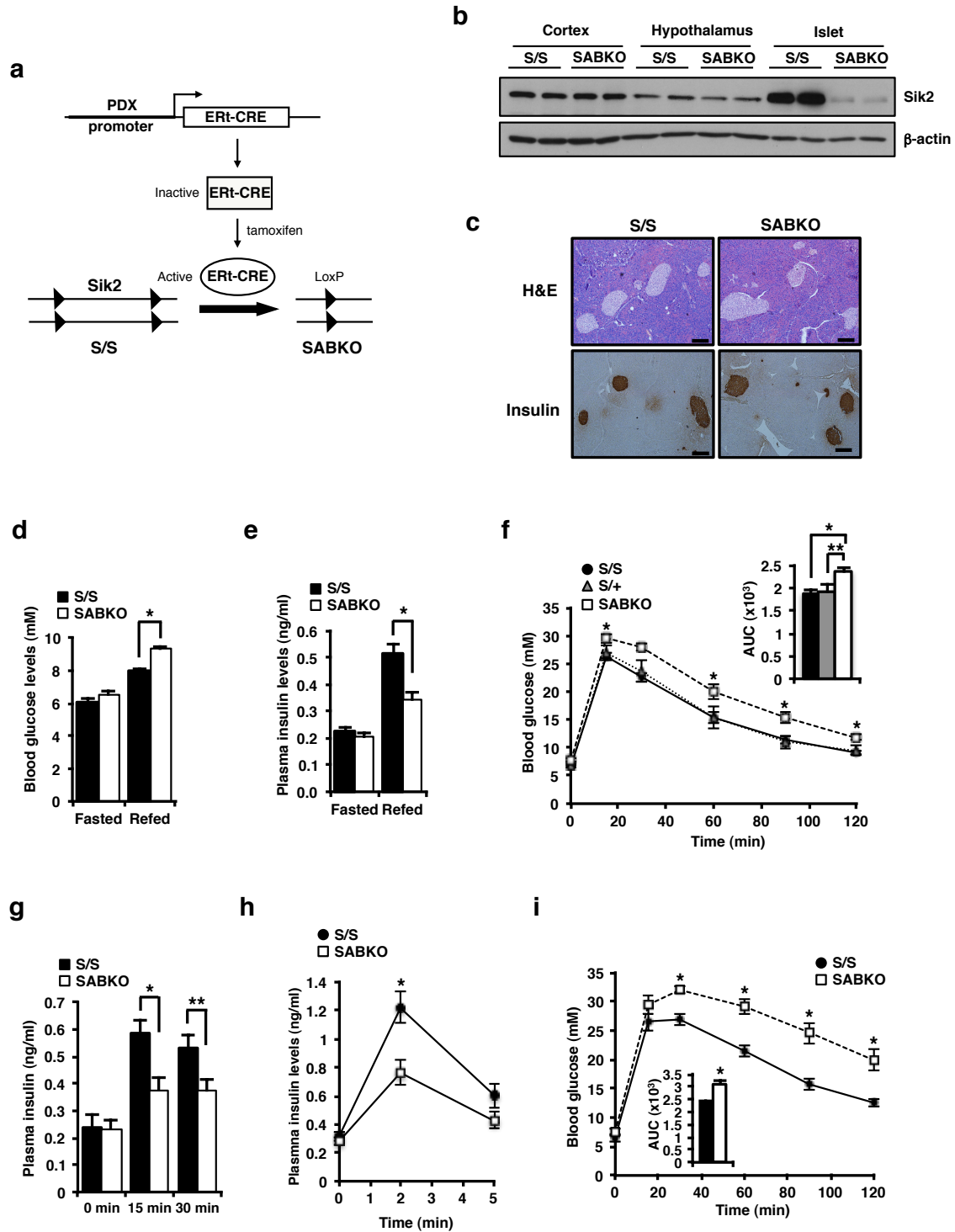


Figure 1. Deletion of *Sik2* in adult beta cells leads to glucose intolerance due to impaired stimulus-dependent insulin secretion. a. Adult *Sik2*^{loxP/loxP} mice carrying the *Pdx1-CreERT2* transgene were injected with tamoxifen to induce b cell specific deletion of

Sik2. b. Western blot analysis of Sik2 levels from extracts of cortex, hypothalamus, and islets in S/S and SABKO mice. c. (Top) Pancreatic sections from control S/S and SABKO mice stained with H&E. Bar = 100 μ m. (Bottom) Immunostaining of pancreatic sections of control S/S and SABKO mice stained with insulin antibody. Bar = 100 μ m. d. Blood glucose levels of fasted and 2 h refed control (S/S, n=31) and SABKO (n=34) mice were measured 1 week after tamoxifen treatment. e. Plasma insulin levels after 2 h of refeeding in control (S/S, n=20) and SABKO (n=12) mice. f. Glucose tolerance test (2 mg glucose/g, IP injection) for tamoxifen-treated S/S (n=31), S/+ (n=6), and SABKO (n=26) mice. Blood glucose concentrations were determined at indicated times. Area under the curves (AUC) is shown at top right. g. Plasma insulin levels at 15 min and 30 min post glucose injection during GTT for control S/S (n=13) and SABKO (n=13) mice. h. Plasma insulin levels at 2 min and 5 min after L-arginine injection (1 mg/g body weight) in S/S (n=12) and SABKO (n=11) mice. i. Glucose tolerance test on control S/S (n=9) and SABKO (n=8) mice after 16 weeks on HFD. Area under the curve (AUC) is shown.

arginine was attenuated in SABKO mice, indicating that loss of Sik2 in beta cells causes an insulin secretion defect *in vivo* (Figure 1h). To provoke an increased functional response from the β cell, control and SABKO mice were fed a high fat diet (HFD) to mimic conditions of human metabolic syndrome. While all animals had indistinguishable body weights and food intake after 17-18 weeks on HFD (Supplementary Fig. S2l and m), SABKO animals had significantly elevated blood glucose and lower plasma insulin levels after refeeding (Supplementary Fig. S2n-o). Interestingly, SABKO mice on HFD also displayed a pronounced deficiency in glucose clearance compared to control animals (Figure

1i), which again correlated with reduced plasma insulin (Supplementary Fig. S2p) and became worse with time on HFD (Supplementary Fig. S2q).

As insulin content in SABKO islets was unchanged (Figure 2a), we suspected that defective insulin secretion may underlie the impaired glucose clearance phenotype. Static glucose-stimulated insulin secretion (GSIS) assays revealed that isolated islets lacking Sik2 secrete ~40% less insulin following stimulation with glucose (Figure 2b). Furthermore, acute inactivation of Sik2 in control islets with lentiviral shRNA targeting Sik2 reduced GSIS to levels observed from SABKO islets (Figure 2b) without affecting content (Supplementary Fig. S3a); similar inhibition of GSIS was observed in islets following treatment with the pan-Sik inhibitor HG-9-91-01 (Figure 2c and S3b). We confirmed these results in the glucose-responsive cell line MIN6, in which Sik2 silencing impaired GSIS (Supplementary Fig. S3c), and overexpression of Sik2 WT but not a kinase-dead Sik2 mutant increased both basal and GSIS without changing insulin content (Figure 2d and S3d and e).

Glucose-stimulated insulin secretion involves oxidation of nutrients and consequent generation of ATP, cell membrane depolarization, extracellular calcium influx, and ultimately insulin granule release¹⁸⁻²⁰. Seahorse assays confirmed normal glycolysis and mitochondrial respiration in Sik2 knockdown MIN6 cells, indicating that the defect in insulin secretion does not result from impaired cellular energetics or metabolism

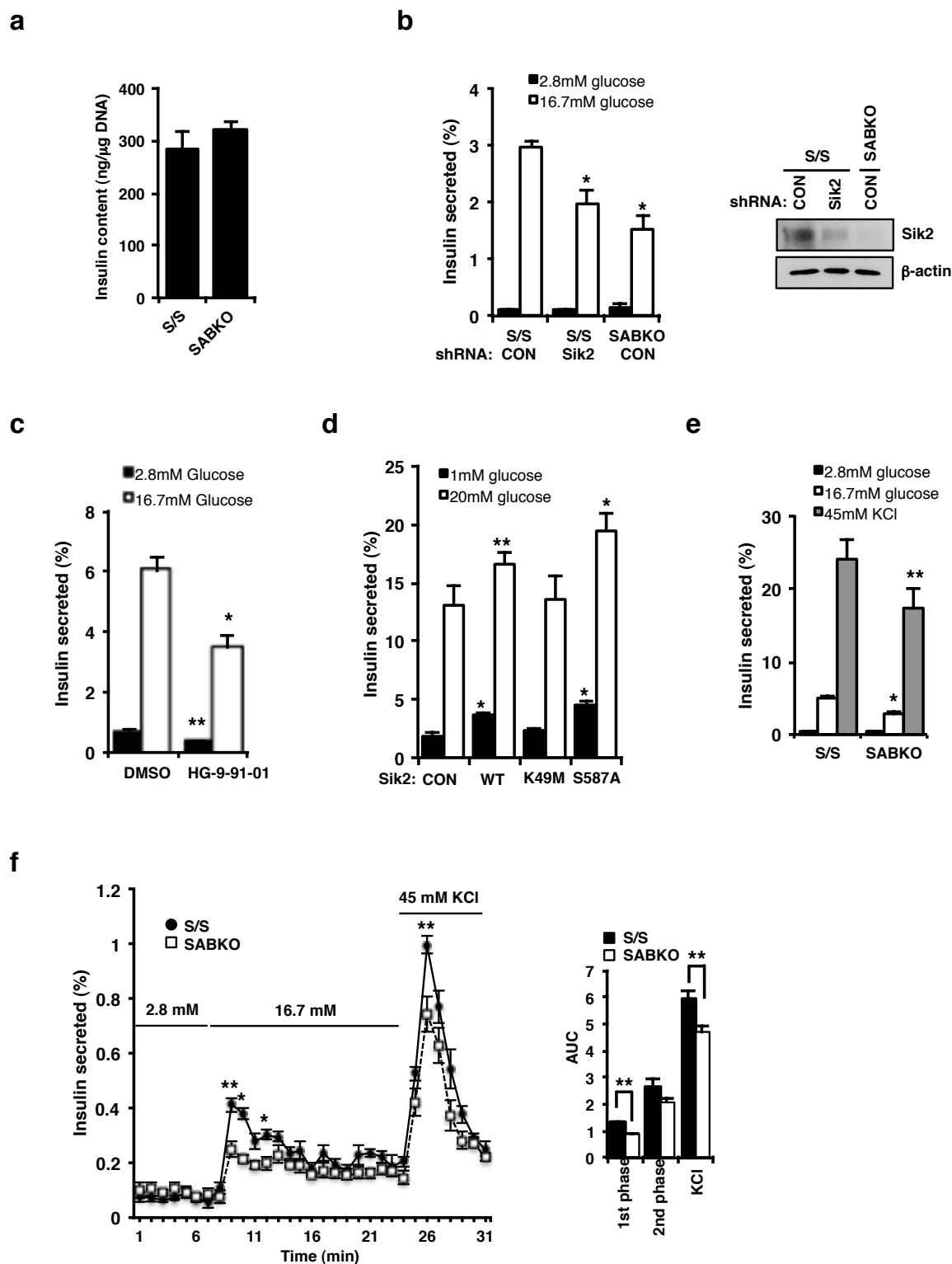


Figure 2. Sik2 is required for glucose-stimulated insulin secretion at a step downstream of depolarization

a. Insulin content (ng insulin/ug DNA) is unchanged in control S/S and SABKO islets. b. (Left) Insulin secretion from S/S and SABKO islets infected with control or Sik2 shRNA. Islets were treated with low (2.8 mM) and high (16.7 mM) glucose for 1 hr. (Right) Western blot analysis of Sik2 levels in islets indicated in the right panel. c. Insulin secretion from mouse islets pre-treated with DMSO or pan-Sik inhibitor HG-9-91-01 (0.5 mM) for 24 hr followed by incubation with low (2.8 mM, 1 hr) and high (16.7 mM, 1hr) glucose in the presence or absence of 0.5 mM HG-9-91-01. d. Insulin secretion from Min6 cells infected with Sik2 WT, kinase inactive (K49M), or constitutively active (S587A) mutant. Cells were treated with low (1 mM) and high (20 mM) glucose for 1 hr. e. Insulin secretion from S/S and SABKO islets treated with low (2.8 mM) and high (16.7 mM) glucose, and 45 mM KCl for 1 hr. f. Glucose stimulated insulin secretion assay by perfusion of 75 islets from control S/S (n=3) and SABKO (n=3) mice. (Left) Percentage of total islet insulin content in each fraction following treatment with indicated amount of glucose or KCl is shown. (Right) Histogram showing area under the curves for perfusion GSIS data is shown, separated into first phase (8-12 min) and second phase (12-24 min) high glucose (16.7 mM) and response to depolarization (45 mM KCl). (Supplementary Fig. S3f and g). Treatment with 45 mM KCl indicated that SABKO beta cells secrete less insulin in response to direct membrane depolarization (Figure 2e). Analysis of secretion dynamics from control and SABKO islets confirmed the defective response to KCl depolarization and revealed a specific requirement for Sik2 during first phase insulin secretion (Figure 2f).

These data prompted us to identify a Sik2 target required for GSIS at a step downstream of mitochondrial metabolism. We identified an LXBS/TXSXXXL motif, the consensus phosphorylation site for Sik2¹⁵, surrounding Ser91 of Cyclin dependent kinase 5 regulatory subunit 1 (Cdk5r1, also known as p35) (Figure 3a). p35 is an activator of the atypical cell

cycle kinase Cdk5, which is enriched in the nervous system and also inhibits insulin secretion by blocking calcium ion influx in the beta cell ²¹⁻²⁴. Sik2 wt, but not a kinase-dead mutant, directly phosphorylates recombinant p35 wt *in vitro* (Figure 3b), which is prevented by mutation of Ser91 to Ala (Figure 3c). Sik2 and p35 form a complex as indicated mutual co-precipitation of the endogenous proteins from both HEK293T and MIN6 cells (Figure 3d, S4a and b). Consistent with a previous report ²¹, silencing p35 enhanced insulin secretion in MIN6 cells cultured in both low and high glucose without affecting insulin content (Figure 3e and S4c). In contrast, overexpression of p35 wt reduced GSIS, an effect that was more pronounced with the Ser91A construct (Figure 3f and S4d).

We noted that the Ser91Ala mutant accumulated to higher levels in this setting, which prompted us to evaluate the role of the phosphorylation at Ser91 by Sik2 in the regulation of p35 levels. Indeed, silencing of Sik2 led to a reduction in P-Ser91 signal and concomitant increase in p35 levels in MIN6 cells (Figure 3g, S4e and f). Furthermore, p35 levels were increased in SABKO islets (Figure 3h). Inhibition of Siks with HG-9-91-01 also increased p35 levels in islets (Supplementary Fig. S4g). As silencing of Ampk alpha1, Lkb1, Sik1 or Sik3 had no effect on p35 levels in MIN6 cells, we conclude that Sik2 has a specific role in regulating p35 levels (Supplementary Fig. S5a-c). Indeed, Sik2-knockdown MIN6 cells showed increased phosphorylation of voltage-dependent calcium channel (VDCC) at Ser783, a critical p35-Cdk5 target which correlates with VDCC inhibition (Figure 3i) ²¹. Consistent with a role for Sik2 in the regulation of p35-Cdk5 activity,

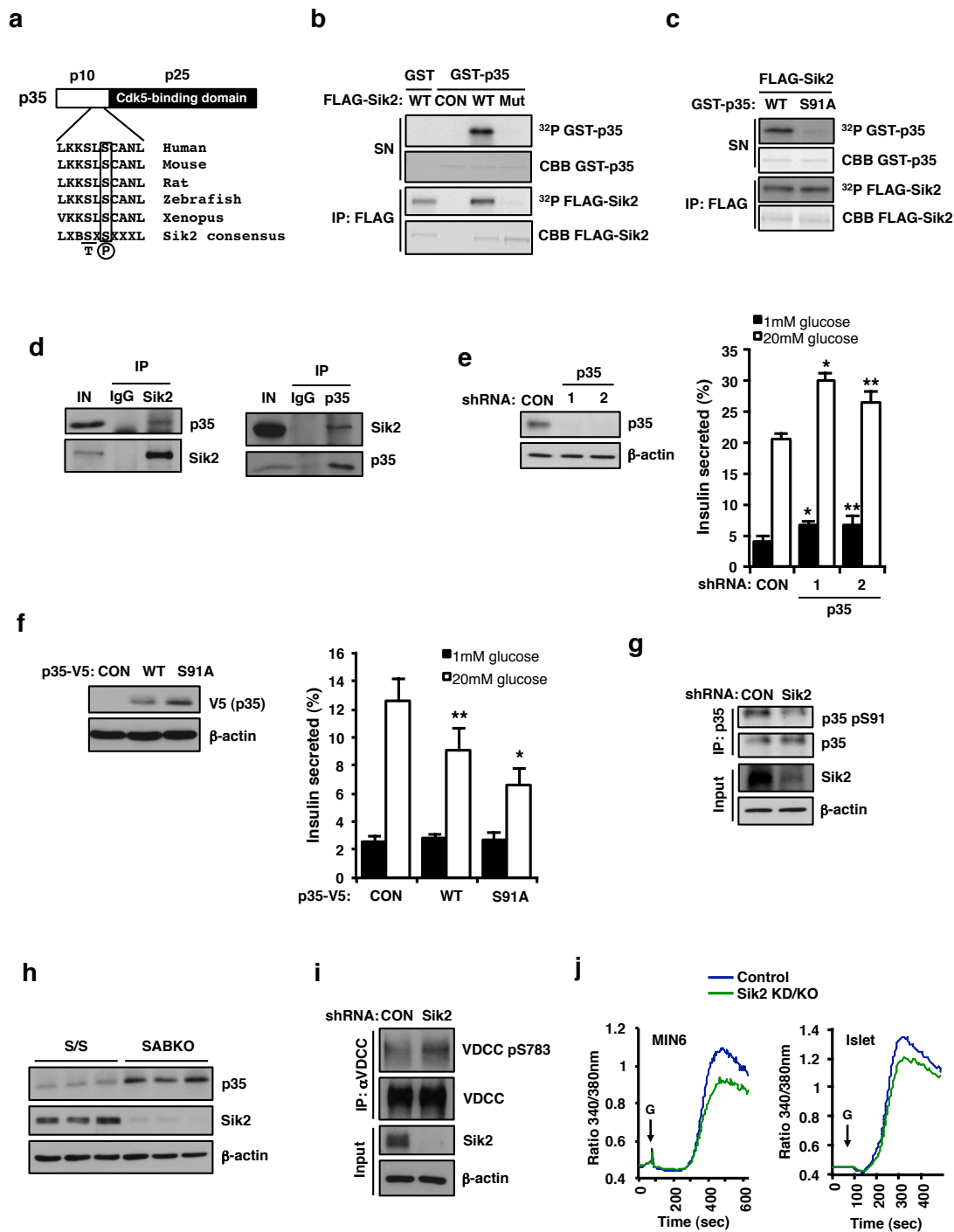


Figure 3. Sik2 is required for calcium ion influx in response to glucose through regulation of p35 phosphorylation and protein levels.

a. Schematic of p35 showing N-terminal p10 domain, C-terminal p25 Cdk5 binding domain and the location of Ser 91. Conservation of consensus Sik2 phosphorylation site at Ser91 of

p35 using standard single letter amino acid nomenclature is shown. B= basic residue, X = any amino acid. b. In vitro kinase assay using FLAG-Sik2 wild type (WT) or K49M kinase dead mutant (Mut) showing incorporation of ^{32}P into full-length GST-p35 in the reaction supernatant. Autophosphorylation and coomassie blue staining of immunoprecipitated FLAG-Sik2 protein remaining on beads is shown in bottom two panels. CON = Empty vector control. c. In vitro kinase assay using immunoprecipitates of FLAG-Sik2 and GST-p35 WT and S91A mutant as substrates. ^{32}P and coomassie blue (CBB) staining of GST-p35 substrates and immunoprecipitated FLAG-Sik2 are shown. d. (Top) Western blot analysis of immunoprecipitates of endogenous Sik2 and IgG control from MIN6 cell extracts for presence of endogenous p35. (Bottom) Western blot analysis of immunoprecipitates of endogenous p35 and IgG control from MIN6 cells. e. (Top) Western blot analysis of p35 levels in MIN6 cells infected with lentivirus expressing non-targeting control (CON) or p35 shRNA. (Bottom) GSIS assay showing percent insulin secretion (normalized to total insulin content) in control or p35 knockdown MIN6 cells cultured in 1 mM (black bars) or 20 mM (white bars) glucose. f. (Top) Western blot analysis of p35-V5 levels in MIN6 cells infected with lentivirus expressing empty vector (CON), p35-V5 WT or S91A. (Bottom) Insulin secretion from indicated Min6 samples in western blot treated with low (1 mM) and high (20 mM) glucose for 1 hr. g. Western blot analysis of pSer91 levels in IPs of endogenous p35 in control or Sik2 shRNA expressing MIN6 cells treated with MG132 (10 μM for 24h). h. Western blot analysis of endogenous p35 levels in SABKO islets. i. Western blot analysis showing pSer783 levels on VDCC immunoprecipitated from control or Sik2 knockdown MIN6 cells. j. (Left) Real time intracellular calcium measurements with Fura-2 dye in control and Sik2 knockdown MIN6 cells cultured in 1 mM glucose were stimulated with 20

mM glucose. Data is from a single experiment performed in triplicate, representative of three experiments. (Right) Real time intracellular calcium measurements using Fura-2 dye in dissociated islet cells from S/S control and SABKO mice. Cells cultured in 2.8 mM glucose were stimulated with 16.7 mM glucose. Data are averaged from n=3 experiments with > 150 total cells per genotype.

Sik2 knockdown MIN6 cells and SABKO islet cells also displayed impaired calcium influx following glucose stimulation (Figure 3j).

To test if Sik2 triggers degradation of p35 following phosphorylation at Ser91, we first monitored p35 levels in the presence of MG132, an inhibitor of the 26S proteasome²⁵. Sik2 overexpression reduced p35 wt levels, an effect that required Sik2 kinase activity and Ser91 of p35 (Figure 4a and 4b). Following MG132 treatment, p35 levels increased even in the presence of wt Sik2, indicating that phosphorylation of p35 at Ser91 by Sik2 leads to p35 turnover by the proteasome (Figure 4c). Consistent with this model, we detected poly-ubiquitination of p35-V5 wt but not the Ser91Ala mutant (Figure 4d), as well as ubiquitination of endogenous p35 that was reduced by Sik2 silencing (Figure 4e). We next used a proteomic approach to identify proteins that interact with p35, and we identified the E3 ubiquitin ligase Pja2²⁶ as a *bona fide* p35 interactor, confirmed by co-immunoprecipitation from MIN6 cell extracts (Figure 4f). Overexpression of FLAG-Pja2

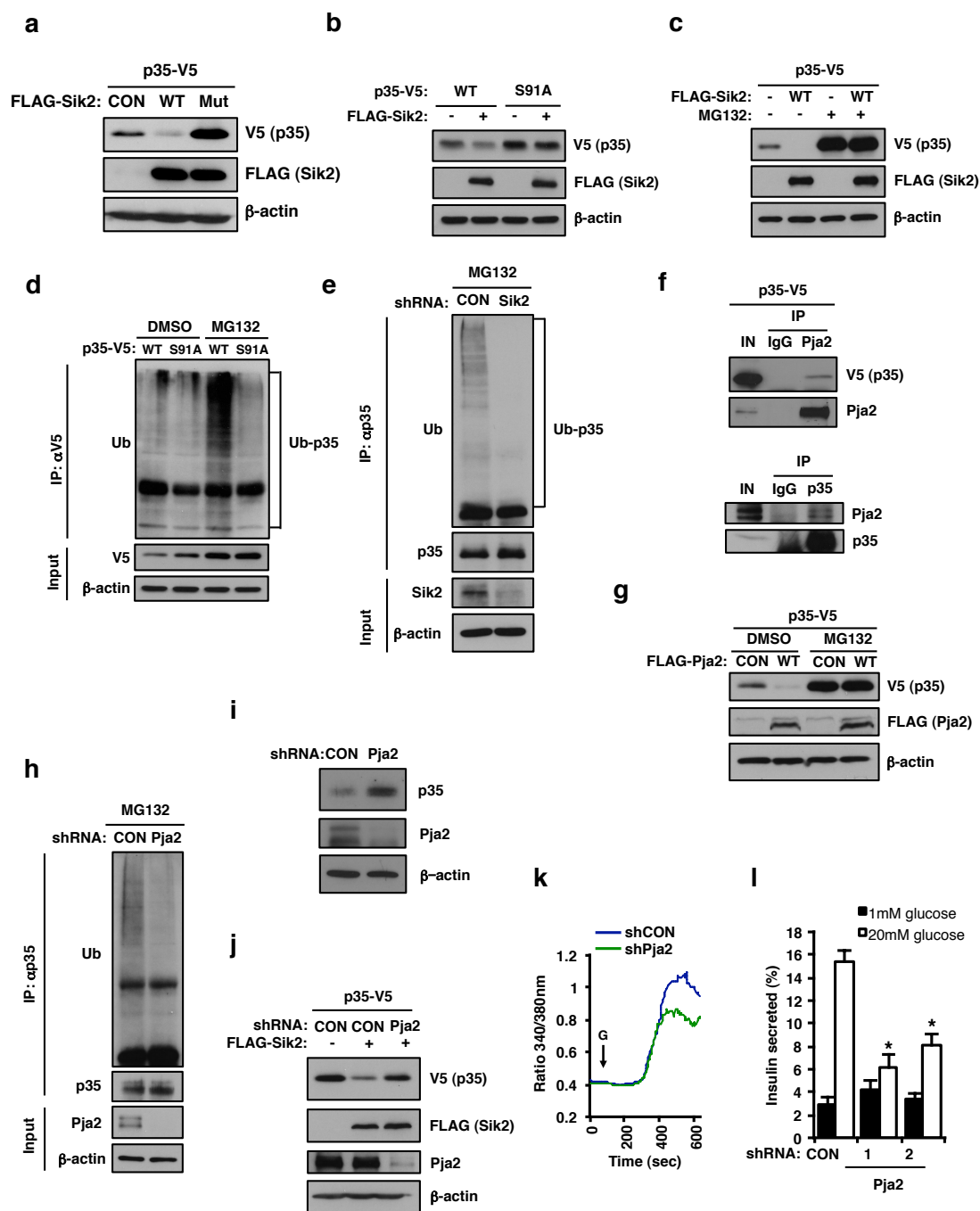


Figure 4. Sik2 promotes phosphorylation-dependent degradation of p35 by the ubiquitin E3 ligase Pja2.

a. Western blot analysis showing levels of p35-V5 in extracts from HEK293T transfectant co-expressing empty vector control (CON), FLAG-Sik2 WT or K49M mutant (Mut). b.

Western blot analysis of p35-V5 and S91A mutant levels in HEK293T cells expressing empty vector control or FLAG-Sik2 cDNA. c. Western blot analysis of p35-V5 levels in extracts from HEK293T transfectants coexpressing empty vector (-) or FLAG-Sik2 WT. The effect of treatment with DMSO control (-) or MG132 proteasome inhibitor (10 μ M for 24 h) is shown. d. Western blot analysis of poly-ubiquitination levels on p35-V5 immunoprecipitated from HEK293T cells expressing p35-V5 WT or S91A mutant cDNA. Treatment with DMSO or MG132 (10 μ M for 24 h) is indicated. e. Western blot analysis showing poly-Ub levels in p35 immunoprecipitates isolated from Min6 cell extracts following infection with control non-silencing (CON) or Sik2 shRNA. Cells were treated with 20 μ M MG132 for 24 h. f. (Top) Western blot analysis of immunoprecipitates of endogenous Pja2 from extracts of HEK293T cells expressing p35-V5. (bottom) Western blot analysis of immunoprecipitates of endogenous p35 and IgG control from MIN6 cell extracts for presence of endogenous Pja2. g. Western blot analysis of p35-V5 levels in extracts from Min6 cells coexpressing empty vector (-) or FLAG-Pja2 WT. The effect of treatment with DMSO control (-) or MG132 proteasome inhibitor (20 μ M for 24 h) is shown. h. Western blot analysis showing poly-Ub levels in p35 immunoprecipitates isolated from Min6 cell extracts following infection with control non-silencing (CON) or Pja2 shRNA. Cells were treated with 20 μ M MG132 for 24 h. i. Western blot analysis of endogenous p35 levels in mouse islets infected with Pjs2 shRNA. j. Western blot analysis of p35-V5 levels in extracts from HEK293T transfectants coexpressing FLAG-Sik2 WT together with or without shRNA targeting Pja2. k. Real time intracellular calcium measurements with Fura-2 dye in control and Pja2 knockdown MIN6 cells cultured in 1 mM glucose were stimulated with 20 mM glucose. Data is from a single experiment performed in triplicate, representative of three

experiments. 1. Insulin secretion in PJA2 knockdown MIN6 cells after treatment with 1 mM and 20 mM glucose.

reduced levels of p35-V5 protein, an effect that was blocked by MG132 treatment (Figure 4g) Silencing of Pja2 decreased p35 poly-ubiquitination and led to an increase in endogenous p35 levels (Figure 4h, 4i, and S6a), and prevented p35 degradation induced by Sik2 overexpression (Figure 4j). Importantly, knockdown of Pja2 in MIN6 cells reduced glucose-stimulate calcium entry (Figure 4k) and reduced GSIS by ~50% (Figure 4l, S6b). Taken together, these data support a direct role for the Sik2-Pja2-p35 cassette in the regulation of calcium-mediated insulin secretion in the beta cell.

The requirement of Sik2 for effective glucose homeostasis prompted us to evaluate the status of Sik2 in animal models of obesity and prediabetes. Western blot analysis of Sik2 protein in islets from ob/ob mice ²⁷ revealed that Sik2 levels were 2.5-fold higher when compared to islets from B6 controls (Figure 5a). Elevated Sik2 levels with a concomitant reduction in p35 were also observed in islets isolated from mice on a high fat diet (HFD), a model of diet-induced obesity (Figure 5b). The common feature of hyperglycemia in the ob/ob and HFD models prompted us to evaluate whether Sik2 is regulated by glucose. Following overnight culture, Sik2 protein levels increased directly as a function of ambient glucose concentration, an effect that was

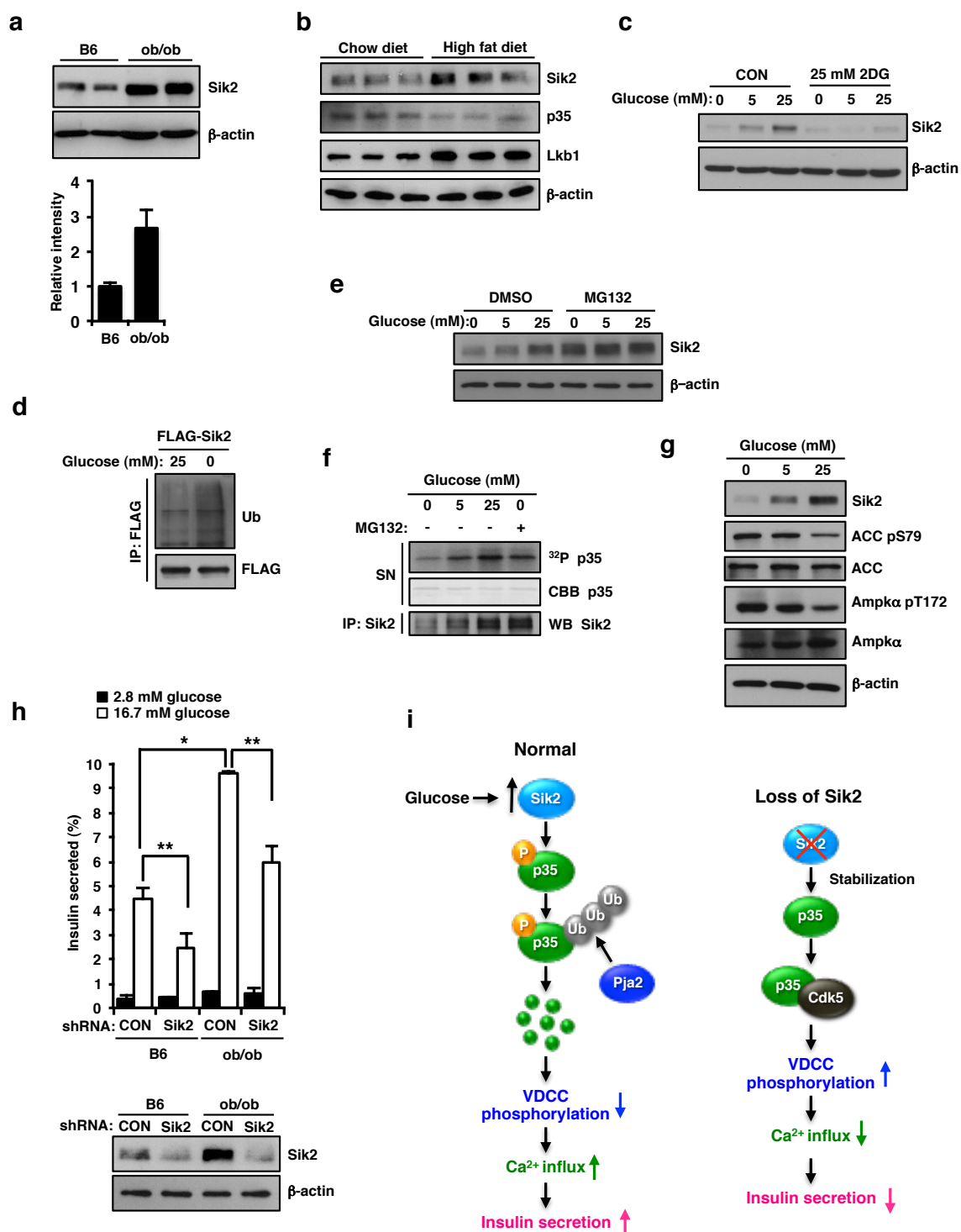


Figure 5. Sik2 is essential for beta cells to meet insulin demand in models of metabolic syndrome.

a. (Top) Western blot analysis of Sik2 protein levels in islets from control C57BL/6J

(“B6”) and B6.V-Lep^{ob}/J (“ob/ob”) mice. (Bottom) Histogram showing relative intensity of Sik2 protein levels normalized to beta actin loading control. b. Western blot analysis for Sik2, p35 and Lkb1 levels in high fat diet-fed animals (20 weeks). c. Western blot analysis of Sik2 levels. Min6 cells were cultured for 15 h in 0, 5 or 25 mM glucose in the presence or absence of 2DG (25 mM). d. Western blot analysis showing poly-Ub levels in FLAG-Sik2 immunoprecipitates isolated from Min6 cell extracts following infection with FLAG-Sik2. Cells were cultured in 0 or 25 mM glucose in the presence of 20 uM MG132 for 15 h. e. Western blot analysis of Sik2 levels. Mouse islets were pre-treated with MG132 (20 uM) or DMSO for 3h, then cultured for 15 h in 0, 5 or 25 mM glucose in the presence or absence of MG132 (20 uM). f. In vitro kinase assay using GST-p35 WT and Sik2 purified from MIN6 cells cultured for 15 h in 0, 5 or 25 mM glucose together with or without MG132 (20 uM). ³²P and coomassie blue (CBB) staining of GST-p35 substrates and immunoprecipitated Sik2 are shown. g. Western blot analysis showing Sik2 and phosphorylation of ACC and Ampka in MIN6 cells cultured for 15 h in 0, 5 or 25 mM glucose. h. (Top) Static GSIS assay using islets from B6 and ob/ob mice infected with non-specific control or Sik2 shRNA lentivirus. (Bottom) Western blot analysis of Sik2 protein levels in islets from parallel samples shown in the top panel. i. Model showing the role of the Sik2-Pja2-p35-Cdk5 pathway in glucose-stimulated insulin secretion.

blocked by treatment with 2- deoxyglucose, an inhibitor of glycolysis (Figure 5c). Treatment with rapamycin, an inhibitor of mTORC1 did not prevent Sik2 accumulation, indicating that protein translation was not required for this effect (Supplementary Fig. S7a). Furthermore, Sik2 mRNA levels were the same in wt and ob/ob mice suggesting that transcription or mRNA stability played no role in this setting (Supplementary Fig. S7b).

In contrast, we detected an increase of poly-ubiquitinated Sik2 species following overnight culture in low glucose (Figure 5d), and an accumulation of Sik2 protein after MG132 treatment of both MIN6 cells and islets (Figure 5e and S7c). As *in vitro* kinase activity present in Sik2 immunoprecipitates correlated with total Sik2 protein levels, we conclude that the intrinsic activity of Sik2 is not regulated by glucose (Figure 5f), and that we conclude that Sik2 levels are controlled post-translationally by ubiquitination. Levels of Ampk protein were unchanged and its activity was reduced in this setting (Figure 5g), consistent with our previous work ¹. Importantly, GSIS assays demonstrated that ob/ob islets hypersecrete insulin, which could be reversed by silencing Sik2 (Figure 5h). As insulin content was also unaffected by Sik2 silencing in this context (Supplementary Fig. S7d), we conclude that functional adaptation of ob/ob islets requires higher levels of Sik2.

Here we have delineated a pathway essential for first phase insulin secretion and for functional compensation in the beta cell wherein p35 levels are coordinated in response to hyperglycemia via the Sik2-Pja2 complex (Figure 5i). Dynamic analyses of islet function following nutrient stimulation has revealed that impaired first phase insulin release is a hallmark of T2D, and progressive impairment of first phase secretion occurs as beta cells fail

to compensate for the increasing demand^{28-30 29-36}. Our results show highlight the potential for targeting the Sik2-Pja2 complex to elicit such compensatory secretory responses from the beta cell for the treatment of insulin deficiency. Pja2 is activated by cAMP second messenger²⁶ which accumulates in the beta cell during feeding in response to incretin stimulation and crosstalk with calcium signaling pathways³⁷.

That the Sik2-p35 pathway is causally linked to enhanced insulin secretion is highlighted by the reversal of hypersecretion following silencing of Sik2 in islets isolated from obese mice. While pharmacological inhibition of all Siks reduced insulin secretion, Sik2 appears to be unique among the Siks in the regulation of p35 levels and insulin secretion, as knockdown of Sik1 and Sik3 had no effect in this regard (Supplementary Fig. S8 a and b). The unaltered Crtc2 phosphorylation and Creb target gene activation in SABKO islets indicates that Sik2 functions to regulate secretory events but not Creb-dependent gene expression or mTOR signaling in the beta cell¹.

Ampk is activated under low energy conditions to mobilize energy stores in all cell types, and in the beta cell acts to restrain protein translation and insulin production^{1,2,38}. Our observation that Sik2 protein accumulates in excess glucose without effect on its intrinsic activity indicates that there is diversity within the Ampk family both in the response to nutrient status and effects on insulin secretion. While Sik2 is ubiquitously expressed, p35 appears exclusively in brain and islets (Supplementary Fig. S8c). Interestingly, p35 levels are also higher in the brain of whole body Sik2 knockout mice (Supplementary Fig. S8d), indicating conservation of this pathway in these two tissues. Indeed, pancreatic beta cells and

neurons share conserved evolutionary ontology and cAMP and calcium-dependent signaling mechanisms ³⁹. Our work provides the first link between two central regulatory pathways, Lkb1-Ampk and p35-Cdk5, in the biology of excitable cells.

Materials and Methods

Mice

Sik2^{loxP/loxP} mice (C57BL6/129sv background) carrying loxP sites flanking exons 5-7 (exon 5 encodes the activation loop) were backcrossed four times onto the FVB/n background, then mated to Pdx-CreERT2 mice (FVB/n background) to generate Sik2^{loxP/loxP}, Pdx-CreERT2⁺ mice and appropriate controls. To induce Sik2 gene ablation, 5 mg of tamoxifen (Sigma) per 40 g body weight was injected IP every second day for a total of 3 injections, as described (Fu et al., 2009). Unless otherwise stated, analyses were performed starting 7-10 days after the last injection. To generate Sik2 whole animal KO mice, animals homozygous for the Sik2 loxP allele were crossed with mice carrying the Hprt-Cre transgene. Tissues were isolated from 6-10 wk old Sik2^{loxP/loxP}; Hprt-Cre⁺ mice and snap frozen in liquid nitrogen before use. All procedures involving mice were approved with the Animal Care Committee of the University of Ottawa. For high fat diet studies, mice were fed normal lab diet or high fat diet (45 kcal% fat; Open Source Diets) for 16-20 weeks as indicated. B6.v-Lepob/J and C57BL/6J mice were purchased from The Jackson Laboratory.

Cell Culture and Reagents

HEK293T and Min6 cells (p. 21-30) were cultured in DMEM + 10% FCS + antibiotics in a humidified atmosphere with 5% CO₂. MIN6 medium was supplemented with 100 uM b-mercaptoethanol. For plasmid transfection, HEK293T were transfected using PEI (Sigma) or Lipofectamine 2000 (Invitrogen). Mouse islets were cultured in RPMI-1640 supplemented with 10% FCS and antibiotics. Rapamycin was from LC Laboratories. Pan-

Sik inhibitor HG-9-91-01 was kindly provided by Dr. Philip Cohen (University of Dundee). Antibodies: Polyclonal Sik2 antiserum “E1” targeting peptide TQYLQHRLQKPNLLPKA was generated with the Covance custom antibody service and used following affinity purification. A second affinity purified Sik2 antibody was kindly provided by Dr. Olga Göransson (Lund University, Sweden). Anti-serum against phospho-p35 (Ser91) and phosphorylated and unphosphorylated peptides of p35 (KKSL(p)SCANLSC and KKSLSCANLSC) were kindly provided by Dr. Shin-ichi Hisanaga (Tokyo Metropolitan University, Japan). Anti-phospho-VDCC (Ser783) was a kind gift from Dr. Kazuhito Tomizawa (Kumamoto University, Japan). Anti-b-actin (AC-15, 1:5000), normal rabbit IgG, anti-FLAG (M2, 1:2000), and anti-VDCC (C1603, 1:500) antibodies were from Sigma. Anti-V5 (46-0705, 1:5000) was from Invitrogen. Anti-p35 (C64B10, 1:1000), ubiquitin (P4D1, 1:2000), phospho-S6K (Thr389) (#9205, 1:000), phospho-rpS6 (Ser235/236) (D57.2.2E, 1:1000), rpS6 (54D2, 1:2000), phospho-eEF2 (Thr56) (#2331, 1:1000), eEF (#2332, 1:2000), phospho-Ampk (Thr172) (#2531, 1:1000), Ampka (F6, 1:2000), phospho-ACC (#3661, 1:1000), and ACC (#3662, 1:1000) were from Cell Signaling Technology. Anti-Lkb1 (Ley 37D/G6, 1:000) and S6K (C-18, 1000), p35 (C-19), and Hsp90a/b (H-114, 1:2000) were from Santa Cruz. Anti-PJA2 (A302-991A, 1:2000) was from Bethyl Laboratories. Crtc2 and phospho-Crtc2 (Ser171 and Ser275) were described previously ⁴⁰.

Glucose tolerance, insulin tests, and immunohistochemistry

Animals were fasted for 16 hr prior to IP injection with 2 mg/g body weight (chow diet) or 1.75 mg/g body weight of glucose (high fat diet), and blood glucose and insulin levels were measured at the indicated times as described ¹. Plasma insulin levels were measured by

ELISA using an ultra-sensitive mouse insulin kit (Crystal Chem). For measurement of fasted and refed blood glucose and insulin levels, mice were fasted for 16 h, then refed for 2 h. For arginine injection, mice were fasted for 16 h, then injected intraperitoneally with 1 mg/g body weight of L-arginine (Sigma). For insulin tolerance test, mice were fasted for 4 h, then injected intraperitoneally with 0.6 U/kg body weight of insulin (NovoRapid, Novo Nordisk).

RNA interference and cDNA plasmids

shRNAs targeting *Sik2* and *Cdk5r1/p35* were packaged in 293T cells cultured in 15 cm dishes using pLKO.1, pCMV8.3 and pMD2.G vectors. Seed sequences of shRNAs (in pLKO.1) were as follows: m/hSik2: CCTGGACTCTTTGATTGTGAA; mCdk5r1#1: CCCACGTTAGTGCTGGTTAAT; mCdk5r1#2: ACGATGGATCCAGGGTAGATG; m/hLKB1: CGCCAAGCTCATCGGCAAGTA; mAmpka1#1: CCCATCTTATAGTTCAACCAT; mSik1: CTGGGACTGAACAAGATCAAA; mSik3: CGGAATATCGTTCATCGTGAT; mPJA2#1: CGGAGAGTATTACCAACTCTT; mPJA2#2: GCCAGTAAGGAAAGCATTGAT. Control shRNA was from Sigma. Virus was concentrated 100X by ultracentrifugation at 28,000 g for 2 hr with a lower 20% sucrose layer. Virus was resuspended at 4°C overnight and stored in aliquots at -80°C for use. For overexpression, cDNA encoding mouse *Sik2* WT, K49M, and S587A as well as mouse p35-V5 WT and S91A was inserted into pDONR211 vector (Invitrogen), then Gateway recombined into pLenti6-V5 vector (Invitrogen) according to the manufacturer's instructions. Cells were infected with lentivirus for 30h-96 h.

Glucose Stimulated Insulin Secretion (GSIS)

GSIS assays were performed as described ¹. Briefly, 24 h after isolation 10 size-matched small and medium islets were cultured in RPMI-1640 were equilibrated with Krebs Ringer Buffer + 2.8 mM glucose for 30 min, prior to incubation in 300 ul KRB + 2.8 mM glucose for 1 hr. The SN was removed and stored, and islets were stimulated with 300 ul of KRB + 16.7 mM glucose for 1 h, followed by incubation with KRB + 45 mM KCl for 1 h (stimulated). All incubation steps were performed at 37°C in a tissue culture incubator. Total insulin was harvested by acid-ethanol extraction overnight. For MIN6 experiments, cells cultured in 48-well (or 96-well) plates in triplicate were washed with 250 ul (100 ul) KRB + 1 mM glucose for 30 min, followed by a second incubation in KRB + 1 mM for 1 h (“unstimulated”). SN was collected and replaced with KRB + 20 mM glucose for 1 h (“stimulated”). Where indicated, 45 mM KCl was used to depolarize cells. Insulin measurements were performed by HTRF assay (Cisbio).

Co-immunoprecipitation, SDS-PAGE and Western Blotting

HEK293T or Min6 cells were lysed in ice cold (all steps were performed at 4 °C) co-IP buffer (20 mM Hepes-KOH, pH 7.5/ 150 mM NaCl/ 2 mM EDTA/ 1% Triton X-100) containing halt protease inhibitor cocktail (Thermo Scientific) and the whole-cell extracts were precleared with Protein A/G agarose (Santa Cruz) for 2 h. Precleared lysates were incubated with 3 mg of anti-Sik2 (E1) antibody or control rabbit IgG overnight, prior to incubation with 30 ml of Protein A/G agarose (50% slurry) for 2 h. After washing 3 times with co-IP buffer, immunoprecipitates were resolved by SDS-PAGE, followed by immunoblot analysis as described ¹. For glucose treatments, islets were starved with 2.8 mM

glucose for 2 h then treated with the indicated concentrations of glucose for 1 h prior to extract preparation.

Measurements of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were performed as described ¹.

In vitro kinase assay

Assays were performed as described in ⁴⁰. Purified GST-p35 WT and S91A proteins were incubated with FLAG-Sik2 (purified from HEK293T cells) immobilized on protein A/G agarose in kinase assay buffer at 30 °C for 20 min prior to SDS-PAGE and autoradiography. Proteins were visualized by SimplyBlue safe stain (Invitrogen).

Quantitative RT-PCR

Was performed on a Mastercycler EP Realplex 2 (Eppendorf) using a QuantiTect SYBR green PCR kit (Qiagen). Relative gene expression was determined by $\Delta\Delta\text{CT}$ normalized to expression of 36b4. Following primer sets were used: mouse Sik1 (5'-GCCTTGACCTCGACTCTGC-3' and 5'-CCCCCAAGTCAGGTGTTCTA-3'), mouse Sik2 (5'-GCAGCTCACAACCCCATTTGTG-3' and 5'-GGACTTCAGGACACCGCATC-3'), mouse Sik3 (5'-TCCTCACCTGGTACCTGACC-3' and 5'-CAGGGTGTTGGAGTCCTTGT-3'), mouse Nr4a2 (5'-CGACCTCTCCGGCCTTTTA-3' and 5'-CGCCGAAATCGTTGTCAGTA-3'), mouse Irs2 (5'-AGCTTTGATTGGCTGTCCTGGAG-3' and 5'-TGGCGATATAGTTGAGGCCGTTGT-3'), mouse 36b4 (5'-CCACGAAAATCTCCAGAGGCAC-3' and 5'-

ATGATCAGCCCGAAGGAGAAGG-3'). Primer sets of Sik1 and Sik3 were designed using Primer3.

Mitochondrial stress test

MIN6 cells were infected with lenti-shRNA targeting Sik2 or non-silencing control on Seahorse XF24 cell culture microplate on day 0. On day 3, cells were starved in 400 uL of Seahorse XF assay medium containing 1 mM glucose for 30 min, and oxygen consumption measured following treatment with 20 mM glucose, 0.5 uM FCCP and rotenone (0.5 uM) at the indicated times. Protein was quantified using BCA (Thermo Scientific) for normalization.

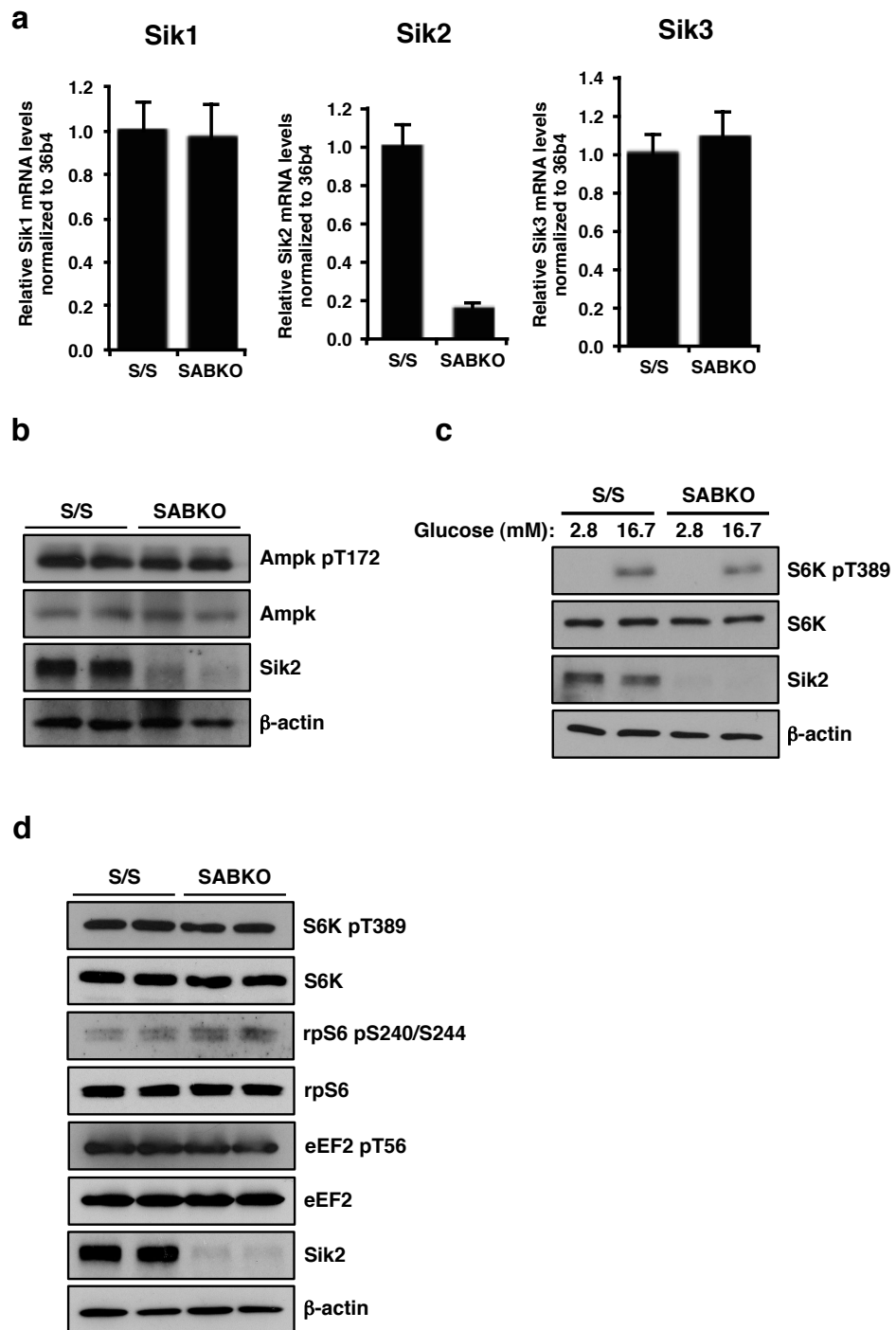
Statistical analyses

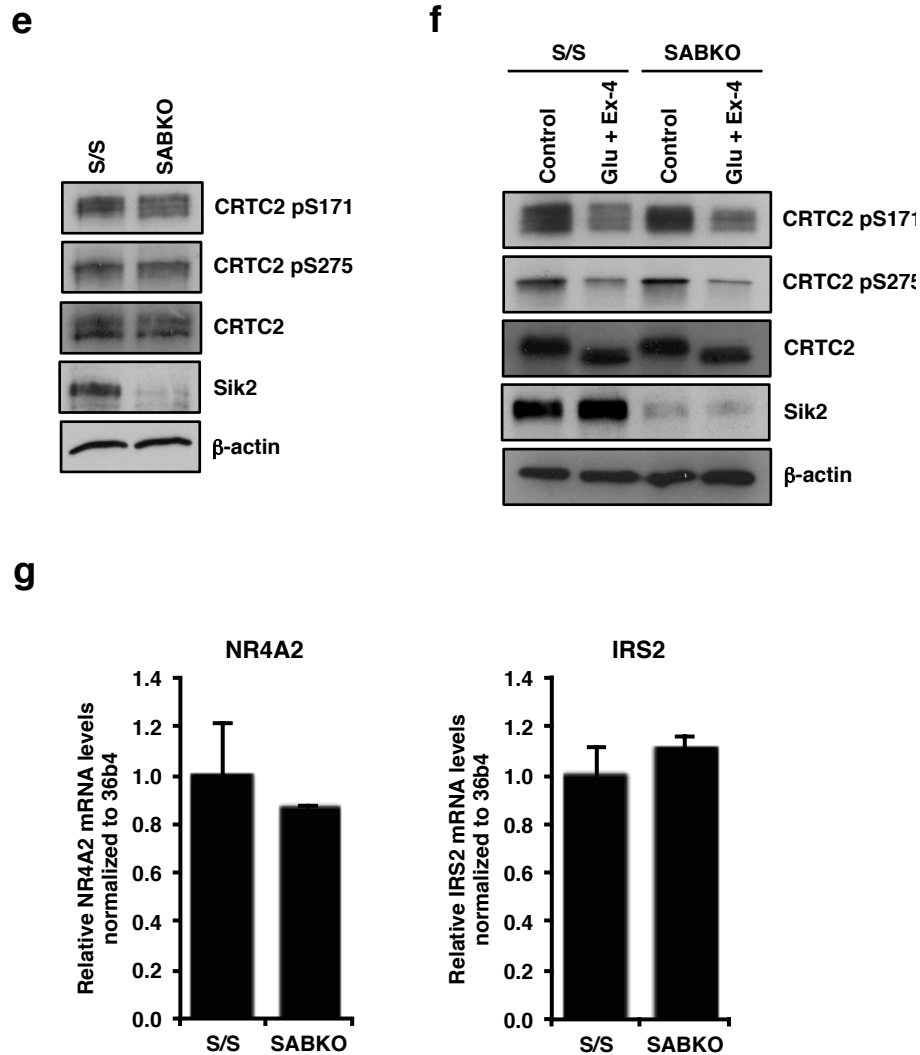
All studies were performed on at least three independent occasions. Results are reported as mean $\pm$ s.e.m. Statistical significance for all data was determined using two-tailed unpaired Student's *t*-test; *p*-values < 0.05 were considered significant. Significance in all figures is indicated as follows: * *p* < 0.01, ** *p* < 0.05.

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Innovation (CFI) to RAS, operating grant from CIHR (MOP-84314) to ACG, by a Japan Society for the Promotion of Science Fellows to JS (10J00366), and Canadian Diabetes Studentship to AF. ACG holds the Canada Research Chair in Functional Proteomics and the Lea Reichmann Chair in Cancer Proteomics. RAS holds the Canada Research Chair in Apoptotic Signaling.

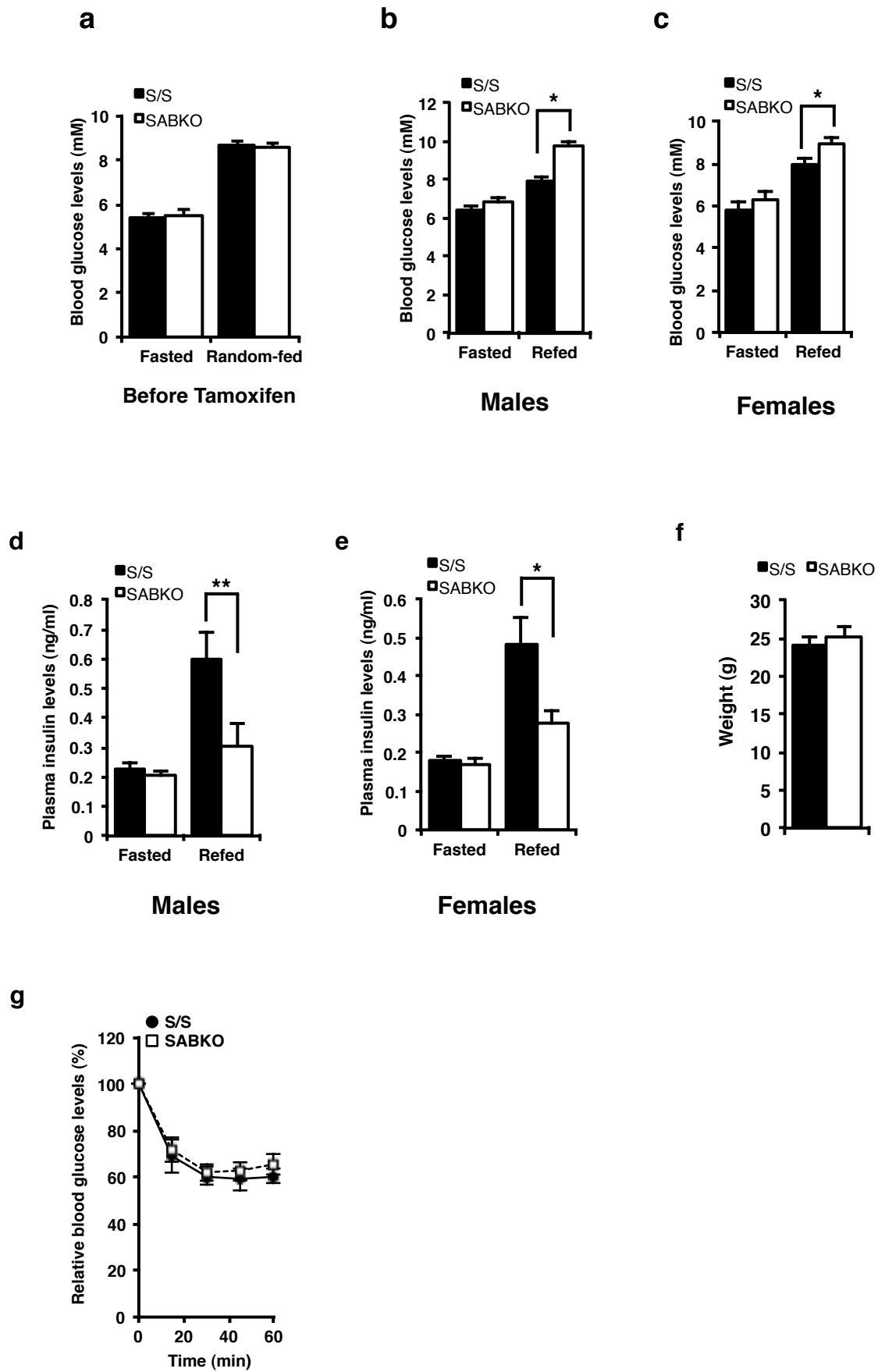
Supplementary Figures



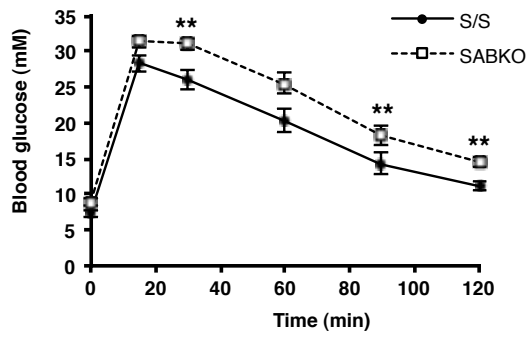


S1. QPCR analysis showing reduction of Sik1, Sik2 and Sik3 mRNA levels in total RNA from islets isolated from S/S and SABKO mice. RNA was pooled from 3 mice per genotype. Sik mRNA levels are normalized to levels of 36B4 mRNA as internal control and are representative of two experiments. b. Western blot analysis showing the phosphorylation status and total protein levels of Ampk in islets from control S/S and SABKO mice. c. Western blot analysis of mTOR signaling pathway in SABKO islets. Western blot analysis of the phosphorylation status of mTORC1 substrate S6 kinase (S6K) at Thr389 in control

S/S and SABKO islets cultured in 2.8 mM or 16.7 mM glucose for 1h. d. Western blots showing levels of S6K pThr389, S6 pSer240/pSer244, and eEF2 pThr56 and their respective total protein controls in islets cultured in RPMI-1640 medium. e. Western blot analysis of Crtc2 phosphorylation at regulatory sites Ser171 and Ser275 in islets from control S/S and SABKO mice. f. Western blot analysis of Crtc2 phosphorylation at regulatory sites Ser171 and Ser275 in islets from control S/S and SABKO mice cultured in low glucose or stimulated with 16.7 mM glucose + 10 nM exendin-4 for 1h. g. QPCR analysis of mRNA levels of Creb target genes Nr4a2 (left) and Irs2 (right) in islets isolated from control S/S and SABKO animals.

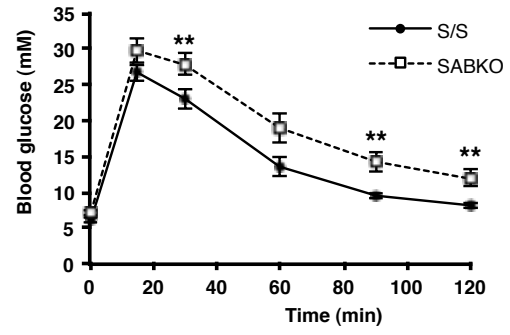


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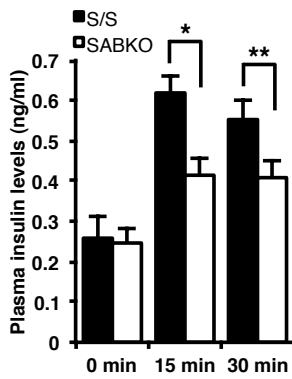
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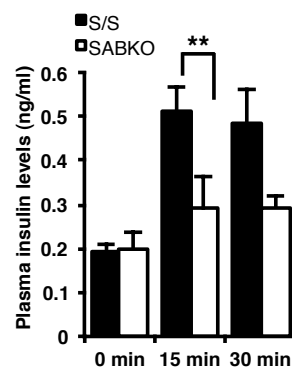
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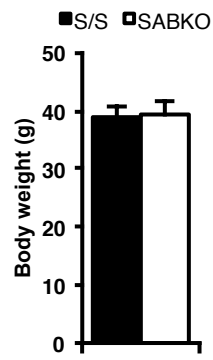
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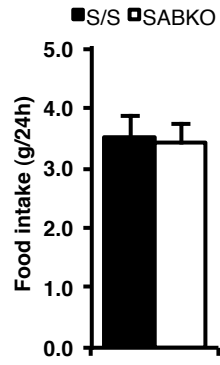


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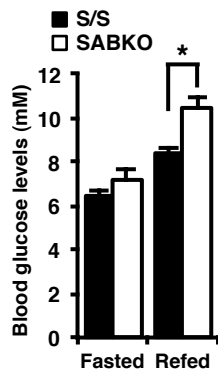
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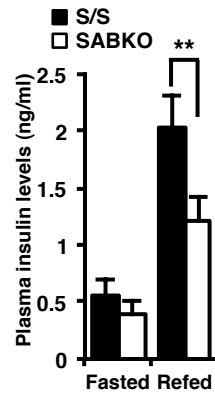
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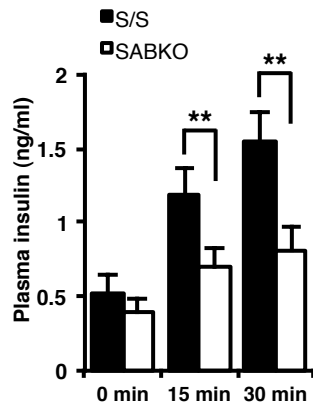
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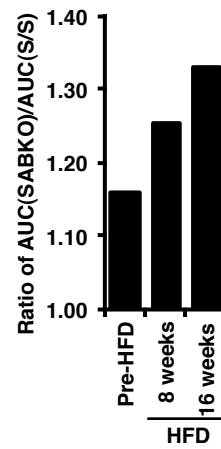
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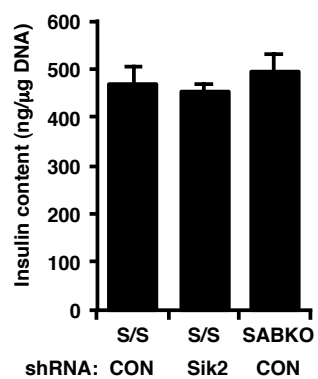
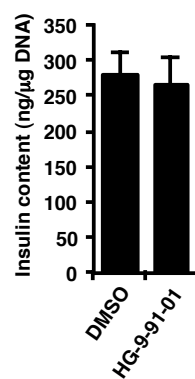
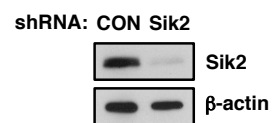
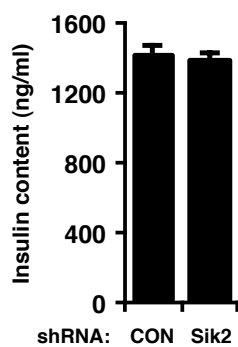
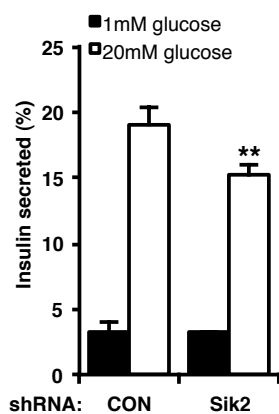
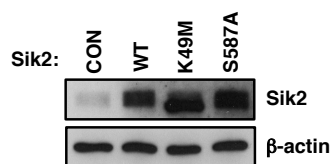
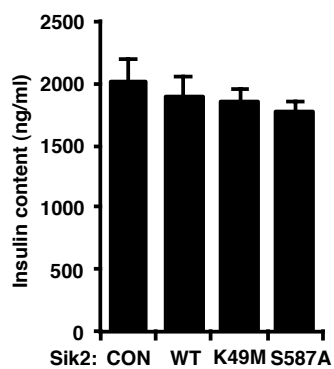
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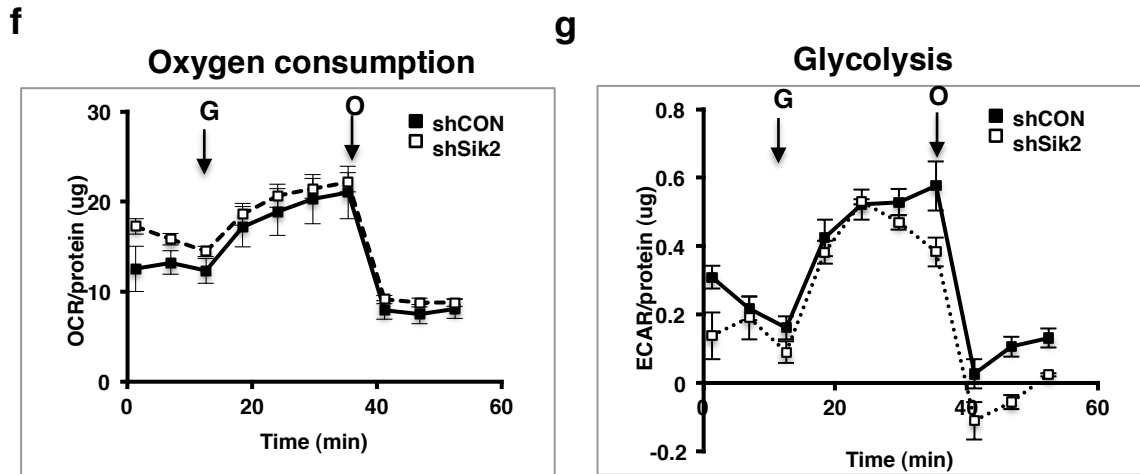


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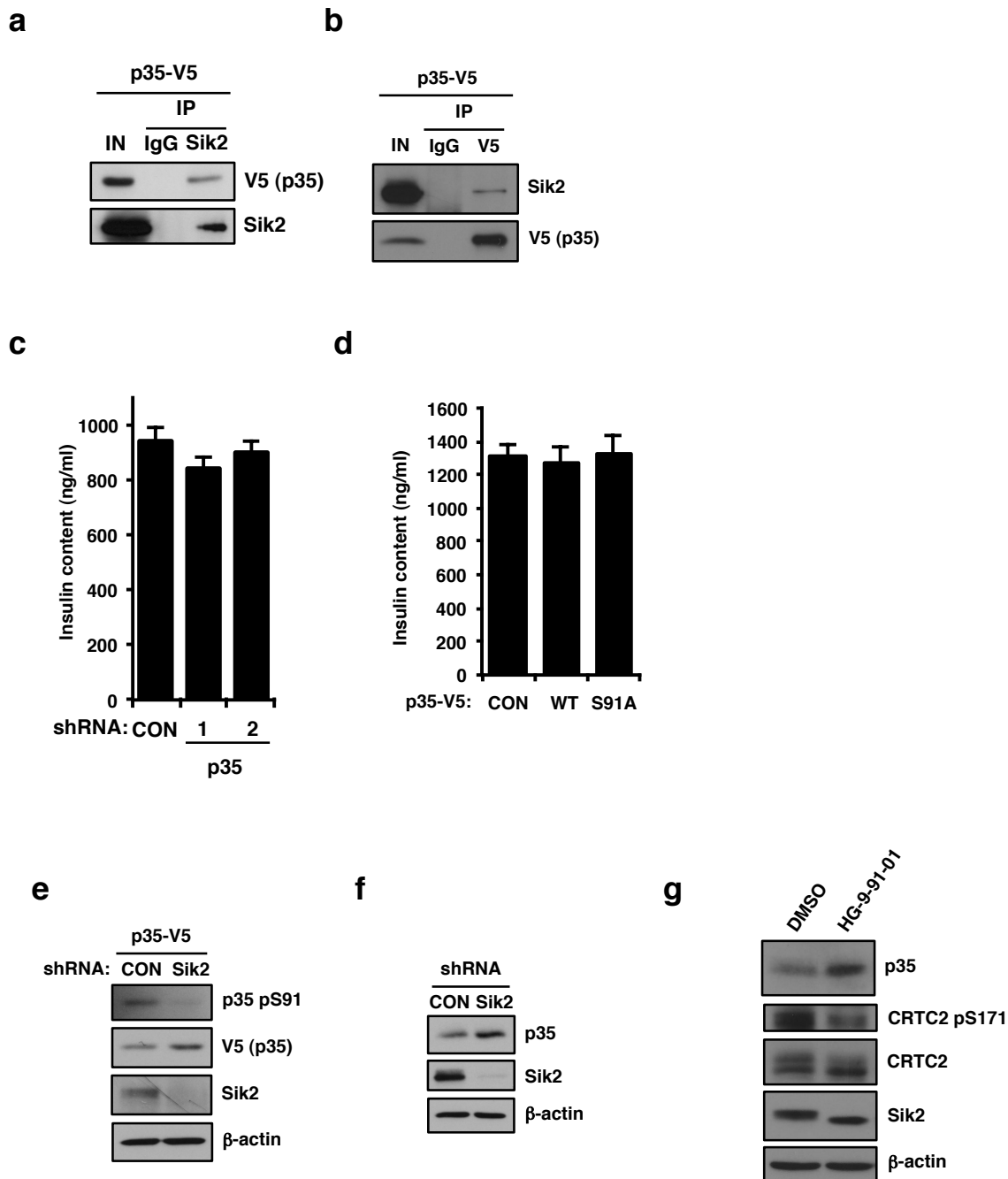


S2. Blood glucose levels of fasted and random fed control (S/S, n=35) and SABKO (n=33) mice were measured before tamoxifen treatment. b, c. Fasted and refed (2 h) blood glucose levels in male S/S (n=17) and SABKO (n=16) (b) and female S/S (n=5) and SABKO (n=5) mice (c). d, e. Fasted and refed (2 h) plasma insulin levels in male S/S (n=14) and SABKO (n=18) (d) and female S/S (n=5) and SABKO (n=5) mice (e). f. Body weights of control (S/S, n=14) and SABKO (n=9) animals at age ≥ 9 weeks. g. Insulin tolerance test in S/S (n=7) and SABKO (n=11) mice. h, i. Glucose tolerance test in male S/S (n=8) and SABKO (n=7) (h) and female S/S (n=8) and SABKO (n=4) mice (i). j, k. Plasma insulin levels during glucose tolerance test in male S/S (n=9) and SABKO (n=9) (j) and female S/S (n=4) and SABKO (n=4) mice (k). l. Body weights of control (S/S, n=14) and SABKO (n=8) animals on high fat diet for 16 weeks. m. Food intake in control (S/S, n=11) and SABKO (n=8) mice after 18 weeks on HFD. n. Blood glucose levels in control (S/S, n=14) and SABKO (n=8) animals on high fat diet for 16 weeks following an overnight fast or 2 h refeeding. o. Plasma insulin levels for animals shown in the left panel. p. Plasma insulin levels at indicated times postinjection of glucose during GTT assay shown in Fig. 1i for control (S/S, n=8) and SABKO (n=8) HFD-fed mice. q. Ratio of AUC from HFD-fed SABKO mice at indicated times to AUC for normal diet (pre-HFD) SABKO mice.

a**b****c****d****e**

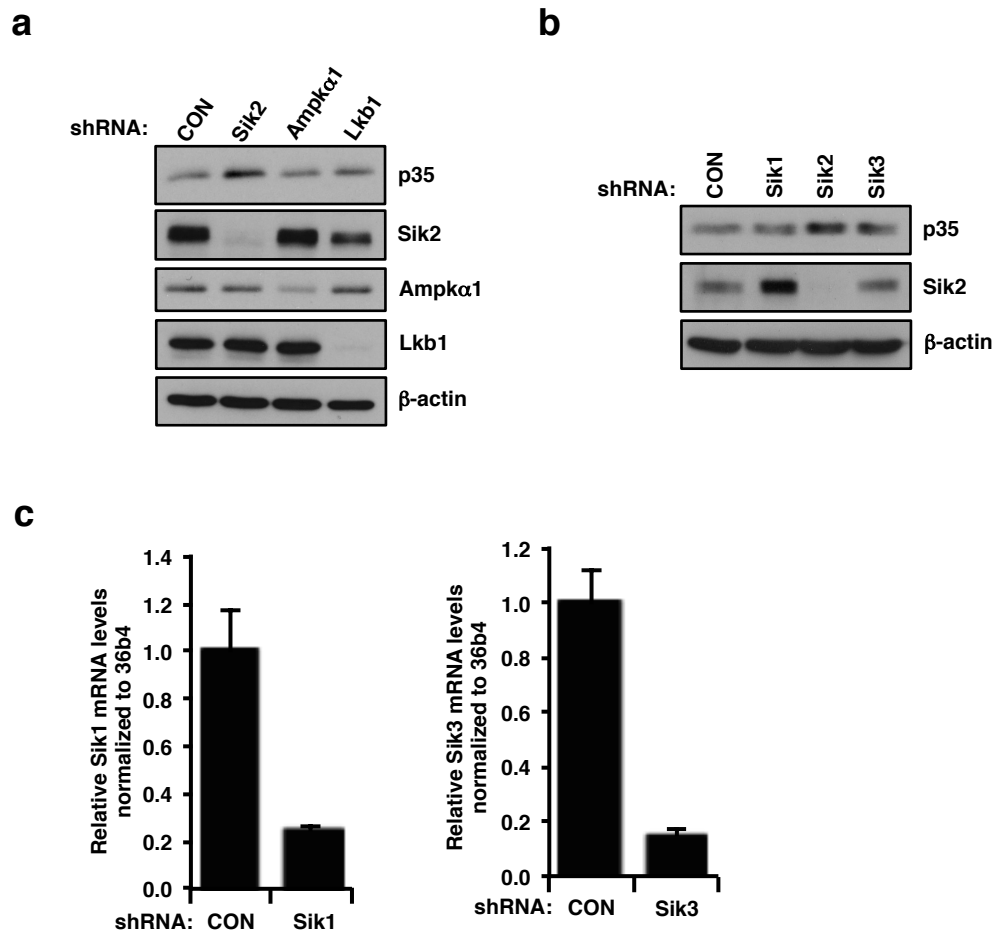


S3. Insulin content from islets shown in Figure 2b. b. Insulin content from islets treated with HG-9-91-1 shown in Figure 2c. c. (left) GSIS assay showing percent insulin secretion (normalized to total insulin content) in control or Sik2 knockdown MIN6 cells cultured in 1 mM (black bars) or 20 mM (white bars) glucose. (middle) Insulin content. (right) Western blot analysis of Sik2 levels in MIN6 cells stably infected with lentivirus expressing non-targeting control (CON) or Sik2 shRNA. d. Western blot showing levels of Sik2 protein in MIN6 cell extracts following infection with lentivirus expressing Sik2 WT, kinase-dead K49M or constitutively active S587A mutants compared to empty vector control. e. Insulin content in MIN6 cells infected with lentivirus encoding an empty vector (CON) or Sik2 WT, K49M, or S587A cDNA. f. Oxygen consumption in control and Sik2 knockdown MIN6 cells during mitochondrial stress test. Oxygen consumption rates are normalized to ug protein. g. Extracellular acidification rate in cells from f, (ECAR, a proxy for glycolytic activity). Effects of addition of glucose (G) and the ATP synthase inhibitor oligomycin (O) are shown.

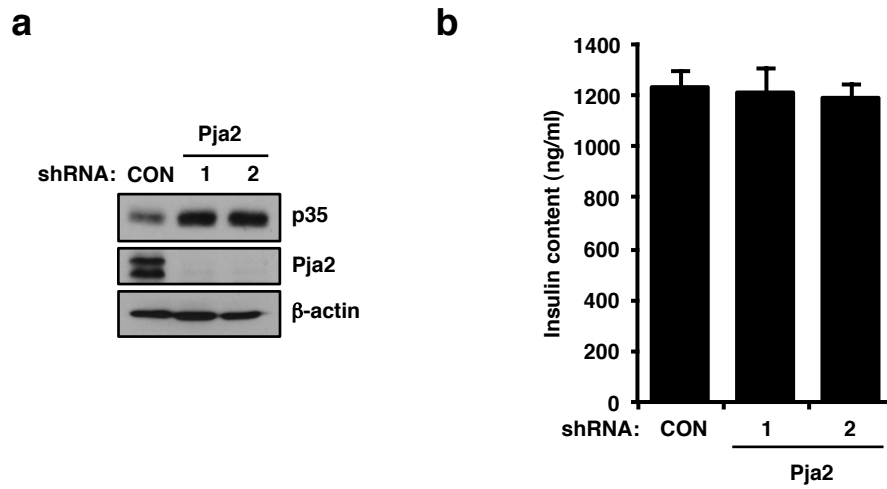


S4. Western blot analysis of immunoprecipitates of endogenous Sik2 from extracts of HEK293T cells expressing p35-V5. **b.** Western blot analysis of immunoprecipitates of p35-V5 and IgG control from extracts of HEK293T cells expressing p35-V5 for presence of endogenous Sik2. **c.** Insulin content from cells shown in Figure 3e. **d.** Insulin content from cells shown in Figure 3f. **e.** Western blot analysis of pSer91 levels on p35-V5 in Min6 cells

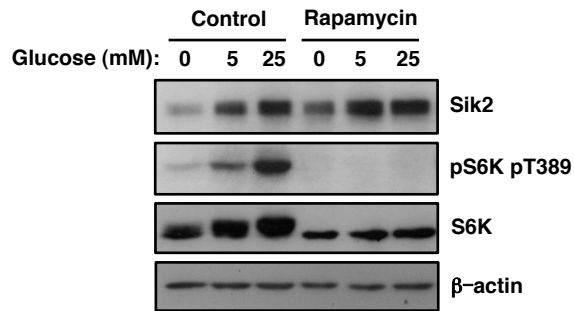
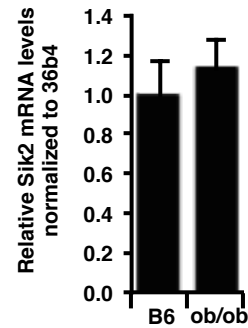
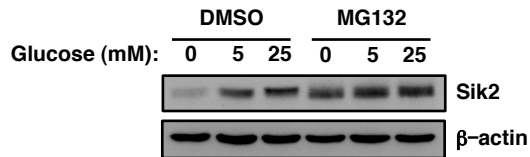
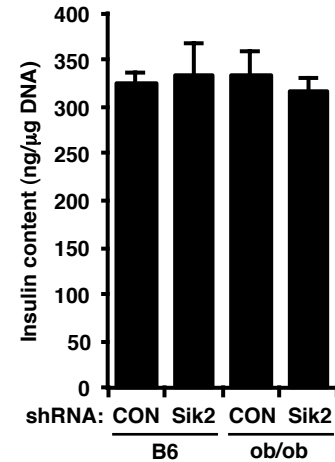
infected with lenti-nonsilencing control (CON) or lenti-Sik2 shRNA with anti-p35 pSer91 antibody. f. Western blot analysis of endogenous p35 levels in Sik2 knockdown MIN6 cells. g. Western blot analysis of p35 expression and Crtc2 phosphorylation in mouse islets treated with DMSO or 0.5 uM HG-9-91-01 for 24 hr.



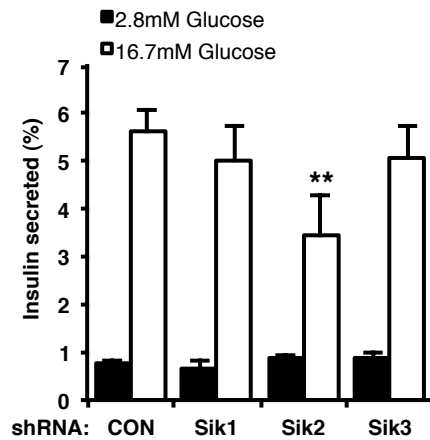
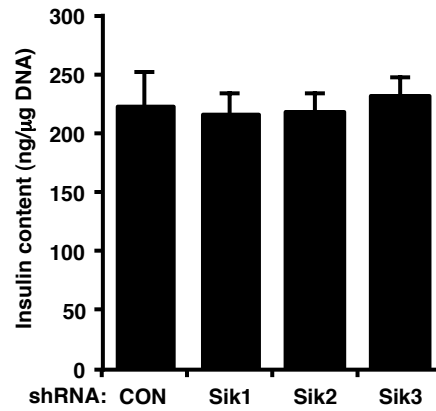
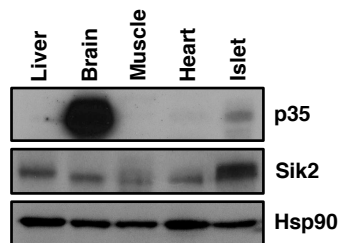
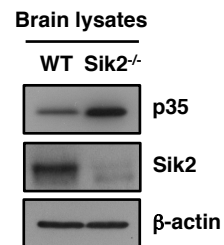
S5. Western blot analysis of p35 levels in Min6 cells infected with lentivirus encoding shRNA targeting Sik2, Ampkα1, Lkb1, or non-silencing control (CON). b. Western blot analysis of p35 and Sik2 levels in MIN6 cells infected with lentivirus encoding non silencing control shRNA (CON) or shRNAs targeting Sik1, Sik2 or Sik3. c. QPCR data showing knockdown of Sik1 and Sik3 mRNAs from experiment shown in b.



S6. Western blot analysis of endogenous p35 levels in Pja2 knockdown MIN6 cells. b. Insulin content in MIN6 cells from Figure 4l that were infected with lentivirus encoding control non-silencing (CON) or two independent shRNAs targeting Pja2.

a**b****c****d**

S7. Western blot analysis of Sik2 levels. MIN6 cells were pretreated with 100 nM rapamycin or EtOH for 3h, then cultured for 15 h in 0, 5 or 25 mM glucose in the presence or absence of 100 nM rapamycin. b. QPCR analysis of Sik2 mRNA levels normalized to 36B4 internal control in islets isolated from control C57BL6/J (B6) and ob/ob mice. c. Western blot analysis of Sik2 levels. MIN6 cells were pre-treated with MG132 (20 μM) or DMSO for 3h, then cultured for 15 h in 0, 5 or 25 mM glucose in the presence or absence of MG132 (20 μM). d. Insulin content in islets isolated from C57BL6/J (B6) or ob/ob mice infected with control non-silencing (CON) or Sik2 shRNA.

a**b****c****d**

S8. Insulin secretion from mouse islets infected with lentivirus encoding control non-silencing (CON), Sik1, Sik2, or Sik3. b. Insulin content from islets shown in a. c. Tissue Western blot showing p35 and Sik2 levels in indicated mouse tissues. d. Western blot analysis showing levels of p35 and Sik2 in brain extracts from WT and Sik2 whole animal knockout mice.

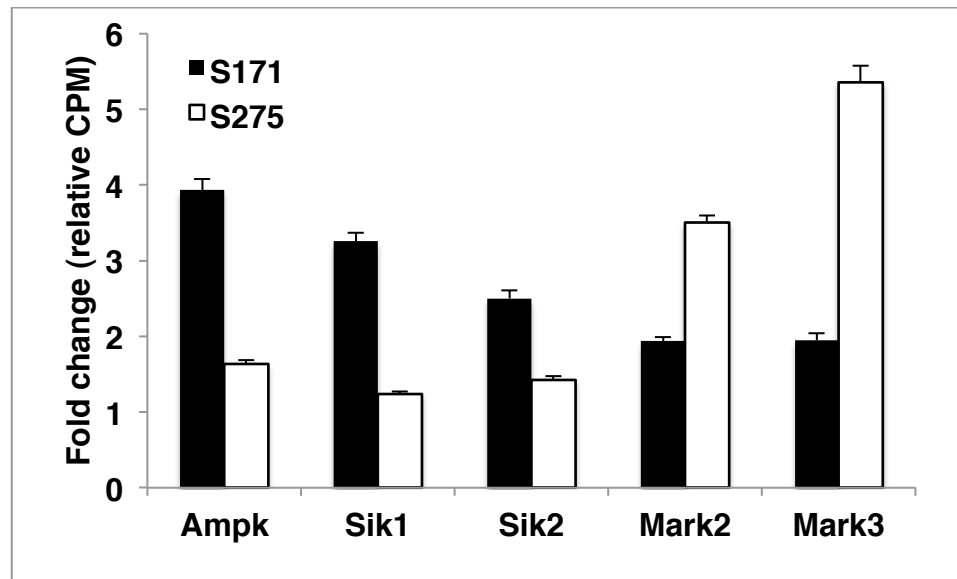
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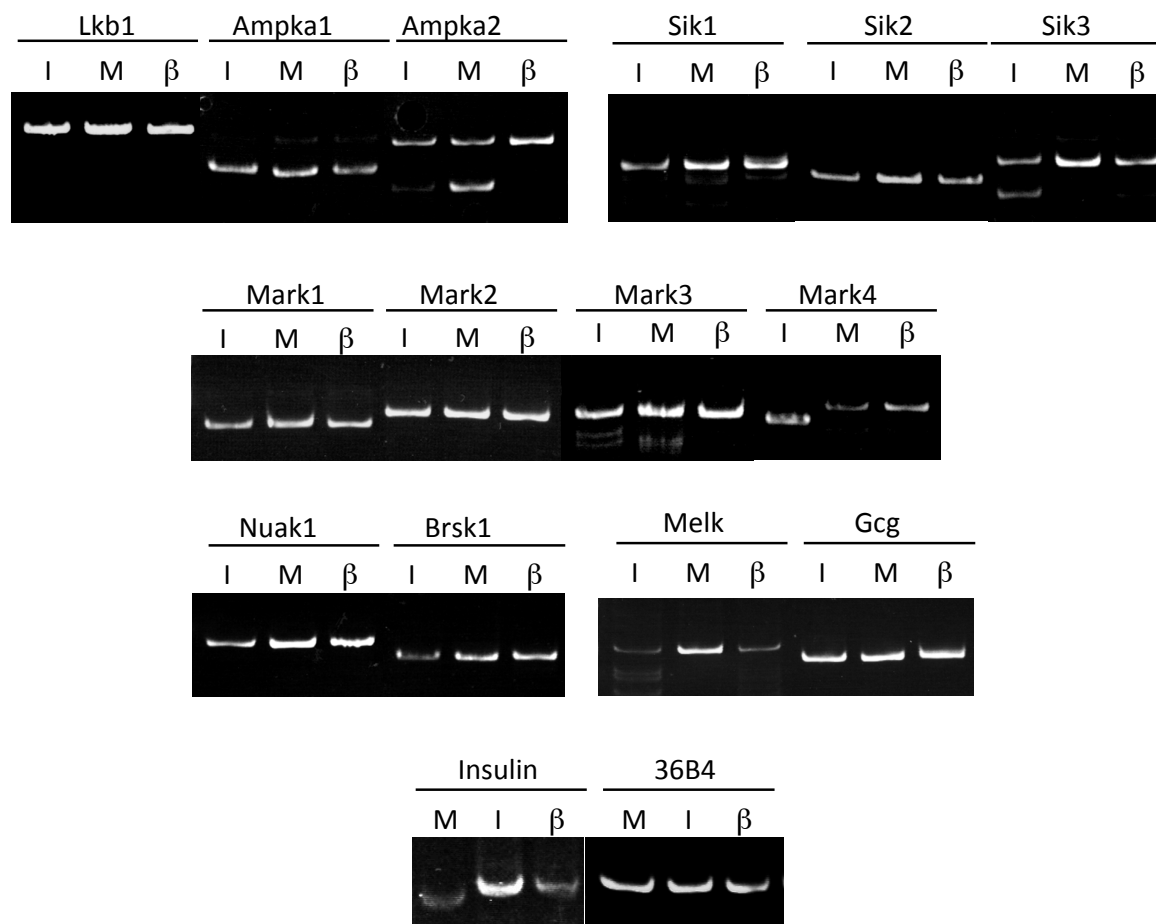
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Appendix 2. Preferential phosphorylation of Crtc2 at Ser171 and Ser275 by different AMP kinases. In vitro phosphorylation of Ser171 and Ser275 containing GST-peptides, reactions were loaded onto P81 paper and read in a scintillation counter, background of each kinase phosphorylation level was subtracted from reactions with substrate, data are pooled from 4 independent experiments.



Appendix 3. Gene expression of AMP kinase family members in pancreatic beta cells.

RT-PCR of RNA extracted from either whole isolated islets (I), MIN6 cells (M) and FACS-purified beta cells (β) using oligos against the coding sequences of LKB1, AMPKalpha1 and 2, Sik1-3, Mark1-4, Nuak1, Brsk1, Melk and endocrine cell markers insulin and glucagon (Gcg) reveals expression of 13 out of 14 AMP kinases in beta cells using 36B4 as a relative total RNA control.



Appendix 4. Depletion of Intranuclear Rodlets in Mouse Models of Diabetes

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Keywords Diabetes . Pancreatic islet . Beta cell . Insulin . Leptin . Intranuclear rodlet

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Abstract. Intranuclear rodlets (INRs) are structures present within the nuclei of human insulin-secreting beta cells of the endocrine pancreas. Their physiological significance, and whether they are altered in disease, is unknown. In the present study, the proportion of pancreatic beta cells containing INRs was examined in mouse models of type II diabetes and in a model with improved beta cell function. To gain insights into the molecular regulators of INR formation, mice with a conditional adult beta cell-specific knockout of the serine/threonine protein kinase Lkb1 (Lkb1 adult beta cell knockout (LABKO) mice) were studied. To investigate INR changes in a pathophysiological context, beta cell INRs were examined in two models of human metabolic syndrome: (1) mice maintained on a high-fat diet and (2) leptin-deficient ob/ob mice. The proportion of beta cells containing INRs was significantly reduced in LABKO mice. This reduction was not mediated by two key downstream effectors of Lkb1, mTor and Mark2. High-fat diet regimen reduced beta cell INR frequency by more than 40%, and leptin-deficient ob/ob mice exhibited a dramatically (19-fold) reduced INR frequency relative to wild-type mice. Taken together, our results support the view that INR formation in pancreatic beta cells is a dynamic and regulated process. The substantial depletion of beta cell INRs in LABKO and diabetic mice suggests their relationship to beta cell function and potential involvement in diabetes pathogenesis.

Introduction.

There is increasing evidence that dysfunction of insulin-secreting beta cells in the pancreatic islets of Langerhans plays a primary role in the pathogenesis of type II diabetes (T2D) [1]. Beta cells are archetypal metabolic sensors and critical regulators of blood

glucose homeostasis. The precise cellular mechanisms through which they achieve this and the factors underlying their failure in diabetes remain to be completely elucidated [1]. The study of beta cell morphology has contributed substantially to our understanding of beta cell function and dysfunction. We recently described the existence of intranuclear rodlets (INRs) in human endocrine pancreas [2]. These structures, which we originally identified in neurons [3], are distinct, nuclear, rod-shaped filamentous structures that stain intensely with a monoclonal antibody generated against the neuron-specific cytoskeletal protein class III beta tubulin (C3T). In pancreas, INRs are found predominantly in beta cells and are completely absent from acinar cells. In humans, they are present in approximately 23% of beta cells and in <5% of alpha-, delta-, and PP cells combined [2]. The physiological significance of beta cell INRs is presently unknown; however, preliminary studies in our laboratory indicate that these are highly dynamic nuclear bodies rather than static protein aggregates.

The molecular mechanisms underlying beta cell INR formation are currently unknown. As an approach to identifying the possible molecular mediators of INR formation in beta cells, we examined INRs in mice with a conditional beta cell-specific knockout of serine/threonine kinase Lkb1 (Lkb1 adult beta cell knockout, LABKO mice) [4]. As a broad-spectrum regulator of beta cell function [5], Lkb1 is an attractive initial candidate for this approach as it regulates many aspects of beta cell structure and function. It achieves this via phosphorylation and consequent activation of members of the 5' AMP-activated protein kinase (AMPK) family [6]. Accordingly, the beta cells of LABKO mice are larger due to the loss of AMPK-mediated suppression of the mammalian target of rapamycin (mTOR) and display what appears to be abnormal cell polarity due to the loss of microtubule affinity-regulating kinase 2 (Mark 2) activity [4, 7]. Functionally, LABKO animals display enhanced

basal and nutrient-stimulated insulin secretion and are resistant to high-fat diet (HFD)-induced impairment of glucose tolerance [4, 7].

In the present study, we first compared the frequency of beta cell INRs in LABKO and control mice maintained on either normal (chow) diet or a HFD, an extensively used model of human metabolic syndrome and an inducer of diabetic transformation in mice [8]. To probe further the status of beta cell INRs in a pathological context, we investigated them in an unrelated mouse model of T2D which has been widely used as a model of the human prediabetic state, the leptin-deficient ob/ob mouse [9].

Materials and Methods.

Mice.

LABKO Mice. Pancreatic tissues from LABKO and control mice reported in the previous study of Fu et al. [4] were employed in the present study. Generation and characterization of LABKO mice, including the absence of Lkb1 in LABKO islets as well as the morphometric analysis of islet and beta cell size, was previously described [4]. Briefly, Lkb1^{loxP/loxP} (L/L; FVB/n background) [10] and Pdx-CreERT2 mice (ICR background; kind gift of D. Melton) were mated to generate Lkb1^{loxP/loxP}, Pdx-CreERT2 (LABKO) mice. The Pdx-CreERT2 mice were backcrossed three times onto FVB/n background and then were intercrossed. To induce Lkb1 gene ablation, 5 mg of tamoxifen (Sigma) per 40 g of body weight mass was injected intraperitoneally every second day for a total of three injections. LABKO and L/L control mice were 10–12 weeks old at the time of killing and showed no significant difference in

their mean body weight (27 ± 0.7 vs. 28 ± 0.8 , respectively, $p=0.36$) or the wet weight of extracted pancreata (0.296 ± 0.017 vs. 0.246 ± 0.060 respectively, $p=0.12$).

Mark2 Knockout Mice. Tissues from 12- to 14-week-old Mark 2/EMK+/- mice were a kind gift from H. Piwnicka-Worms. The generation of these mice and their characterization has been described previously [11].

ob/ob Mice Leptin-null, male, 11-week-old ob/ob mice and their wild type C57/B6 littermate controls were purchased from Jackson Labs. The ob/ob mice were visibly obese and had significantly elevated fasted blood glucose levels (10.9 ± 0.8 mM compared to 6.6 ± 0.5 mM in controls, $p < 0.003$). Mice were killed at 12 weeks of age following an overnight fast.

Treatments.

For HFD studies, mice were fed normal laboratory chow diet or 45% of total caloric intake derived from fat (HFD; Open Source Diets) for 16 weeks. Body weights of LABKO (34.8 ± 2.5 g) and L/L (35.1 ± 2.3 g) mice were not significantly different from one another ($p=0.91$). However, they were significantly higher than their respective counterparts on a normal chow diet ($p=0.00005$ for LABKO and $p=0.02$ for L/L). Rapamycin (0.6 mg/kg; LC Laboratories) was injected every second day for a total of three injections starting 1 day after the last tamoxifen injection. All procedures involving mice were approved by the Animal Care Committee of the University of Ottawa.

Immunostaining and Image Analysis.

Pancreata from LABKO and their L/L controls were isolated after perfusion with 4% paraformaldehyde in PBS, weighed, and stored in fixative overnight at RT. Pancreata from ob/ob and their C57/B6 controls were immersion-fixed in 10% neutral-buffered formalin for 1 week at RT. Tissues were embedded in paraffin and 4- μ m sections prepared. For the detection of INRs and beta cells, sections were stained with a mixture of anti-C3T (mouse monoclonal SDL.3D10, 1:100, Sigma, St Louis, MO, USA) and an antibody to insulin (rabbit polyclonal H-86, 1:600; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Secondary antibodies for immunofluorescence detection were as follows: AlexaFluor 488 goat anti-mouse IgG (H+L) and AlexaFluor 594 donkey anti-rabbit IgG (H+L) (Molecular Probes). Sections were mounted using Vectashield mounting medium with DAPI as a nuclear counterstain and examined using a Zeiss Axioplan II fluorescence microscope (Carl Zeiss Microscopy, Germany) with attached AxioCam digital camera. For each mouse, images of pancreatic islets (10–50 per mouse) were acquired using three-color multidimensional acquisition. Blinded, manual counts of beta cell nuclei and INR containing beta cell nuclei were performed. Data were analyzed by ANOVA using SigmaStat (Systat Software, Inc.) statistical software and presented as mean INR frequency $\pm$ SEM.

Results.

Appearance of INRs in Mouse Islets is Similar to Human Islets.

INRs were present in beta cell nuclei of all mice examined (Fig. 1). Consistent with our observations in human islets [2], INRs were predominantly rod-shaped (Fig. 1b, arrow). In some cells, they appeared as single, intensely stained dots (Fig. 1b, arrowhead). In these instances, gradual transition through the z-plane of the optical section resulted in a continuous, uninterrupted shift in the position of the dot in the XY plane, as would be expected for rods oriented at an oblique angle with respect to the plane of the optical section. Also consistent with our human studies, INRs were rarely observed within the nuclei of insulin-negative islet cells and never in acinar cells.

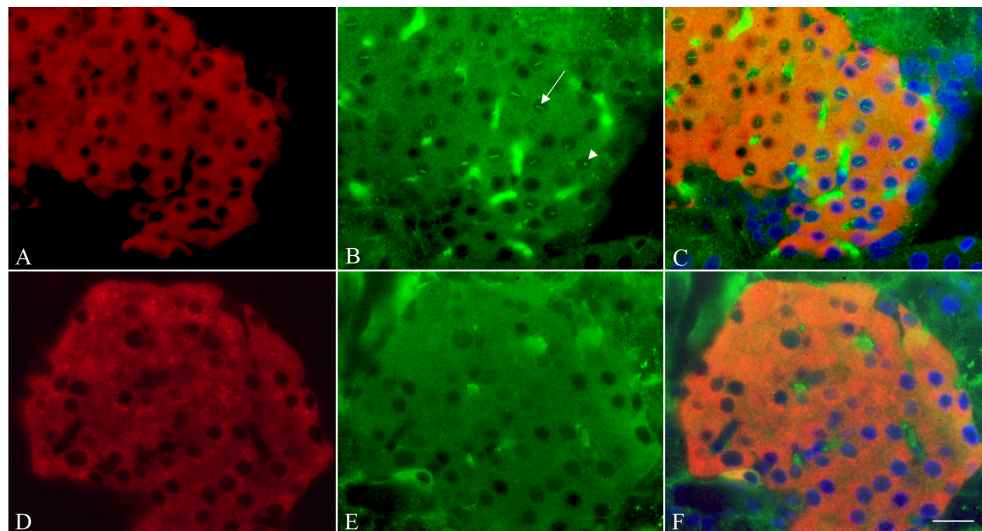


Figure. 1. Islets of Langerhans in L/L (a–c) and LABKO (d–f) mice double-immunostained for insulin (red) (a, d) and C3T (green) (b, e). Merged images shown with DAPI nuclear counterstain (blue) in c, f. In L/L mice, multiple INRs are visible in beta cells as rod-shaped intranuclear structures (arrow in b) or as dot-shaped profiles viewed “end-on” (arrowhead in b). No INRs are visible in the LABKO islet. Bar in F=20 μ M.

Beta Cell INR Frequency is Reduced in LABKO Mice.

In order to examine the potential effects of *Lkb1* on INR formation, beta cell INR frequency was compared in the islets of LABKO and control (L/L) mice. As shown in Fig. 2, beta cell INR frequency was reduced by more than twofold in LABKO mice compared to L/L controls under both normal diet ($14.3 \pm 4.6\%$ vs. $35.8 \pm 1.6\%$, respectively, $p < 0.005$, $n=3$) and HFD conditions ($2.8 \pm 0.2\%$ vs. $21.3 \pm 1.7\%$, respectively, $p < 0.01$, $n=3$).

*Beta Cell INR Depletion in LABKO Mice Is Not Mediated By the *Lkb1* Effectors mTOR or Mark2.*

Loss of inhibition of mTOR and loss of activation of Mark2 have been previously reported as key downstream effectors of the *Lkb1*-null beta cell phenotype [4, 7]. Injections of the mTor inhibitor rapamycin were previously shown to reverse the beta cell hypertrophy phenotype of LABKO mice [4]. Treatment with rapamycin resulted in a further reduction in beta cell INRs ($15.4 \pm 8.4\%$ vehicle vs. $5.4 \pm 2.8\%$ rapamycin, $p < 0.05$, $n=4$; Fig. 3a); thus, the decrease in INRs in LABKO mice does not involve cell size or increased mTor activity.

To determine whether the loss of activation of Mark2 was responsible for INR depletion in LABKO mice, INRs were examined in Mark2 knockout mice and their respective controls. As shown in Fig. 3b, Mark 2 knockout mice exhibited no alterations in the proportion of INR-containing beta cells ($11.8 \pm 3.3\%$ Mark2+/+, $8.7 \pm 2.8\%$ Mark2+/-, $11.3 \pm 3.9\%$ Mark2-/-).

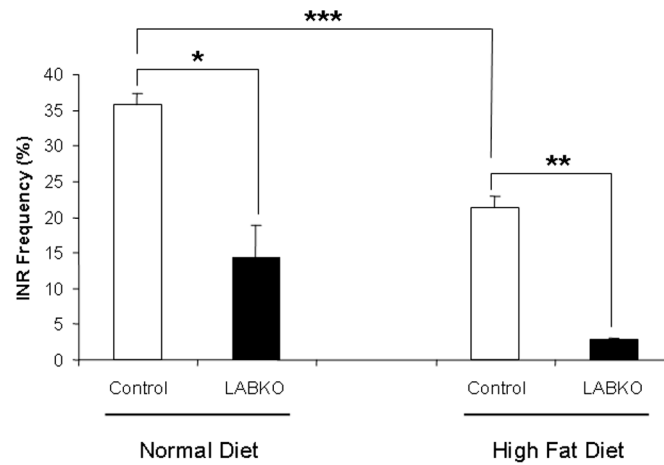


Figure 2. The proportion of beta cells containing INRs (INR frequency) is significantly reduced in LABKO vs. L/L (control) mice under both ND (* $p < 0.05$) and HFD (** $p < 0.01$) conditions. In L/L (control) mice, those maintained on a HFD show significantly reduced INR frequency vs. those on a ND (*** $p < 0.005$). Data shown are mean $\pm$ SEM; $n=3$ in each group.

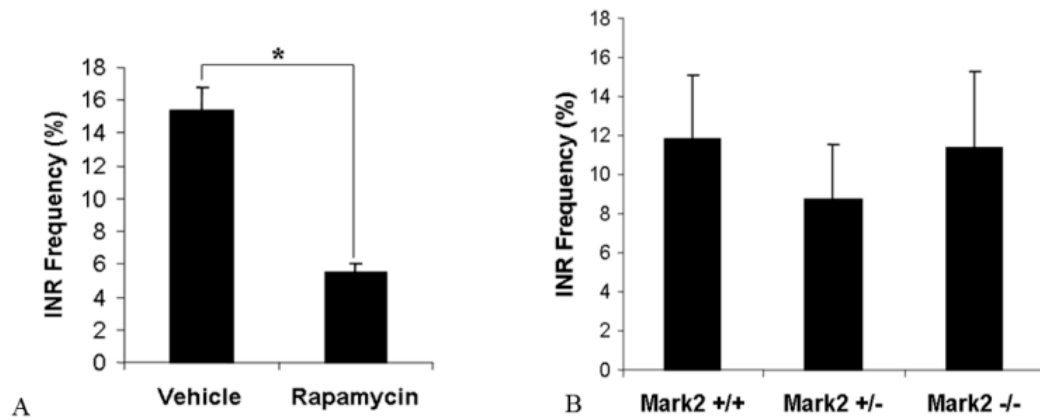


Figure 3. A The proportion of beta cells containing INRs (INR frequency) is reduced in LABKO (* $p < 0.05$) mice treated with rapamycin. Data shown are mean $\pm$ SEM; $n=3$ in each group. **B** The proportion of beta cells containing INRs (INR frequency) is not significantly different among Mark2 control (Mark2+/+), heterozygous (Mark2+/-), and homozygous (Mark2-/-) Mark2 knockout mice. Data shown are mean $\pm$ SEM; $n=4$ in each group.

Beta Cell INRs are Depleted in Normal Mice Maintained on a HFD.

L/L control mice were maintained on either a normal diet or a HFD for a period of 16 weeks. As expected, HFD mice developed features reminiscent of human metabolic syndrome, as has been described in detail previously [4]. They displayed a >40% reduction in INR content compared to normal diet group ($21.3 \pm 1.7\%$ vs. $35.8 \pm 1.6\%$, $p < 0.005$, $n = 3$; Fig. 2).

Beta Cell INRs are Dramatically Reduced in ob/ob Mice.

The reduction of beta cell INR frequency associated with HFD-induced metabolic syndrome prompted us to investigate beta cell INR prevalence in another mouse model of diabetes, the leptin-deficient ob/ob mouse. As shown in Fig. 4a, the islets of 12-week-old hyperglycemic, obese ob/ob mice exhibited a dramatic depletion of beta cell INRs relative to controls (Fig. 4a). Quantitative analysis (Fig. 4b) revealed a 19-fold reduction ($32.6 \pm 2.5\%$ vs. $1.7 \pm 0.4\%$, respectively, $p < 0.001$, $n = 4$).

Discussion

INRs are a prominent yet poorly understood morphological feature of pancreatic beta cell nuclei. This study extends our previous demonstration of INRs in human pancreatic islet cells [2], confirming their presence in mouse beta cells.

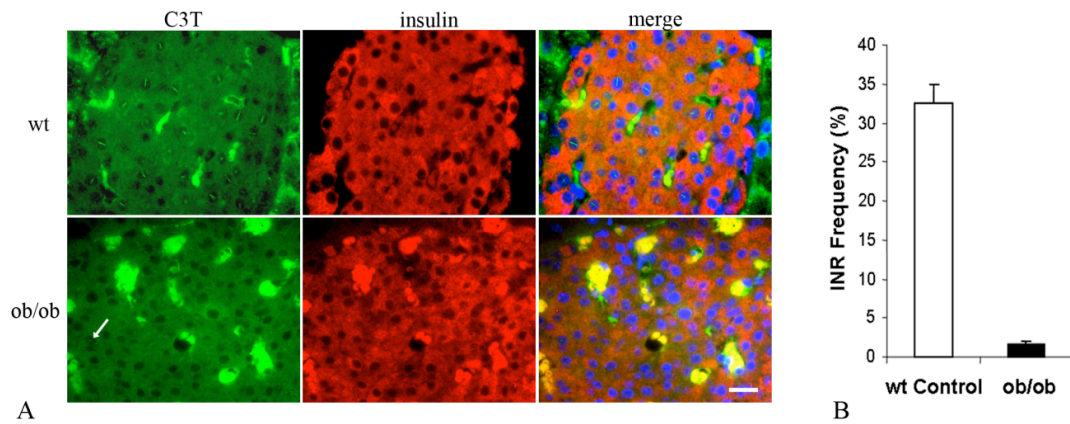


Figure 4. A Islets of Langerhans in wild-type (wt, top row) and ob/ob (bottom row) mice double-immunostained for insulin (red) and C3T (green). Merged images shown with DAPI nuclear counterstain (blue). In wild-type mice, multiple INRs are visible in beta cells as rodshaped intranuclear structures. Only a single INR (arrow) is seen in the ob/ob islet. Bar, 30 μ M. **B** Quantitative analysis reveals a marked reduction in INR frequency in ob/ob mice compared to wild-type controls ($p < 0.001$). Data shown are mean $\pm$ SEM; $n = 4$ in each group.

Moreover, we demonstrate that the presence of INRs in beta cells is regulated, and we provide evidence that beta cell INRs may be of pathophysiological relevance to diabetes. These new observations will provide a framework for a detailed analysis of INR dynamics using additional mouse models of diabetes with varying beta cell function imposed by genetic or pharmacological manipulation.

In search of molecular regulators of INR formation, we demonstrated a significant loss of INRs in LABKO mice relative to controls under both normal diet and HFD conditions. This suggested that INR formation in beta cells might be regulated by Lkb1 or by one or more of its downstream effectors in the AMPK family [6]. However, INR depletion in LABKO beta cells was not mediated by the two key Lkb1 effectors, mTOR or Mark2. It is noteworthy that the Lkb1-induced suppression of microtubule polymerization is mediated through the activation of Mark2. Thus, although INRs are immunoreactive for the beta-tubulin subtype C3T, their depletion in LABKO mice does not appear to be related to the Lkb1-mediated regulation of microtubule polymerization [12]. Furthermore, although INR depletion is characteristic of LABKO, ob/ob, and HFD mice, the latter two models actually have elevated levels of beta cell Lkb1 [4]. This suggests that the immediate effector of INR depletion does not lie directly within the Lkb1 signaling pathway but instead that INR changes in LABKO mice are an indirect consequence of the functional alterations induced in beta cells by Lkb1 depletion. Thus, it is possible that INR depletion in LABKO mice results from some feature common to all three models examined here. Potential candidates currently being tested include enhanced beta cell insulin production or secretion, islet neogenesis, or islet hyperplasia [4, 7].

We have previously reported a dramatic loss of INRs in human postmortem brain samples derived from Alzheimer's disease patients relative to age-matched controls [13]. Here, we extend the potential pathological relevance of INRs to diabetes by demonstrating a significant reduction in beta cell INRs in the HFD-induced disease paradigm and even more dramatically in the leptin-deficient ob/ob mouse model of diabetes. Both the HFD and the ob/ob mice are models of obesity-related insulin resistance and hyperglycemia. It is possible that underlying metabolic changes of pathophysiological relevance are the modulators of INR frequency in ob/ob and HFD mice. In this context, it will be interesting to determine whether INR depletion can be reversed in these models by exogenous leptin administration or reversion to a normal diet, respectively. In addition, it will be important to examine the effects of clinically relevant therapeutics on beta cell INRs in these models.

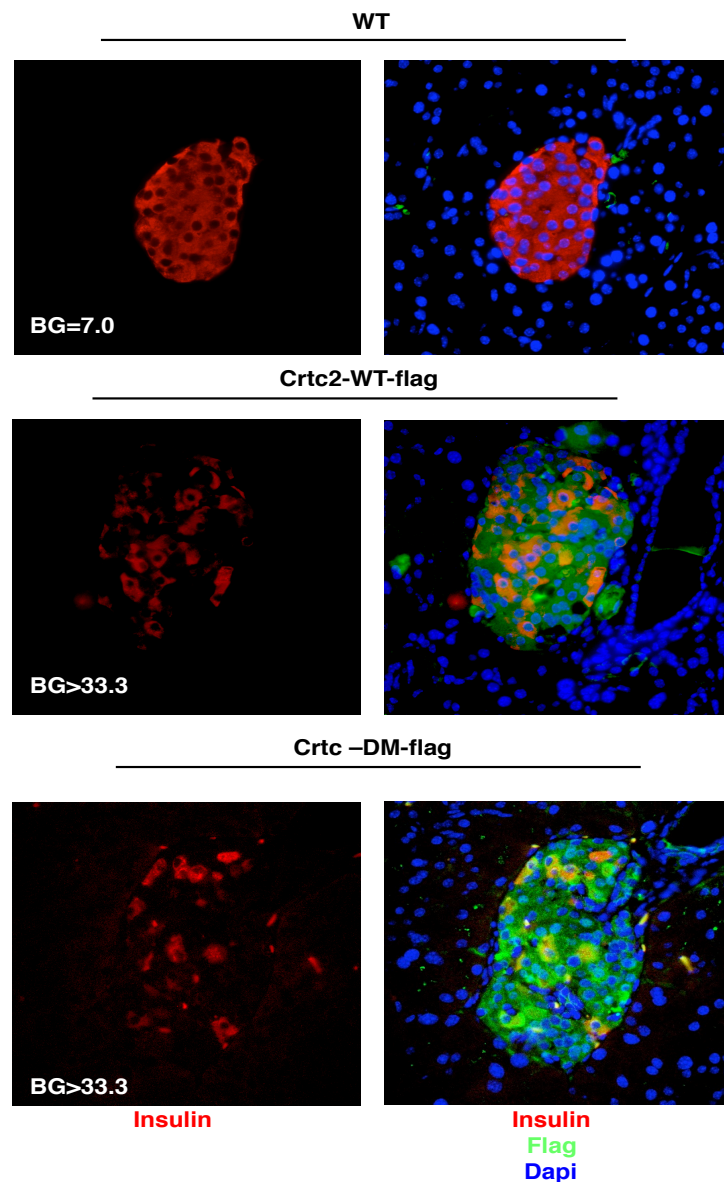
The results of the present study represent the first report of the regulation of INR frequency in pancreatic beta cells. In light of considerable evidence for beta cell dysfunction in the pathogenesis of T2D [14], our results introduce INRs as a new marker of beta cell function with the potential to further our understanding of beta cell biology in both normal and diabetic conditions.

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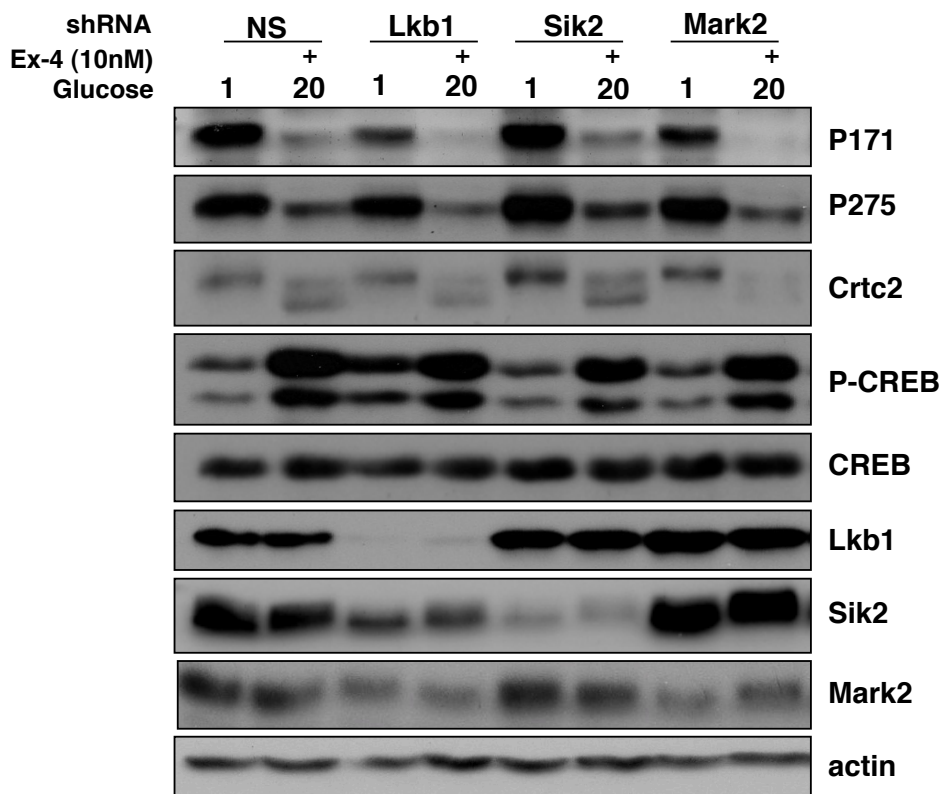
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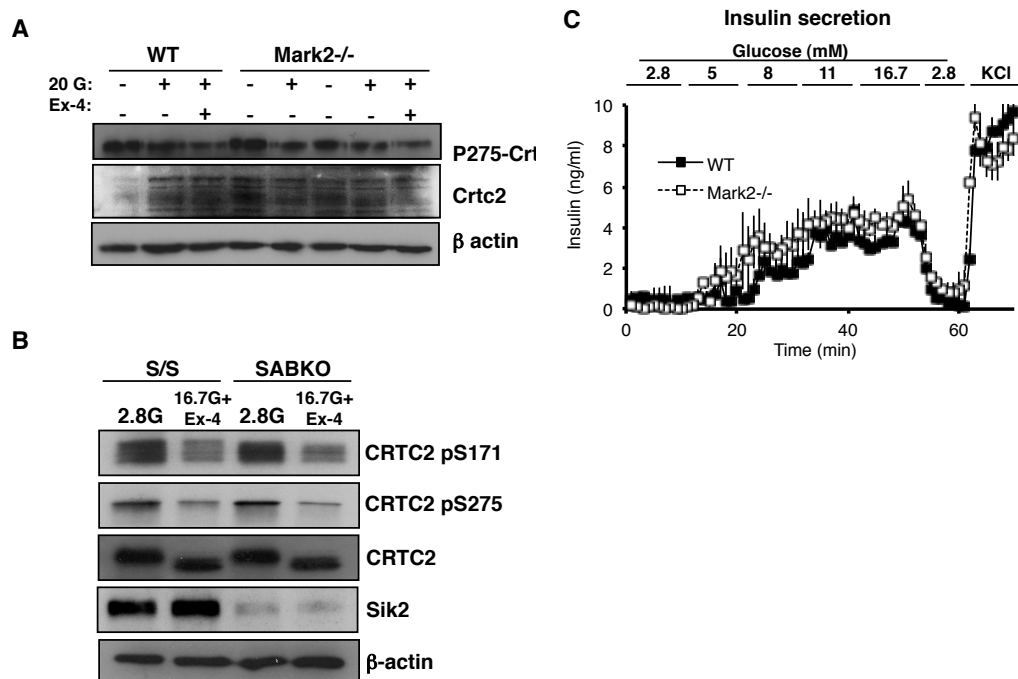
Appendix 5. Reduced insulin staining and elevated blood glucose in 2 mice with Crtc2 overexpression in beta cells. Images of immunofluorescence staining using antibodies against insulin (red) and flag (green) from WT, WT-Crtc2 or double mutant (S171A and S275A) DM-Crtc2 driven by the Pdx1 promoter pancreas sections. Mice were endpointed at 7 weeks of age due the development of severe hyperglycemia, a control WT littermate was taken for comparison. BG = blood glucose at a random fed point just prior to sacrifice.



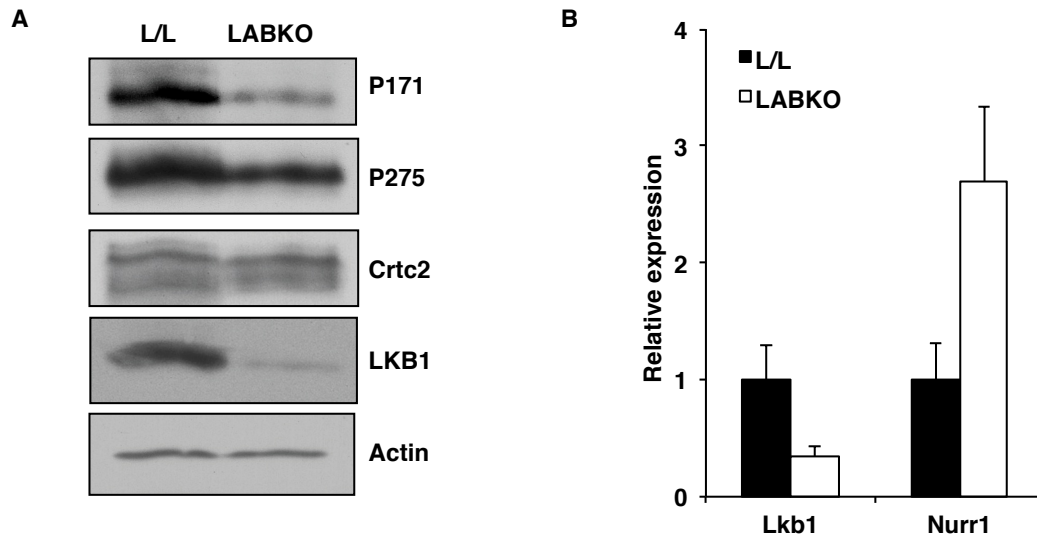
Appendix 6. Increased Crtc2-Creb activity in Lkb1 knockdown MIN6 cells is not due to loss of Sik2 or Mark2. Western blot analysis of protein extract from non-silencing (NS) control, Lkb1, Sik2 and Mark2 shRNA lentiviral-infected MIN6 cells. All cells were starved in 1 mM glucose and then either starved for an additional 1hr or treated for 1 hr with 10 nM exendin-4 and 20 mM glucose for 1 hr. Antibodies against Lkb1 (Santa Cruz; 1:1000), P-171-Crtc2 (1:1000), P-275-Crtc2 (1:2000), total Crtc2 (1:1000), P-Creb (1:1000), total Creb (1:1000), Sik2 (1:1000), Mark2 (1:5000) and actin (Sigma, 1:10,000) were used. Experiment performed by C. Eberhard.



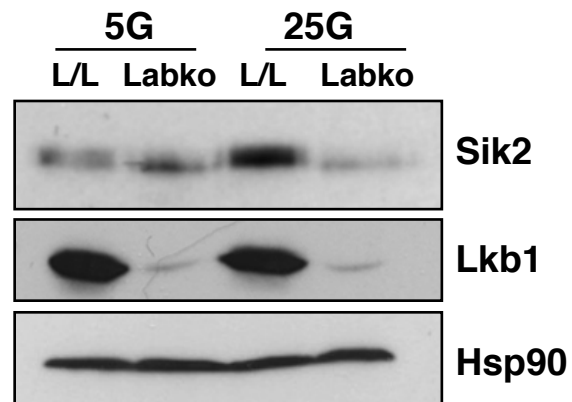
Appendix 7. Mark2 and Sik2 are not required for Crtc2 phosphorylation in islets and Mark2 knockout does not affect insulin secretion in isolated islets. A. Western blotting analysis on extracts of 50 islets per lane from wild-type (WT) or Mark2 ^{-/-} mice. Islets were starved in 2.8 mM glucose for 1 hr and then harvested or treated with 20 mM glucose and 10 nM exendin-4 as indicated, antibodies against phosphorylated serine275- Crtc2 (1:1000), total Crtc2 (1:1000) and actin (Sigma, 1:10,000) as a loading control. B. Western blot analysis on islets from S/S control or SABKO mice, starved in 2.8 mM glucose for 1 hr and harvested or treated for an additional hr in 16.7 mM glucose and 10 nM exendin-4. Antibodies were used against Sik2 (1:1000), phosphorylated serine171-Crtc2 (1:1000), phosphorylated serine275-Crtc2, total Crtc2, total Crtc2 and actin. C. Measurement of insulin secretion in 50 perfused islets per sample in triplicate from WT and Mark2 knockout mice, fractions were collected at 1ml/min and insulin was read using an htrf immunoassay (Cisbio).



Appendix 8. Lkb1 deletion enhances Crtc2-Creb activation in islets. A. Western blot analysis on islet protein extracts from L/L and LABKO mice. Antibodies against P-171-Crtc2, P-275-Crtc2, total Crtc2, Lkb1 and actin were used as described above. B. QPCR analysis of RNA extracted from L/L and LABKO islets using primers against Lkb1 and Nurr1 genes, for each gene expression is graphed relative to control L/L levels and all expression values were normalized to 36B4 as a total RNA input control (p=0.008).

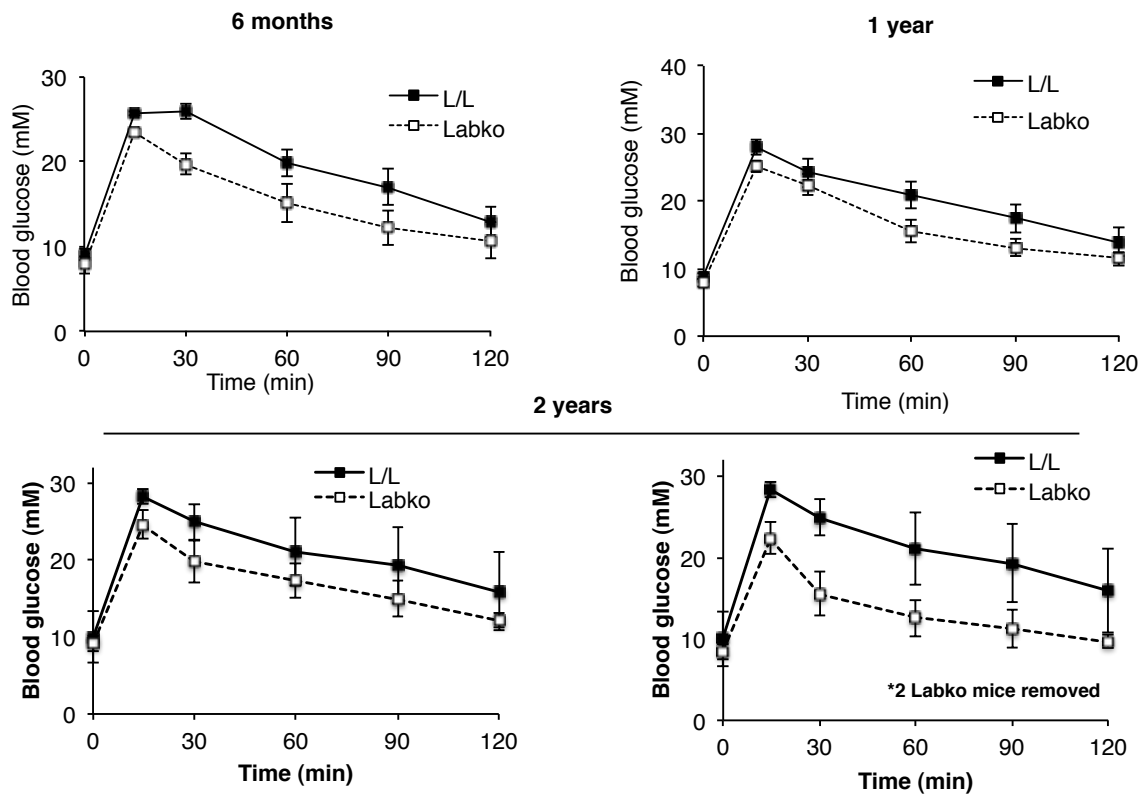


Appendix 9. Sik2 stabilization requires Lkb1 and glucose. Western blot analysis of protein extracts from L/L and LABKO islets (50 islets/lane) starved in 5 mM glucose for 15 hours and then harvested or treated with 25 mM glucose for another 15 hours. Antibodies against Sik2, Lkb1 and Hsp90 (Santa Cruz, 1:1000) were used as described above.



Appendix 10. LABKO mice maintain improved glucose clearance up to 2 years

following Lkb1 deletion. Glucose tolerance tests (GTTs) were performed at 6 months (top left), 1 year (top right) and 2 years (bottom panels) following tamoxifen-induced Lkb1 deletion. For all GTTs, mice were fasted overnight and then 2 mg/kg glucose was intraperitoneally injected and blood glucose levels were recorded over 2 hours. At 2 year time point mice were harvested Lkb1 levels were assessed by immunofluorescence on pancreas sections. Of the LABKO mice, 2 had noticeable Lkb1 expression in islets and were removed from the GTT on the right bottom panel.



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Seminar award for 1st place, University of Ottawa, 2010 (\$200)
Faculty of Medicine travel grants, University of Ottawa 2010, 2011 (\$500 x 2)
NIH-NIDDK Keystone Symposia travel scholarship, 2010 (\$1000)
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Seminar Abstracts:

A Fu, C Reeks, A Hardy, X-Q Dai, PE MacDonald, MB Wheeler, and RA Sreaton. MB Wheeler, and RA Sreaton. LKB1 depletion enhances insulin secretion despite impairing glucose metabolism. Boston-Ithica islet club meeting. Woodshole, MA. April 2013.

A Fu, C Reeks, A Hardy, X-Q Dai, PE MacDonald, MB Wheeler, and RA Sreaton. MB Wheeler, and RA Sreaton. Role of LKB1 in regulating insulin secretion and beta cell proliferation. Boston-Ithaca islet club meeting. Burlington, Vermont. April 2012.

A Fu, C Reeks, Y He, N Bardeesy, R Touyz, MB Wheeler, and RA Sreaton. Role of LKB1 in regulating insulin. University of Ottawa, Biochemistry symposia, Feb 2012.

A Fu, AC-H Ng, C Depatie, N Wijesekara, Y He, G-S Wang, N Bardeesy, FW Scott, RM Touyz, MB Wheeler, and RA Sreaton. Loss of LKB1 in adult b cells increases b cell mass and enhances glucose tolerance in mice. Children's Hospital of Eastern Ontario (CHEO) Research symposia, Ottawa, Oct 2011.

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Seminar series and journal club participation:

Cell Signalling seminars – University of Ottawa, presented 1 seminar (2008-2010)
Ottawa beta cell group – Ottawa Health Research Institute, 2 years participation, presented 3 seminars (2009-2011)
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CHEO Research institute work in progress presentations, presented 2 times per year (2007-2013).

Poster Abstracts:

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A Fu, C Reeks, A Hardy, X-Q Dai, PE MacDonald, MB Wheeler, and RA Screaton. LKB1 couples glucose metabolism and cellular excitability to insulin secretion. FASEB AMPK research conference. Oct 2012. Pacific Grove, California, USA.

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