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The Role of the CRTC Family of CREB Coactivators in Glucose Homeostasis

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

Meghan Fennell Hogan

Committee in charge:

Professor Marc R. Montminy, Chair
Professor Randolph Y. Hampton
Professor Jerrold M. Olefsky
Professor Lorraine Pillus
Professor Maike Sander

2013

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Chair

University of California, San Diego

2013

Dedication

This thesis is dedicated to the staff and campers at the Clara Barton Camp for girls with diabetes.

I never imagined as a child that I would study diabetes, and this work is a thanks to all that I learned about myself and my disease as a camper, and a testament to the changing face of children with diabetes.

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It is difficult to express the gratitude that I have for my family, especially my parents who have always encouraged me to wonder, research, and keep learning and going. While they have been thousands of miles away in Rhode Island, my St. James-by-the-Sea family has kept me buoyed with mandatory choir rehearsals on Thursday nights, hugs, and lots of food.

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Chapter 2 has excerpts from Ravnskjaer K, Hogan MF, Lackey D, Tora L, Dent SYR, Olefsky J, Montminy M. KAT2B and WDR5 Mediate Epigenetic Effects of Glucagon on Gluconeogenesis. J Clin Invest. (2013); doi: 10.1172/JCI69035. Only experiments that I conducted or contributed to are included. Permission to use this work as part of my dissertation has been obtained from all co-authors.

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ABSTRACT OF THE DISSERTATION

The Role of the CRTC Family of CREB Coactivators in Glucose Homeostasis

by

Meghan Fennell Hogan

Doctor of Philosophy in Biological Sciences

University of California, San Diego, 2013

Professor Marc R. Montminy, Chair

Obesity and its associated health issues are reaching epidemic levels worldwide. There are many reasons for this increase in metabolic diseases including environmental and lifestyle changes. However, there are also many biological mechanisms that shift the energy homeostasis of a system to a

dysregulated state, often resulting in obesity or diabetes. One of these mechanisms that contribute to the rise of blood glucose levels and eventual failing of insulin producing beta-cells in the pancreas is hepatic gluconeogenesis. The hormones that regulate this process include insulin and glucagon. Glucagon acts through the secondary messenger cAMP, stimulating the cAMP Response Element Binding Protein (CREB) and its associated co-activators, the CRTCs to enhance gluconeogenic enzyme transcription. It has been clear that CRTC2 plays a role in this process, but this dissertation provides evidence for overlapping roles of CRTC2 and CRTC3, explaining past observations regarding modest phenotypes of CRTC2 null mice. Additionally, we show here that chronic expression of a liver expressed, constitutively active CRTC2 through an adeno-associated virus that upregulated hepatic gluconeogenesis is able to lead to an insulin resistant phenotype that is eventually compensated for by the upregulation of *IRS2*, a CREB target gene.

Chapter 1 Introduction

1.1 Energy Metabolism

Metabolism involves all the processes that break down (catabolism) or build up (anabolism) various metabolites in a system. These metabolites are used for storage, energy, and making the building blocks of life, such as nucleic acids, amino acids, proteins, enzymes, and tissues, that compose the body itself. When scientists discuss a metabolic disorder, this most often refers to a problem with the metabolism of energy balance: the energy spent must equal the energy brought into the system, often counted in caloric input and output.

The body is remarkably good at maintaining energy balance of a system: approximately 70% of calories are expended on maintenance of the body and its organs, keeping them working at their peak efficiency, called the resting metabolic rate. Around 20% of calories go towards supplying energy for physical activity and the remaining 10% go to maintaining body heat, a process called thermogenesis. The body is surprisingly accurate (about 99%) at keeping the body weight constant, and it is the 1-2% of intake that ends up causing shifts in metabolism over time, resulting in obesity and changes in energy homeostasis.

1.2 Regulation of Energy Metabolism

There are many ways of regulating metabolism, one of the most basic being that of nutrient regulation. The body is able to recognize periods of scarcity compared to periods of plenty and in turn regulates the control of calories burned and the energy sources used to maintain basal metabolic rates. It is exceedingly uncommon for humans to consume the same amount of nutrients each day and every day brings about different levels of activity that need to be supported by energy burning. Despite this uneven consumption and spending, body weight remains remarkable stable, most likely due to the existence of systems that maintain energy homeostasis throughout the body (Yamada and Katagiri, 2007). This maintenance necessitates that there is communication between various tissues to "collect" information on the state of nutrients and responding appropriately to changes in fuel availability. The brain/central nervous system (CNS) is the master regulator of this process, gathering information from peripheral tissues and then sending signals to regulate the metabolic mechanisms in those tissues (Marshall, 2006; Yamada et al., 2008).

In order to adapt to cycles of nutrient availability throughout a standard 24-hour period, energy metabolism in animals has itself evolved to be cyclical (Hatori et al., 2012). These cell-autonomous circadian rhythms are based on interlocking feedback circuits in which multiple proteins, including BMAL1,

CLOCK, NPAS2, and ROR act as transcriptional activators, while PER, CRY, and REV-ERB act as inhibitors. These proteins promote transcription of various target genes, all in cyclical rhythms, contributing to the maintenance of energy homeostasis (Reddy and O'Neill, 2010).

Several of the most well known regulatory pathways for maintaining energy balance are hormonal pathways: primarily insulin and glucagon. These hormonal pathways rely on intra-tissue communication, as the hormone is produced in one tissue (such as the pancreas) it then binds to particular receptors on various other tissues modulate transcription of a variety of other proteins (Yamada and Katagiri, 2007). For example, insulin is produced in the β-cells in the islets of the pancreas and is secreted when glucose levels are increased. The insulin signaling pathways themselves synchronize growth and development, fertility, and lifespan through three interwoven levels: proximal signaling, intermediate signaling, and distal signaling (Biddinger and Kahn, 2006).

Through intermediate signaling, insulin regulates the PI 3-Kinase (that subsequently activates the kinase AKT); the Mitogen Activated Protein Kinase pathway (which phosphorylates substrates in the cytoplasm to mediate the proliferative effects of insulin); and the transcription factor sterol regulatory element binding protein (SREBP)-1c which is capable of activating fatty acid biosynthesis, regulation of gluconeogenesis, and adipocyte differentiation

(Biddinger and Kahn, 2006). Looking at a molecular level, insulin regulates many other pathways including protein synthesis in muscle and liver; lipid synthesis in liver and adipose tissue; glycolysis and glucose storage in the muscle and liver; and most importantly to the work described in this dissertation, the inhibition of gluconeogenesis in the liver (Cheng et al., 2010).

Standing opposite to insulin is the hormone glucagon, which is secreted by α-cells in the pancreatic islet in response to a decrease in blood glucose concentrations, especially during fasting. Glucagon mainly affects the liver and adipose tissue (Fritsche et al., 2008). In the liver, glucagon will bind to its G-protein coupled receptor, resulting in the activation of adenylyl cyclase that will catalyze the production of cAMP. cAMP in turn will bind the regulatory subunits of protein kinase A (PKA), resulting in activation of the kinase comparable to that of AKT/PKB for insulin signaling (Altarejos and Montminy, 2011; Fritsche et al., 2008).

The hormonal inputs in mediating metabolic energy balance are often important in intra-tissue/organ pathways. These hormonal communication pathways lead to intermediate pathways that are able to directly control energy metabolism, known as the signaling pathways. Signaling pathways include the aforementioned PI3-Kinase pathway, the sirtuin (SIRT-1) pathway, the mammalian target of rapamycin (mTOR), and the AMPK pathway. These signaling pathways all act as modifiers by phosphorylation or de-acetylation of

downstream targets to ensure that feeding or fasting signals are integrated and transmitted to the cells themselves.

mTOR integrates inputs from upstream pathways, including that of insulin, and amino acid availability. mTOR is also capable of sensing cellular nutrient, oxygen, and energy levels (Tokunaga et al., 2004). AKT (activated by the PI3 Kinase pathway) in turn phosphorylates and inhibits TSC2, which inhibits mTOR. When no longer inhibited, mTOR along with the protein raptor (making up mTORC1), regulates protein synthesis by phosphorylating and activating S6Kinase, which can increase ribosomal biogenesis (Biddinger and Kahn, 2006).

It has also been suggested that mTORC1 plays an important role in lipid synthesis through promotion of the cleavage which activates SREBP1c (Cheng et al., 2010; Peterson et al., 2011). Additionally, mTORC2 participates in the storage of glycogen by activating AKT, which inhibits GSK3β, enhancing the activation of glycogen synthase. It is also thought that because of the upstream role of AKT, mTORC2 may directly participate in the suppression of gluconeogenesis (Zoncu et al., 2010).

The AMPK pathway senses cellular energy levels through allosteric activation of AMPK by rising levels of AMP from reduced ATP levels. This allosteric activation triggers a signaling cascade, through the phosphorylation of many downstream targets, including two from the mTOR pathway (Marshall,

2006). AMPK mediates effects of known adipokines in regulating food intake, body weight, and glucose and lipid metabolism. AMPK itself is mainly regulated by the master regulator, LKB1, and in the liver is very active in controlling gluconeogenesis (Cantó and Auwerx, 2010; Kahn et al., 2005; Shackelford and Shaw, 2009). AMPK uses phosphorylation to mediate the effects of transcriptional regulators of gluconeogenesis, inhibiting the upregulation of transcription of important gluconeogenic enzymes (Cantó and Auwerx, 2010).

In the end, all of these pathways act through the activation or repression of transcription factors, which bind to certain DNA sequences to enhance the transcription of a particular gene. Examples of those that act in energy balance include FoxO1, PGC1α, HNF4 α, ChREBP, SREBP1, and most important for this dissertation, CREB and the CRTC family (Cantó and Auwerx, 2010). These transcription factors are either activated or repressed based on which signaling pathway is involved, uniting the information about nutritional status of the environment with the cells that will be able to respond appropriately to maintain energy balance.

1.3 Obesity and Dysregulation of Energy Balance

The increase in the rates of obesity has been triggered by environmental and lifestyle changes driven by the overconsumption of energy-dense foods and sedentary lifestyle. These changes create a chronic

imbalance of energy that lead to dysregulation in metabolic homeostasis (Muoio and Newgard, 2006). Many organisms have evolved to survive the more dangerous periods of starvation or scarcity, and now that humans live in times where there is little caloric restriction, these evolutionary mechanisms are “too much of a good thing.”

This idea that humans evolved to store excess energy as fat in times of abundance to deal with periodic episodes of famine is known as the “thrifty gene” hypothesis (Neel, 1962; Spiegelman and Flier, 2001). One of the first thrifty genes to be identified was the gene for leptin—a cytokine secreted from the adipocyte and signals to the hypothalamus. Under normal conditions, leptin increases energy expenditure by increasing sympathetic tone, which stimulates lipolysis and fatty acid oxidation (Altarejos and Montminy, 2011). In mice with genetic inactivation of leptin, ob/ob mice, the protein is biologically inactive, and the obesity is corrected with infusion of an active version of leptin itself. Not only does leptin act as a thrifty protein, but the receptor to which it binds also plays an important role in the nutritional feedback loop from adipose tissues to the brain. If the signaling receptor of leptin is deleted, the mice are obese, but no amount of added leptin is able to correct the phenotype (Spiegelman and Flier, 2001).

Not all cases of obesity are due solely to changes in the environment and nutrient consumption. Several studies have found that diverse

mechanisms throughout the body can contribute to the development of obesity. One such example is endoplasmic reticulum (ER) stress, also known as the unfolded protein response (UPR). ER stress occurs when there is an imbalance between the protein folding capacity and the protein load, resulting in a buildup of unfolded proteins. The expected outcome of the UPR is essentially adaptation to new functional demands and a restoration of ER homeostasis. However, under prolonged ER stress, this homeostasis may not be reached. Under conditions of excess nutrients, the hypothalamus, liver, adipose, muscles, and β-cells all experience the UPR in a cell autonomous and context-dependent manner, but there are similarities in outcomes. The UPR can cause insulin and leptin resistance, alter protein secretion, modify membrane receptor expression, can contribute to β-cell apoptosis, and lead to obesity and inflammation (Cnop et al., 2012).

Increased inflammatory responses links the UPR and ER stress response with obesity and insulin signaling. An important initiator of the inflammatory response (including elevated pro-inflammatory cytokines, activation of the JNK/activator protein 1 and IKK/NF-κB) is adipose tissue that contains macrophages and lymphocytes. Under inflammatory responses, the adipocytes can release several cytokines, macrophage migration inhibitory factor, and of course leptin and adiponectin, which can promote insulin sensitivity. In obese conditions, a consequence of chronically inflamed

adipose tissue is a shift in the energy homeostasis' set point, eventually resulting in insulin resistance and difficulties with euglycemic control (Samuel and Shulman, 2012; Schenk et al., 2008).

1.4 Insulin Resistance and Diabetes

The evolution from obesity to type II diabetes is often characterized by the development of insulin resistance, followed by increases in fasting blood glucose levels (Barthel and Schmoll, 2003; Kahn et al., 2006; Saltiel, 2012; Saltiel and Kahn, 2001). There are a variety of biological mechanisms that can lead to insulin resistance, including inflammation, ER stress and the unfolded protein response, and dysregulation of lipids (Flamment et al., 2012; Muoio and Newgard, 2006; Samuel and Shulman, 2012; Schenk et al., 2008; Shoelson et al., 2006). It is often unclear how these processes are linked to each other, but once a tissue has lost its sensitivity to insulin signaling, the progression to a multi-tissue insulin resistant state to type II diabetes may occur through intra-tissue communication.

While we often think of diabetes as being a disease of the pancreas with dysfunctional islets/β-cells, it really involves multiple tissues, including the brain, skeletal muscle, adipose tissue, and liver (Kahn et al., 2006). The lack of insulin or insufficient insulin production itself of course plays a role in the development of diabetes, but there are many other mechanisms throughout

the body that are able to contribute to insulin resistance and the eventual development of type II diabetes.

The brain of course, plays an important role with the CNS that integrates signals about nutritional availability and anti-obesity signals from leptin to modulate food intake (Elmquist et al., 2005). In addition to the knowledge that the brain integrates information about nutrient availability and feeding signals, the brain itself contains insulin receptor signaling proteins, which when knocked out in a neuron-specific manner, generate animals that have increased appetite and therefore obesity, with impaired ability to inhibit hepatic gluconeogenesis (Biddinger and Kahn, 2006). When the globally deleted insulin receptor mouse model had IR restored in the brain, there was only partial rescue from diabetic ketoacidosis. Interestingly, restoration in the liver and pancreas only delayed the development of diabetes, indicating that insulin action in the brain is required to prevent the development of diabetes (LeRoith and Gavrilova, 2006).

The skeletal muscles are an important part of glucose uptake, accounting for 70-90% of disposal of oral glucose intake, and insulin resistance in the muscle is one of the earliest defects in type II diabetes (Biddinger and Kahn, 2006). Muscle insulin receptor knockout mice (MIRKO) lead to impaired insulin signaling and decreased insulin dependent glucose transport, meanwhile without developing systematic insulin resistance

(Brüning et al., 1998). The importance of the muscle in glucose metabolism was shown again in mice expressing dominant negative Igf1r and muscle-specific knockout of Glut4, but point to various compensatory mechanisms by the adipose tissue and also the suppression of gluconeogenesis in the liver prevent a full blown diabetic phenotype (Nandi et al., 2004).

One of the key tissues involved in insulin resistance and progression to diabetes is that of the liver. The liver is essential for tissues that rely only on glucose for energy sources, such as the brain and red blood cells during times of fasting when the liver is the source of de novo glucose production (gluconeogenesis) (Consoli, 1992). Hepatic gluconeogenesis is maintained in the liver under tight control by two major hormones: glucagon and insulin. During times of scarcity or little consumed glucose, glucagon will activate the gluconeogenic engine, but upon exposure to caloric sources, insulin signaling shuts down the program. This encourages glucose uptake into the liver itself, muscles, and adipose tissue. When insulin signaling and control become less effective (often the case in insulin resistance), the gluconeogenic engine is no longer under extremely tight control (Barthel and Schmoll, 2003; Biddinger and Kahn, 2006; Consoli, 1992). It is in fasting blood glucose levels that we observe this slipping control, due to increasing hepatic glucose production, leading to type II diabetes (Consoli, 1992).

1.5 cAMP Response Element Binding Protein (CREB) Family

Once a protein is translated, it must undergo various forms of modification before it is a fully functional protein. These forms of modification may involve the protein structure itself, as in building disulfide bridges, but often take the form of various functional groups being added to specific amino acid residues such as hydroxylation, glycosylation, phosphorylation and adenylylation. Phosphorylation of transcription factors is commonly used as a cellular signal regarding nutrient availability, cell stress, and environment. The first transcription factor shown to be regulated by phosphorylation was the cyclic AMP response element (CRE)-binding protein (CREB), and its related family members activating transcription factor 1 (ATF1) and CRE-modulator (CREM) (Mayr and Montminy, 2001).

The structures of CREB and its family members share significant organization. All contain a biparte amino terminal transactivation domain (TAD), containing a central kinase inducible domain (KID) and one of two glutamine rich regions (Q2). The KID region contains the important Serine133 on which phosphorylation depends, and it is here that KIX domain CBP/p300 (discussed later) binds most strongly. CREB and its family members also contain a carboxyl-terminal basic leucine zipper domain (bZIP) that acts as the dimerization domain for CREB, and also the DNA binding domain to CRE sites (see Figure 1.1) (Altarejos and Montminy, 2011).

CREB is phosphorylated in response to a variety of signals including growth factors, osmotic stress, ultraviolet radiation, and to hormonal stimulation that increases the concentration of the secondary messenger cAMP (Altarejos and Montminy, 2011). Increases in cAMP occur in response to activation of G-protein-coupled receptors linked to stimulatory G proteins (composed of α, β, and γ subunits), which in turn leads to the activation of adenylyl cyclase (AC), which catalyzes cAMP from ATP. cAMP then binds to the regulatory subunits of the hetero-tetrameric protein kinase A (PKA), whereby the catalytic subunits passively diffuse into the nucleus, where they phosphorylate CREB at Serine 133 (Figure 1.2) (Gonzalez and Montminy, 1989). This diffusion requires about 30 minutes to 1 hour and correlates well to the typical burst-attenuation-recovery gene expression with the phosphorylation of CREB followed by its dephosphorylation (Hagiwara et al., 1992; 1993; Michael et al., 2000).

Under basal conditions, CREB binds as a dimer to the conserved palindromic cAMP responsive element DNA sequence TGACGTCA or to a half sequence TGACG/CGTCA, often found in the promoter proximal regions within 250 bp of the transcriptional start site of approximately 5,000 genes (Altarejos and Montminy, 2011). Phosphorylation of CREB at Ser133 promotes the recruitment of the transcriptional coactivator, CREB Binding Protein (CBP) and its paralog p300, with which transcription typically peaks

after about 30 minutes of stimulation (Arias et al., 1994; Chrivia et al., 1993; Mayr and Montminy, 2001). CREB and CBP/p300 interact through well-defined domains known as the KID (Kinase Inducible Domain in CREB) and KIX (domain of three helices in CBP/p300 that bind to KID) through the phosphorylation of Ser133 (Radhakrishnan et al., 1997). Interestingly, the phosphorylation of Ser133 does not appear to be sufficient in increasing target gene expression. In fact, only approximately 100 CREB target genes are upregulated under exposure to cAMP, the subset differs between tissue types, and the recruitment of CBP/p300 was predictive for cAMP induction (Zhang et al., 2005).

1.6 CRTC (CREB-Regulated Transcriptional Co-activator) Family

Using high-throughput screening methods for modulators of CRE-Luciferase activity, our lab and others identified a family of coactivators (CRTCs or TORCs) that maximize CREB activity when bound with its bZIP domain (Conkright et al., 2003; Iourgenko et al., 2003). The CRTC family has very similar structures, all containing an amino-terminal CREB-binding domain (CBD), a central regulatory domain (REG) and a carboxyl-terminal transactivation domain (TAD) (Altarejos and Montminy, 2011). (See Figure 1.1) Not only are the three family members structurally conserved, but the CRTCs appear to be evolutionarily conserved as well—functional homologues

have been identified from mammals to *Caenorhabditis elegans* and in the fruit fly, *Drosophila melanogaster* (Mair et al., 2011; Wang et al., 2008).

Under basal conditions, the CRTCs are phosphorylated by SIKs and sequestered in the cytoplasm by association with 14-3-3 proteins. Triggering of the cAMP pathways promote the PKA dependent inhibition of SIKs, leading to the dephosphorylation of CRTC2. Additionally, the CRTCs are responsive to calcium signaling as well, with calcium triggering the calcineurin-mediated dephosphorylation, much like with the SIKs. The 14-3-3 proteins then release the protein, and the CRTC is translocated to the nucleus (see Figure 1.3). CRTC binds to the bZIP domain of CREB in the presence of the CRE in the DNA. The unbound state of the bZIP domain of CREB is largely unstructured and therefore unable to interfere with the helical CREB binding domain of CRTC2 (Bittinger et al., 2004; Dentin et al., 2008; Koo et al., 2005; Luo et al., 2012; Screaton et al., 2004; Wang et al., 2010; 2009).

While conserved from worms to humans, the mammalian CRTC family is composed of three members, with slightly different functions primarily due to the location of expression. CRTC1 is highly expressed in certain regions of the brain (prefrontal cortex, cerebellum, and hypothalamus) (Conkright et al., 2003). Mice with a knockout of CRTC1 are obese, hyperphagic, and leptin resistant, and thus CRTC1 appears to have a role in appetite suppression (Altarejos et al., 2008). CRTC2 is ubiquitously expressed throughout the body,

but has primarily been identified with roles of gluconeogenesis and hepatic insulin sensitivity (Dentin et al., 2007; Saberi et al., 2009; Wang et al., 2010). Similarly, CRTC3 is expressed throughout the body, but has primarily been identified with increasing insulin resistance in adipose tissue and energy balance through catecholamine signaling (Song et al., 2010).

1.7 Closing Remarks

CREB is one of the best-studied transcription factors, and yet much remains unknown about its regulation and function. With the discovery of its family of co-regulators, much more has become understood about CREB's roles in a variety of model organisms and in different tissues. As an important player in the cAMP-signaling pathway, CREB and the CRTCs are essential in understanding more about the regulation of gluconeogenesis. In this dissertation, I present evidence that CRTC2 and CRTC3 have overlapping roles in mammalian liver in regulating hepatic gluconeogenesis. Additionally, the constitutively active form of hepatic CRTC2 is sufficient to cause insulin resistance followed by compensation of the phenotype by upregulation of IRS2. The results presented here will hopefully further the study of CREB and the CRTC family in further understanding of mechanisms behind insulin resistance, hyperglycemia, and type II diabetes.

1.8 Figures

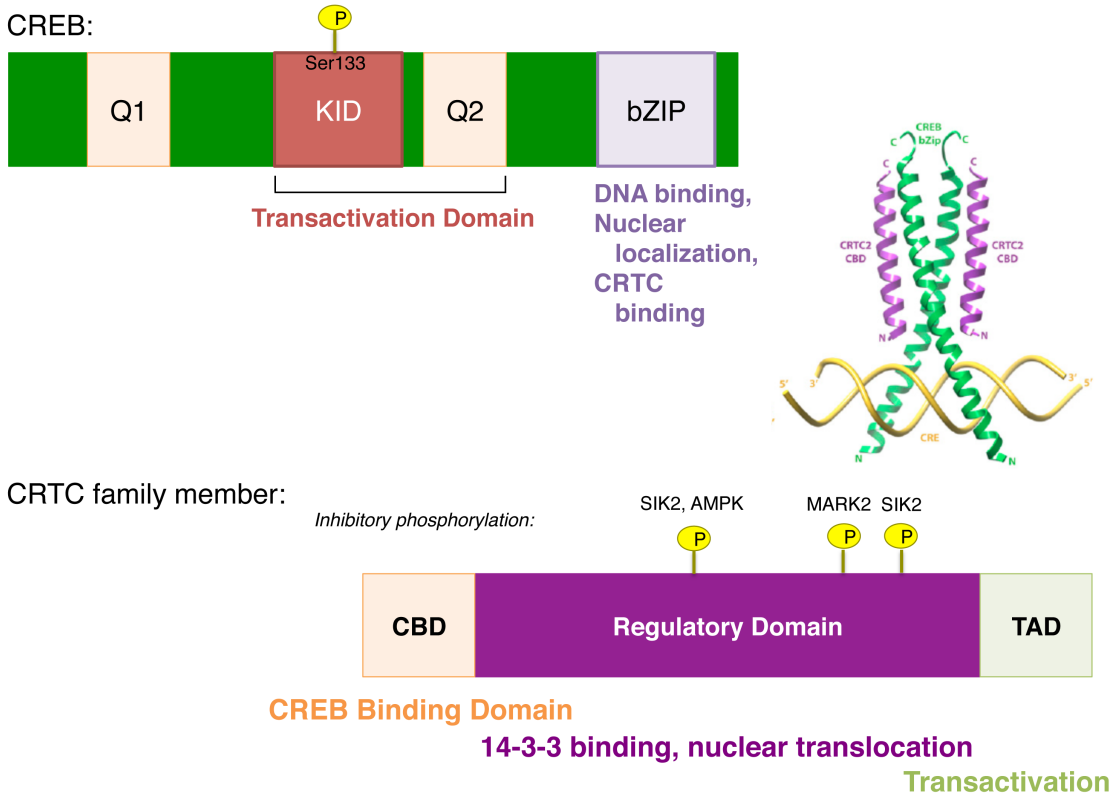

Figure 1.1 Modular organization of CREB and CRTC and structural model of CREB and CRTC2 binding. CREB (top) contains two glutamine rich domains (Q1 and Q2), a kinase inducible domain (KID) that contains Ser133, which, when phosphorylated, promotes an interaction with CBP/p300. The C terminal basic Leucine zipper domain (bZIP) promotes binding to the DNA with CRE site, and to the cAMP-regulated transcriptional coactivators (CRTCs). The CRTC (bottom) family shares significant sequence homology, containing an N-terminal CREB Binding Domain, a central regulatory domwin which contains multiple phosphorylation sites which contribute to binding with the 14-3-3 proteins. These Serines can be phosphorylated by a variety of kinases, noted above the phosphorylation groups. Inspired by (Altarejos and Montminy, 2011). CREB and CRTC2 bind together (middle) to a CRE site in a 2:2:1 stoichiometric ratio through the CRTC2 CBD and CREB bZIP. From (Luo et al., 2012).

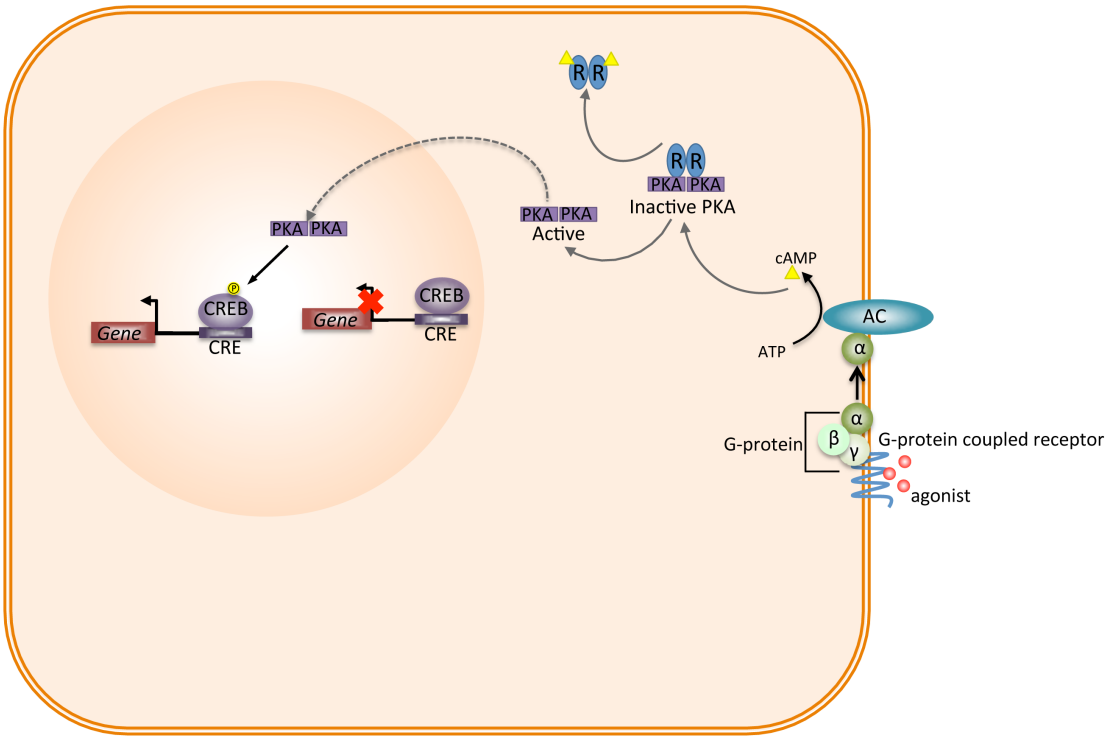

Figure 1.2 CREB stimulation by cAMP. When an agonist (such as glucagon) binds to the G-protein coupled receptors, which are linked to the G-proteins, comprised of an α, β, and γ subunits. The α subunit leads to the activation of adenylyl cyclase (AC), which catalyzes ATP to cAMP. cAMP then binds to the regulatory domains (R) of the inactive PKA, allowing the catalytic subunit to passively diffuse (dotted arrow) into the nucleus, the rate limiting step of the reaction. The active PKA then phosphorylates CREB at Ser133, which is then able to promote gene expression at promoters containing CREs. Based on (Altarejos and Montminy, 2011).

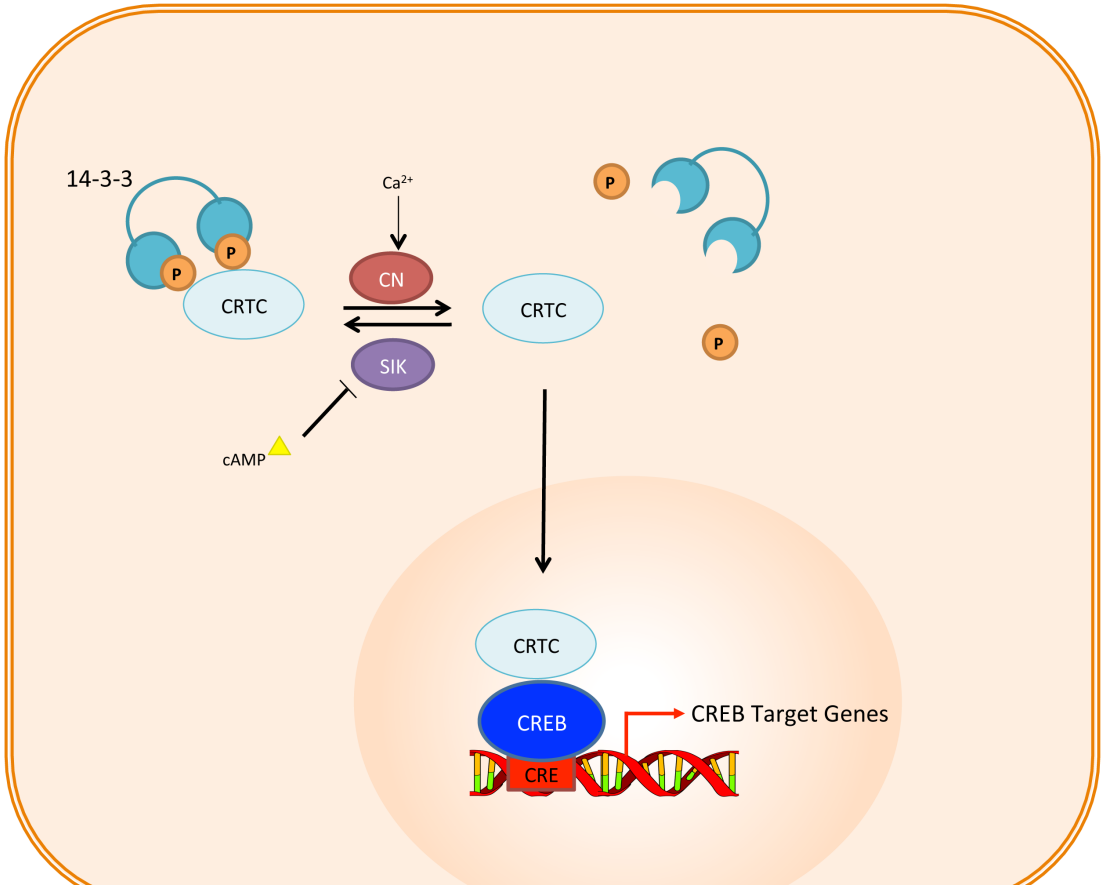

Figure 1.3 CRTC dephosphorylation modulates shuttling into the nucleus. cAMP and calcium regulate the localization of cAMP-regulated transcriptional coactivators. Under basal conditions, CRTCs are sequestered in the cytoplasm through binding with 14-3-3 proteins. cAMP and Ca2+ signals promote dephosphorylation through the inhibition of salt-inducible kinases (SIKs) and induction of the phosphatase calcineurin (CN). Dephosphorylated CRTC translocates to the nucleus, where it binds to CREB and stimulates its activity. Based on (Altarejos and Montminy, 2011).

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Chapter 2 The Role of CRTC3 in Gluconeogenesis

2.1 Abstract

During fasting, increases in glucagon lead to the induction of gluconeogenic genes through the activation of CREB and its coactivator CRTC2. Previous studies have shown the importance of CRTC2 through the use of adenoviral short hairpin RNAi, but targeted disruption of CRTC2 has only modest effects on gluconeogenic compared to acute CRTC2 knockdown. The difference between the chronic and acute loss of CRTC2 may be explained by the presence of an additional family member, CRTC3. We evaluated the potential role of CRTC3, a closely related family member, in compensation for CRTC2. We show here that CRTC3 is expressed in the liver, is active under fasting conditions, and contributes to gluconeogenic gene expression. Our results suggest that while CRTC2 may be the primary coactivator responsible for induction of gluconeogenic genes in response to glucagon, CRTC3 also appears to be important in this setting. Thus CRTC2 and CRTC3 may have overlapping roles in the regulation of glucose production by the liver during fasting.

2.2 Introduction

Despite alternating periods of caloric abundance and scarcity, blood glucose levels remain remarkably constant in normal systems, maintaining energy balance for glucose dependent tissues such as the brain. This important control is upheld by balancing the production of glucose by the liver (gluconeogenesis and glycogenolysis) during fasting conditions with the uptake of glucose into peripheral tissues during fed conditions, both controlled by insulin signaling. Early phases of type 2 diabetes are marked by insulin resistance, and an eventual inability of insulin to inhibit hepatic gluconeogenesis, resulting in elevated fasting blood glucose (Barthel and Schmoll, 2003; Biddinger and Kahn, 2006; Saltiel and Kahn, 2001; Samuel and Shulman, 2012).

Under fasting conditions, increases in glucagon bring about the transcription of hepatic gluconeogenic genes such as *PCK1* and *G6Pase*, in part due to increases in the secondary messenger cAMP (Herzig et al., 2001). Increases in cAMP result in the activation of protein kinase A (PKA), whereby the catalytic subunits of PKA diffuse into the nucleus, phosphorylate cAMP Response Element Binding protein (CREB), and promote recruitment of CBP/p300 that in turn recruits RNA polymerase II complexes (Chrivia et al., 1993; Hagiwara et al., 1993).

A second cAMP regulated pathway also appears to modulate gene expression. Under basal conditions, the CREB family of coactivators (CRTCs) is phosphorylated by SIKs and sequestered in the cytoplasm by association with 14-3-3 proteins. Triggering of the cAMP pathways promote the PKA dependent inhibition of SIKs, leading to the dephosphorylation of CRTC2. The 14-3-3 proteins then release the protein, and CRTC2 is translocated to the nucleus. CRTC2 binds to the bZIP domain of CREB in the presence of the CRE site in the DNA. The unbound state of the bZIP domain of CREB is largely unstructured and therefore unable to interfere with the helical CREB binding domain of CRTC2 (Dentin et al., 2008; Koo et al., 2005; Luo et al., 2012; Screaton et al., 2004; Wang et al., 2010; 2009).

Previous studies have shown that an adenoviral RNAi knockdown of CRTC2 in liver decreases fasting induced gluconeogenic genes, resulting in lowered fasting glycaemia (Koo et al., 2005; Saberi et al., 2009). Similarly, knockout of CRTC2 in isolated hepatocytes shows decreased glucose production (Le Lay et al., 2009; Wang et al., 2010). Although we and others have observed decreases in gluconeogenic genes with adenoviral knockdown of CRTC2, the CRTC2 knockout mice have a more modest phenotype under normal chow conditions, perhaps due to mouse strain differences, and differences in the knockout strategies (Le Lay et al., 2009; Wang et al., 2010), but possibly due to compensation by other family members.

While CRTC2 is the most highly expressed family member in the liver, CRTC3, is also ubiquitously expressed in tissues including the liver. CRTC3 has been found to promote obesity by down-regulating β-adregeneric receptor signaling in adipose tissue. $CRTC3^{-/-}$ mice are resistant to obesity, have lower fasting blood glucose under high fat diet conditions, and are more insulin sensitive (Song et al., 2010). Because the animals are full body knockout mice, it is unclear whether the metabolic phenotype is due only to the lack of CRTC3 in adipose tissue, or if the deficiency of CRTC3 in the liver also contributes to lower blood glucose levels.

These observations led me to test whether CRTC2 and CRTC3 have overlapping roles in the liver. Supporting this idea, the two family members share significant sequence homology (see Figure 2.1a), especially at the regulatory phosphorylation sites that mediate sequestration in the cytoplasm by the 14-3-3 proteins. We found that CRTC3 is active in hepatocytes under glucagon stimulation, contributes to CREB activity, and stimulates gluconeogenic gene expression, as well as glucose production under fasting conditions.

2.3 Results

Role of CRTC3 in gluconeogenesis

In order to determine whether CRTC3 responds to fasting signals, we used primary hepatocytes cultured from wild type, *CRTC2*$^{-/-}$, and *CRTC3*$^{-/-}$ mice. Following exposure to glucagon (100nmol/L) CRTC2 and CRTC3 are dephosphorylated (Figure 2.1b). Dephosphorylation promotes nuclear entry, and the CRTC family is active (Ravnskjaer et al., 2007; Screaton et al., 2004). Indeed, in Figure 2.1c, glucagon promotes nuclear shuttling of both CRTC2 and CRTC3, and both family members are cytoplasmic under basal conditions.

We wondered whether CRTC3 binds to CREB sites over gluconeogenic genes. In chromatin immunoprecipitation (ChIP) assays of wild type and *CRTC3*$^{-/-}$ primary hepatocytes, CRTC3 was recruited to CREB binding sites over the PCK1 and G6Pase promoters (Figure 2.1d).

CRTC3 is required for activation of the gluconeogenic program in hepatocytes

Because CRTC3 is recruited to CREB target genes, we questioned whether the loss of CRTC3 reduces CREB activity. Towards this end, we used an adenoviral 8xCRE-Luciferase reporter (Suzuki et al., 1996) in hepatocytes cultured from *CRTC3*$^{-/-}$ mice (Figure 2.2a). Indeed, CRE-

luciferase activity was substantially decreased in response to glucagon stimulation in CRTC3$^{-/-}$ relative to control cells.

Because CRTC3 appears to be necessary for CREB activity as measured through the luciferase assay, we wondered if CRTC3 was also required for the transcription of CREB target and gluconeogenic genes. Using adenoviral shRNAi of CRTC2 and CRTC3 alone and in combination in primary hepatocytes we saw that there was an attenuation of transcription of both *NR4A1* and *PCK1* (Figure 2.2b and S1). While CRTC3i is less effective in lowering expression of NR4A1 and PCK1, it is also notable that the protein amounts of CRTC2 are increased, perhaps compensating for the loss of CRTC3 (S1). Also supporting this idea of compensation, while the knockdowns of both CRTCs individually decrease mRNA expression, the combinations of the two (CRTC2i & CRTC3i) further reduce transcription of *NR4A1* and *PCK1*.

To further test the importance of CRTC3 in gluconeogenesis, we examined whether overexpression or a phosphorylation defective, constitutively active CRTC3 (CRTC3S$_{162}$A) upregulates CREB activity. By using a human hepatoma cell line (HepG2) and transient transfection, we found over-expression of WT CRTC3 and CRTC3S$_{162}$A increase the activity of both CREB- and G6Pase-luciferase reporters (Figure 2.2c). Interestingly, cells with CRTC3 or CRTC3S$_{162}$A showed induction in the reporter activity under

non-stimulated, basal conditions. Consistent with the results in HepG2 cells, when hepatocytes were infected with an adenoviral version of the constitutively active, dephosphorylation defective CRTC3 (Ad.CRTC3-$Ser_{162,275}$Ala), expression of gluconeogenic genes PCK1 and G6Pase, and CREB target gene NR4A1 were induced (Figure 2.2d).

CRTC3 promotes hepatic glucose production *in vivo*

In previous studies we noted that *CRTC3*$^{-/-}$ (Song et al., 2010) we found that *CRTC3*$^{-/-}$ mice have slightly lower fasting blood glucoses relative to their wild type littermates (Figure 2.3a), an effect that was attributed to decreases in adiposity. To determine whether CRTC3 participates directly in gluconeogenic gene expression, we performed immunoblot studies on liver extracts from overnight fasted and ad libitum fed mice (Figure 2.3b). Notably, both CRTC2 and CRTC3 were dephosphorylated indicating activity in the liver, the site of hepatic gluconeogenesis.

To evaluate CRTC3 activity in liver, we generated a *CRTC3* conditional knockout mouse (Figure S2). Indeed, when crossed with an albumin driven Cre-Recombinase mouse, we were able to breed a liver specific knockout (LC3KO). Similar to CRTC2$^{-/-}$ mice (Wang et al., 2010), the LC3KO mice, after 5 weeks on a 60% HFD, show significant differences in fasting blood glucoses (Figure 2.3c), and have increased glucose tolerance (Figure 2.3d).

2.4 Discussion

As a mechanism through which the body modulates fasting blood glucose levels, hepatic gluconeogenesis and its control are important to energy balance and metabolism. During insulin resistance, hepatic glucose output is up regulated, contributing to rising blood glucose levels that eventually lead to islet failure and type II diabetes. While CRTC2 has long been identified as an important player in hepatic gluconeogenesis, it has been unclear whether its relative CRTC3 has had any additional role. The results here suggest that CRTC3 contributes to the control of hepatic gluconeogenesis through its effects on hepatic CREB activity.

Using primary cultured hepatocytes, we are able to see similar effects of glucagon on CRTC2 and CRTC3. Under stimulated conditions, both CRTC family members become dephosphorylated and translocate to the nucleus. This indicates that both CRTC2 and CRTC3 are active and nuclear in the liver during fasting conditions (stimulation by glucagon). This is furthered by the observation that CRTC3 is recruited to known CREB target and gluconeogenic genes in hepatocytes, suggesting that CRTC3 is indeed active in the gluconeogenic program. Similarly, CRE-luciferase reporter activity is down regulated in CRTC3$^{-/-}$ hepatocytes signifying CRTC3's importance to CREB activity. By quantifying *PCK1* expression using knockdown RNAi for both CRTC2 and 3, we see the additive effect of having both CRTC2 and CRTC3

knocked down in hepatocytes. This supports the idea that the two family members may have similar or overlapping roles in the liver. We have also shown that overexpression of CRTC3 or the constitutively active CRTC3 greatly increases CRE-Luciferase activity, and the transcription of gluconeogenic enzymes even under basal conditions.

In vivo, we see that CRTC3 is dephosphorylated in the liver under fasting, denoting activity in conditions where hepatic gluconeogenesis is important. Using a CRTC3 conditional knockout mouse specific for the liver (LC3KO), we observed that under a high fat diet, LC3KO mice had lower fasting blood glucose levels and increased glucose tolerance, furthering our hypothesis that CRTC3 is involved in the gluconeogenic program in the liver.

Interestingly, it appears from the data presented here that CRTC3 may play a secondary role to that of CRTC2. In hepatocytes, with knockdown of CRTC3, the amount of CRTC2 is increased. This can also be seen in the hepatocytes from the CRTC3$^{-/-}$ mice, and the reverse in CRTC2$^{-/-}$ mice, in which CRTC3 is increased. These observations showing increases of the opposite family member may be showing compensation within the metabolic system. This hypothesis may explain the modest phenotype noted in the LC3KO mice, where only under the challenge of HFD do we see differences in fasting blood glucose—it is possible that CRTC2 is compensating for the loss of CRTC3. Additionally, this may explain the modest phenotype of CRTC2$^{-/-}$

mice, which may have to do with compensation from CRTC3 that until this time has not been recognized as a contributor to hepatic gluconeogenic ability (see Supplementary Figure 3).

In order to fully determine the contributions of CRTC2 and CRTC3 to hepatic gluconeogenesis, we need to generate mice from a CRTC2$^{-/-}$, LC3KO cross. Because CRTC2$^{-/-}$, CRTC3$^{-/-}$ mice are embryonic lethal, it has not yet been possible to look at mice with no CRTC contribution to the gluconeogenic program. Further studies should also elucidate if there are differences within specificities of CRTCs—does CRTC3 bind more closely to the CRE site in the promoter of *G6Pase* than it does with *PCK1*? Does each CRTC mediate effects on specific gene targets differently, or is the compensation provided by one family member in the absence of another is sufficient to rescue expression decreases of all gluconeogenic genes? It will only be when we can eliminate both CRTC2 and CRTC3 individually and together in the liver specifically that we will be able to tease apart the answers to these questions.

2.5 Materials and Methods

Animals

Mice were housed in colony cages in a temperature controlled environment under a 12 hour light/dark cycle with free access to water and a standard chow diet (Lab Diet 5001). Where indicated, mice were fed a high-fat diet (60% kcal%, Research Diets Incorporated, D12492). Unless indicated, studies were performed using 8-12 week old C57Bl/6J males from Jackson Laboratories (Bar Harbor, ME; <000664>). CRTC2$^{-/-}$, and CRTC3$^{-/-}$ mice were described previously (Song et al., 2010; Wang et al., 2010). CRTC3 conditional knockout mice were generated from embryonic stem cells from the International Knockout Mouse Consortium, injected into C57BL/6 blastocytes. The resulting chimeras were crossed with wild-type animals, and resulting exon 4 targeted heterozygotes were crossed to obtain exon 4 targeted mice. These mice were crossed with whole body FLP mice to generate CRTC3 floxed mice. The floxed mice were then crossed with Alb-Cre Recombinase to breed liver specific CRTC3 knockout mice (LC3KO), see Supplementary Figure 2. We obtained approval for mouse studies from the Salk Institute Institutional Animal Care and Use Committee.

Genotyping

Mouse genotypes for CRTC2$^{-/-}$, CRTC3$^{-/-}$, and LC3KO were determined using tail biopsies using real-time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN).

In vivo analysis

Glucose tolerance was tested by an intraperitoneal injection of 16 hr fasted mice with 1g/kg glucose followed by blood glucose measurements every 15-30 minutes as indicated for two hours. For determination of fasting blood glucose, mice were fasted overnight in alpha-dry cages for 16 hours with free access to water. Refed blood glucose values were determined after 5 hours of free access to food. Blood glucose values were determined using a LifeScan One Touch Ultra glucometer.

Immunofluorescence

Live hepatocytes were plated in glass chamber slides (BD #354114), and treated as indicated. Slides were then fixed for 10 minutes in 4% PFA in PBS, and then blocked for 30 minutes in PBS with 1% donkey serum (JAX #017-000-001) and 1% BSA (Millipore #81-066-4). Primary antibodies were incubated overnight in PBS, and secondary antibodies linked with Alexa-Fluor -488 (donkey anti rabbit) or -568 (donkey anti rabbit) (Life Technologies) and DAPI were incubated for 2 hours. Slides were then mounted with 70% glycerol and PBS, and images taken using a Confocal Zeiss 780.

In vitro analysis

HepG2 cells were cultured in Ham's F12 media containing 10% FBS (HyClone), 100mg/mL penicillin-streptomycin. HepG2 cells were transiently transfected with 3000ng DNA using X-tremeGENE HP (Roche #06366244001) and Opti-MEM (Life Technologies #31985088). Primary hepatocytes were derived from C57BL/6J, CRTC2$^{-/-}$, or CRTC3$^{-/-}$ mice as indicated, and maintained in serum free Medium 199 (Dentin et al., 2007). Knockdown and overexpression studies in hepatocytes were done by infecting cells at 2-5 pfu/cell with previously described adenoviruses ((Dentin et al., 2008; Song et al., 2010). All adenoviral RNAi knockdowns were carried out for 60hrs. Hepatocytes were stimulated with glucagon (100nmol/L, Sigma-Aldrich, G2044). We determined luciferase and β-galactosidase activities as previously reported (Conkright et al., 2003).

Gene expression

Total cellular RNA from whole liver or from primary hepatocytes were extracted using the RNeasy kit (Qiagen #74104) and cDNA was generated using the iScript select system (BioRad #170-8840). cDNA was quantified on a LightCycler 480 instrument with 2x SYBR Green (Roche). Gene expression data was presented relative to expression of housekeeping genes such as RPL32.

Protein analysis

Total protein from whole liver or primary hepatocytes was extracted in a RIPA buffer containing protease and phosphatase inhibitors, 0.25% DOC and 0.1% SDS. Proteins were quantified using the Bradford Bio-Rad Protein Assay Kit and separated using SDS-PAGE.

Chromatin Immunoprecipitation

Primary hepatocytes were cross-linked for 10 minutes in 1% formaldehyde directly in their culture dishes, and then the reaction stopped by the addition of glycine. Nuclei were isolated with a hypotonic lysis buffer and dounce homogenization. Resulting chromatin was precleared and incubated with the CRTC3 antibody overnight in the presence of protease, phosphatase, and HDAC inhibitors. The chromatin was then precipitated, purified, and quantified by PCR (LightCycler 480, Roche) and presented as percent of input chromatin.

Antibodies

Antibodies used for immunoblotting, immunofluorescence, and ChIP are in alphabetical order: CRTC3 (1:1000, CST #2720), KDEL (1:1000, Enzo #ADI-SPA-827), Hsp90 (1:5000, SCBt #33755). For CRTC2 (1:5000), CREB (1:5000) and pCREB (Ser133, 1:5000), rabbit polyclonal antibodies were raised against respective antigens (Salk Institute).

Statistical Analysis

Data are presented as means ± s.e.m. Statistical differences for one factor between two groups were determined by use of an unpaired Student's *t*-test. For one factor between more than two groups, a one-way analysis of variance (ANOVA) test was performed. We considered values of $p<0.05$ to be statistically significant.

2.6 Figures

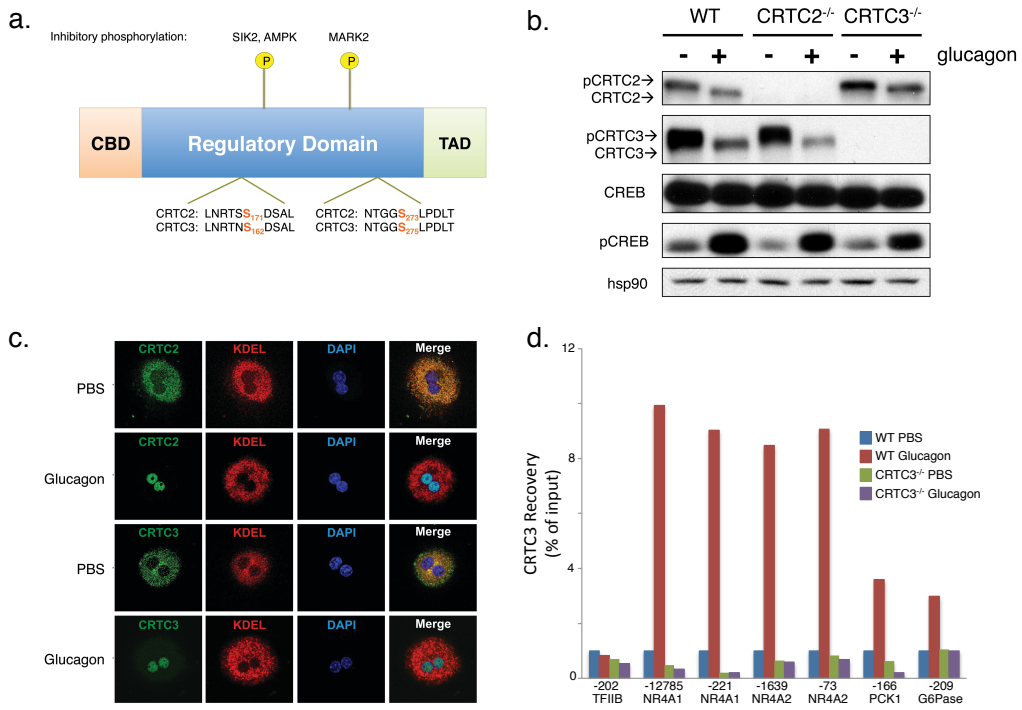


Figure 2.1 The role of CRTC3 in gluconeogenesis
a. Domain structure of the CRTC family, containing an N-terminal CREB Binding Domain (CBD), a regulatory domain, and a C-terminal Transactivation Domain (TAD). Phosphorylation of the indicated serines (by SIK and AMPK, and by MARK2) promotes 14-3-3 protein binding to keep CRTC sequestered in the cytoplasm. Based on (Altarejos and Montminy, 2011) **b.** Protein lysates from primary hepatocytes from *CRTC2*$^{-/-}$, *CRTC3*$^{-/-}$, and wild type control mice stimulated by glucagon (100nmol/L) or PBS. **c.** Primary wild-type hepatocytes stimulated with glucagon (100nmol/L) or PBS as indicated. KDEL is used as an ER localization marker, and DAPI as a nuclear marker. **d.** Chromatin Immunoprecipitation assay of CRTC3 occupancy of *TfIIb* (control) or CREB sites (location noted above gene names) in gluconeogenic genes in response to glucagon (100nmol/L).

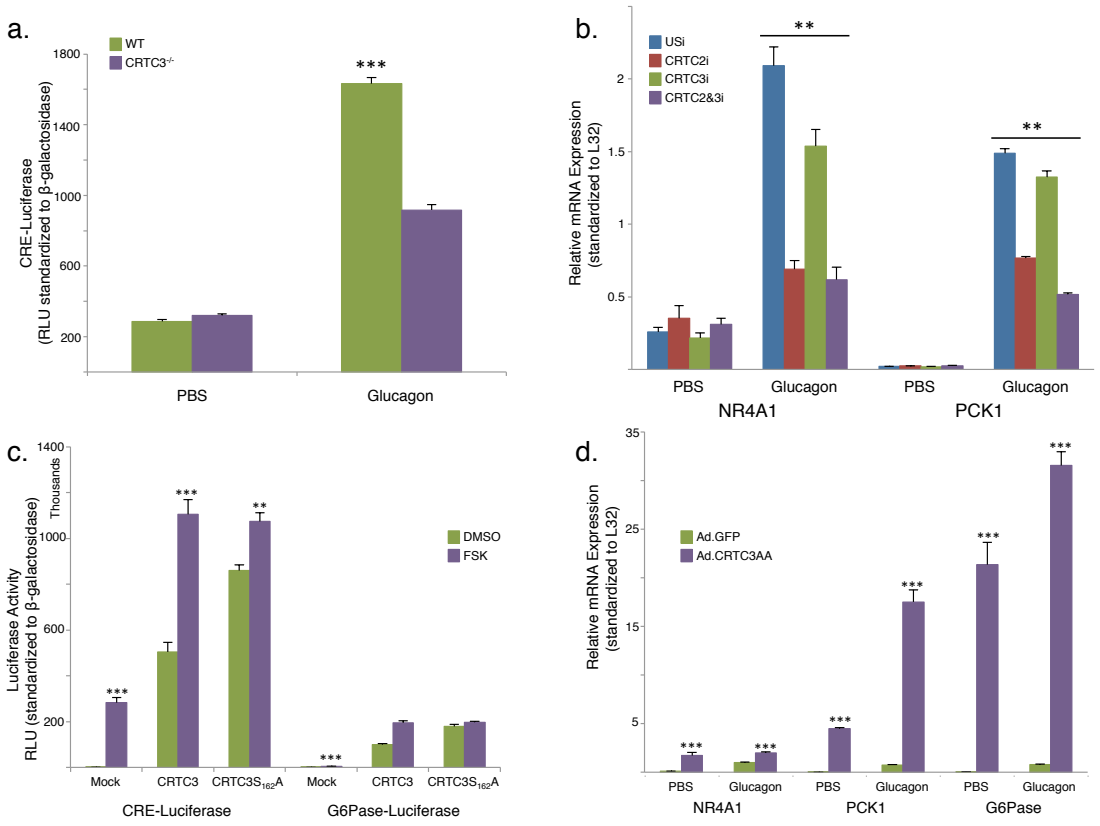


Figure 2.2 CRTC3 is required for activation of the gluconeogenic program in hepatocytes.

a. CRE-Luciferase activity of primary hepatocytes from *CRTC3*$^{-/-}$ and littermate wild type control mouse, standardized to β-galactosidase (co-infected with adenoviral CRE-luciferase). **b.** *NR4A1* and *Pck1* mRNA levels from primary hepatocytes depleted for CRTC2 and CRTC3 alone or in combination through the use of adenoviral shRNA in response to glucagon (100nmol/L). **c.** Transient assay of HepG2 cells transfected with wild-type CRTC3 or constitutively active, phosphorylation defective CRTC3-S162A, and reporters CRE-luciferase, G6Pase-luciferase, and RSV-β-galactosidase, stimulated with 10μM forskolin (FSK) or the vehicle DMSO. Luciferase activity is normalized to β-galactosidase activity. **d.** mRNA levels for *NR4A1*, *PCK1*, and *G6Pase* from primary hepatocytes infected with an adenoviral wild-type CRTC3 stimulated with glucagon (100nmol/L).

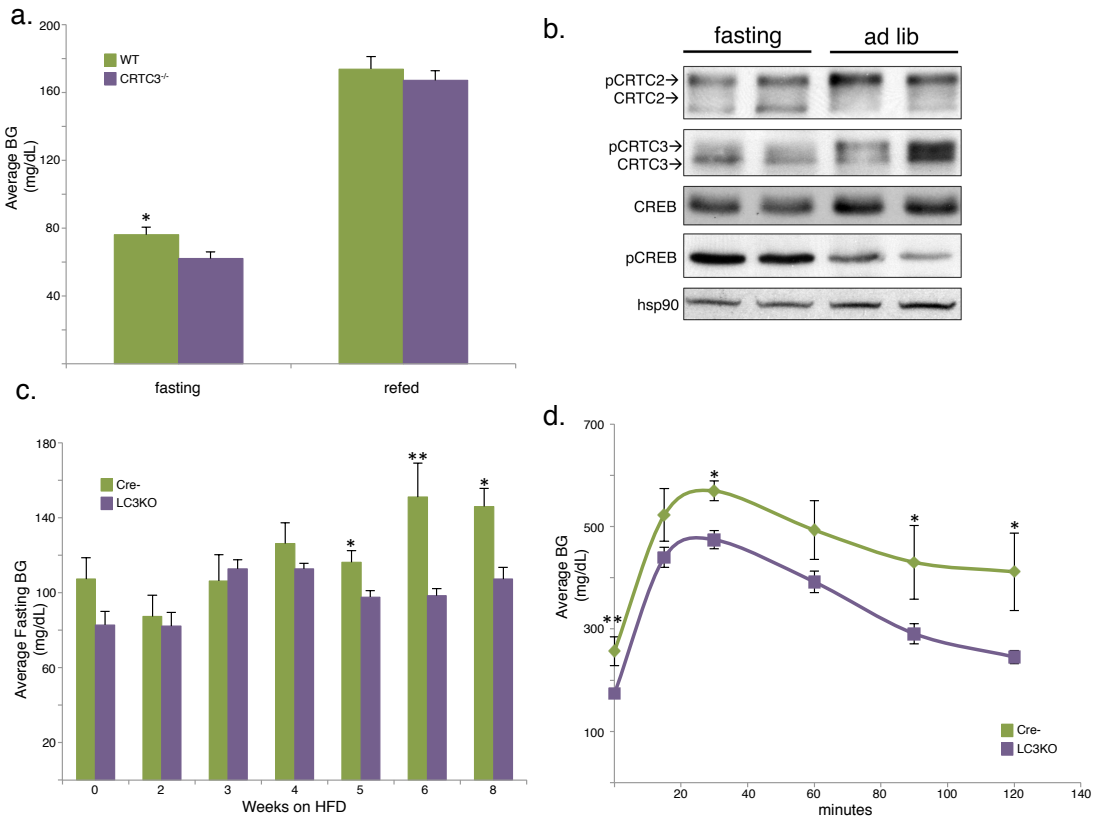
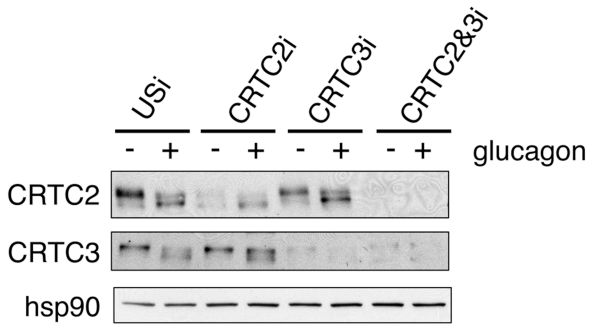
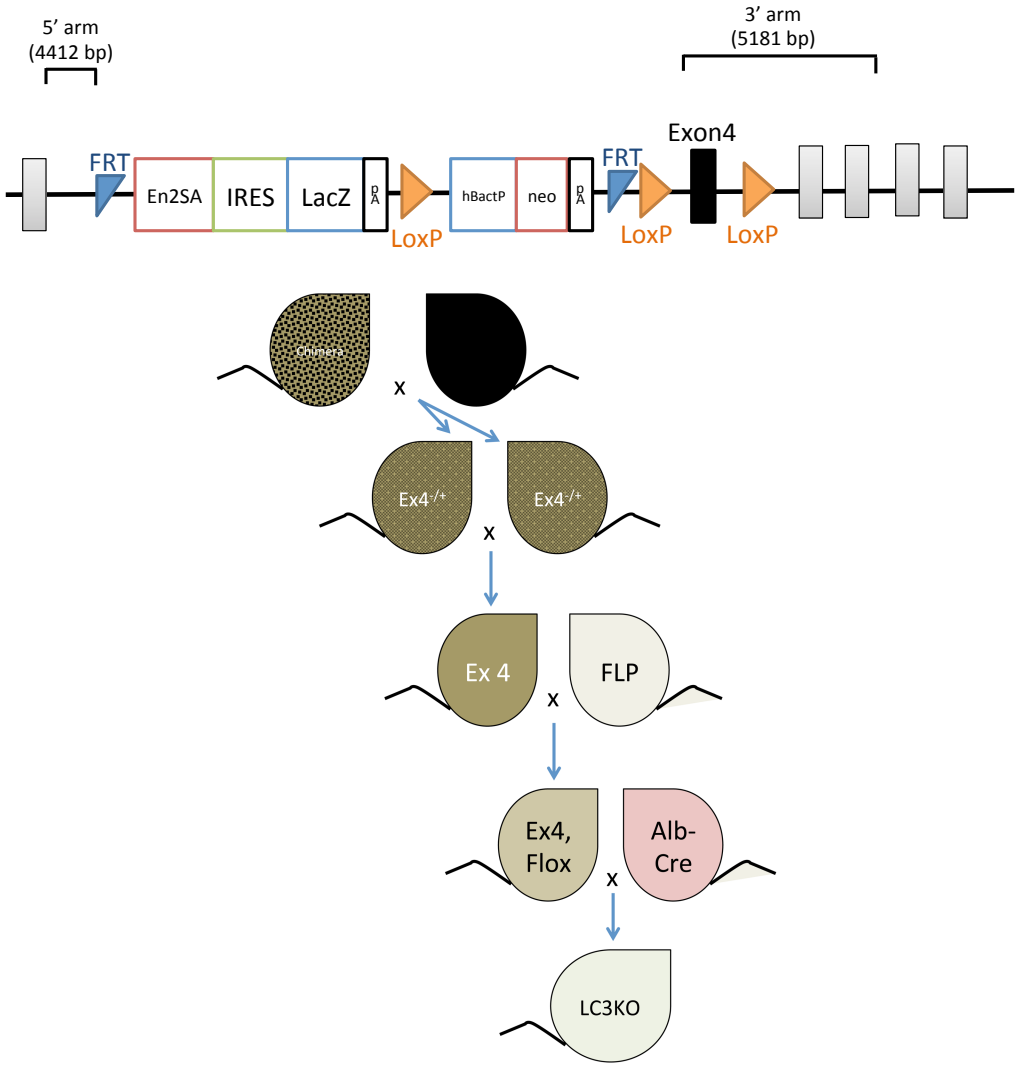


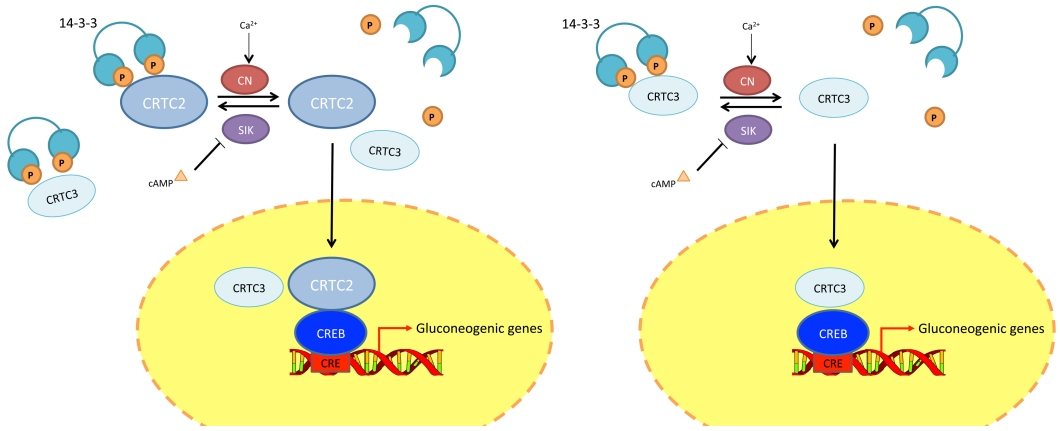
Figure 2.3 CRTC3 promotes hepatic glucose production in vivo. **a.** Average overnight fasting blood glucoses for *CRTC3*$^{-/-}$ mice and WT littermate controls. N=4 for WT, 5 for *CRTC3*$^{-/-}$. Mice were refed for 5 hours. **b.** Protein lysates from livers of 10-week-old WT mice fasted overnight or allowed free access to food. **c.** Average overnight fasting blood glucoses for LC3KO (*CRTC3* liver specific knock out mice) and littermate controls. N=4 for controls, 10 for LC3KO. **d.** Glucose Tolerance Test of LC3KO mice and littermate controls (Cre-Recombinase negative). Mice were on 60% HFD for 10 weeks. N=4 for controls, 10 for LC3KO.



Supplementary Figure 2.1. Protein lysates from primary hepatocytes with infected with adenoviral shRNA. Cells were stimulated with glucagon (100nmol/L) for one hour.



Supplementary Figure 2.2. CRTC3 Conditional Knockout targeting vector from IKMC (image available: http://www.knockoutmouse.org/martsearch/project/84669) and schematic to generate liver specific CRTC3 knock out mice (LC3KO).



Supplementary Figure 2.3. Model for CRTC3 action in gluconeogenesis. Under normal conditions, CRTC3 plays a secondary role to CRTC2, which binds with CREB in the nucleus after release from 14-3-3 proteins (left). It is in the absence of CRTC2 that CRTC3 plays a role in regulating hepatic gluconeogenesis (right).

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2.8 Acknowledgements

Chapter 2 has excerpts from Ravnskjaer K, Hogan MF, Lackey D, Tora L, Dent SYR, Olefsky J, Montminy M. KAT2B and WDR5 Mediate Epigenetic Effects of Glucagon on Gluconeogenesis. J Clin Invest. (2013); doi: 10.1172/JCI69035. Only experiments that I conducted or contributed to are included. Permission to use this work as part of my dissertation has been obtained from all co-authors.

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Chapter 3 Chronic Hepatic Expression of CRTC2 Causes Insulin Resistance, but is Partially Compensated

3.1 Abstract

In the past twenty years, obesity and type II diabetes rates have risen dramatically. To this day, science has elucidated many mechanisms that lead to insulin resistance, a prominent event before the development of diabetes. Often these events and mechanisms lead to one another in an interconnected web. Frequently after insulin resistance is detected, increased hepatic gluconeogenesis is observed. Normally, hepatic gluconeogenesis occurs in response to signals by cAMP and induction of gluconeogenic genes by the activation of CREB and the CRTC family. During times of abundant nutrient resources, insulin signaling shuts down gluconeogenesis, but during hepatic insulin resistance, this regulation is not effective. This chapter of this thesis shows that chronic hepatic expression of a constitutively active CRTC2 through the use of an adeno-associated virus leads to insulin resistance. Interestingly, *in* vivo compensation of the insulin resistance phenotype was observed due to the elevated expression of IRS2 in the liver.

3.2 Introduction

Over the past twenty years, the incidence of obesity and type 2 diabetes has risen dramatically to the point where major health groups such as the World Health Organization have stated that metabolic disorders are epidemic, with over 1.4 billion people overweight, and 347 million people diagnosed with diabetes worldwide. While the changes in lifestyles, such as decreased activity levels and increased caloric consumption do correlate with the appearance of obesity, science has paid a great deal of attention to identify the mechanisms that cause obesity. The progression of obesity to type 2 diabetes is often characterized by the development of insulin resistance and increases in fasting blood glucose levels (Barthel and Schmoll, 2003; Kahn et al., 2006; Saltiel, 2012; Saltiel and Kahn, 2001)

There are many different processes that can lead to insulin resistance, including inflammation, ER stress and the unfolded protein response, disregulation of lipids, and increases in hepatic gluconeogenesis (Flamment et al., 2012; Michael et al., 2000; Muoio and Newgard, 2006; Samuel and Shulman, 2012; Schenk et al., 2008; Shoelson et al., 2006). It is often unclear how these processes are linked to each other, but once a tissue has lost some of its responsiveness to insulin signaling, the progression to a multi-tissue insulin resistant state to type II diabetes may occur. While we often think of diabetes as being a disease of the pancreas with dysfunctional islets/β-cells, it

really involves multiple tissues, including the brain, skeletal muscle, adipose tissue, and liver (Kahn et al., 2006).

The body is remarkably successful at maintaining nearly constant blood glucose levels. This homeostasis is essential for tissues that rely on glucose as energy sources, such as the brain and red blood cells (Wang et al., 2006). During times of fasting or scarcity, the liver enters into a process known as gluconeogenesis: de novo glucose generation. Upon exposure to other nutrient sources, insulin signaling shuts down the gluconeogenic program to encourage glucose uptake into liver, muscles, and adipose tissue. It is due to insulin signaling that these programs are so tightly controlled, and in obesity we often observe metabolic changes in the whole body, in the end leading to decreased insulin sensitivity (Biddinger and Kahn, 2006; Saltiel and Kahn, 2001).

Hepatic gluconeogenesis relies on fasting signals, translated to the cell through glucagon released by the α-cells in the pancreas. Glucagon binds to G-protein coupled proteins receptors (GPCRs), which are linked to stimulatory G proteins, which then release their α-subunits, leading to the activation of adenylyl cyclase (AC). AC then catalyzes the synthesis of cyclic AMP (cAMP) from the energy source ATP. cAMP binds to the regulatory subunit of protein kinase A (PKA), prompting the dissociation of the catalytic subunit from their regulatory subunits which can later diffuse into the nucleus. It is at this point

that the cAMP-responsive binding protein (CREB) is phosphorylated by PKA while still bound to CRE-sites on the DNA, promoting the recruitment of CREB binding protein (CBP) and p300. Those promote the recruitment of RNA Polymerase II, finally leading to the transcription of gluconeogenic enzymes such as *PCK1* and *G6Pase* (Chrivia et al., 1993; Hagiwara et al., 1993; Herzig et al., 2001).

It is well understood that the family of cAMP Regulated Transcriptional Coactivators (CRTC) in combination with CREB to cooperatively boost transcription of certain transcriptional targets. Elevated levels of cAMP lead to the inactivation of the salt inducible kinases (SIKs) that are known to phosphorylate CRTCs. Once dephosphorylated, CRTCs are no longer sequestered in the cytoplasm by 14-3-3 proteins, and move by active transport to the nucleus, where they bind with the bZIP domain of CREB, increasing the occupancy of CREB on binding sites (Dentin et al., 2008; Koo et al., 2005; Luo et al., 2012; Screaton et al., 2004; Wang et al., 2010).

According to the results of the previous chapter of this thesis, CRTC2 appears to be the major coactivator of CREB in the liver, directly responding to fasting signals, with some contribution from CRTC3. Given the importance of CRTC2 in gluconeogenesis, we wondered whether the chronic expression of CRTC2 in the liver would be sufficient to increase hepatic gluconeogenesis

rates, subsequently leading to insulin resistance, that spreads to tissues other than the liver.

3.3 Results

CRTC2S$_{171,275}$A expresses the active form of CRTC2

To investigate whether the adeno-associated virus (AAV.CRTC2AA) expresses a constitutively active, phosphorylation defective version of CRTC2 (CRTC2S$_{171,275}$A, see Figure 3.1a, hereafter called CC for *C*onstitutively active *C*RTC2) we performed an immunoblot experiment in primary hepatocytes derived from CC or control (AAV.Empty, see Figure 3.1a) mice (Figure 3.1b). Under basal conditions (vehicle treated) we observed a greater amount of dephosphorylated CRTC2 in the CC hepatocytes relative to the control hepatocytes. Supporting these results, CC hepatocytes under basal conditions show stronger staining for CRTC2 in comparison to control hepatocytes with under stimulated conditions (glucagon treated) (Figure 3.1c).

Previous studies have linked CRTC2 activity to gluconeogenesis and glucose homeostasis (Koo et al., 2005; Le Lay et al., 2009; Wang et al., 2010; 2009), prompting us to examine whether AAV.CRTC2AA would increase hepatic gluconeogenesis. Examining CC and control hepatocytes under basal (Figure 3.2a) and stimulated (Figure 3.2b) conditions, we found that the transcription of CREB target genes, such as the typical gluconeogenic enzymes *PCK1* and *G6Pase*, and*NR4A1*, were up-regulated.

To ensure that the virus infection was specifically occurring in the liver and therefore is linked to the expression of FLAG-CRTC2AA, we performed

immunoprecipitation studies in a variety of tissues from infected mice (Figure 3.2c). FLAG-CRTC2AA was detected only in the livers of CC animals, indicating that the method of injection, serotype of the virus, and promoter are specific to the liver. Similarly, when we assayed liver tissues from mice by qPCR for wild type CRTC2 and the virally derived FLAG-tagged CRTC2, we found that the FLAG.CRTC2 mRNA was only present in the CC mice. The total amount of CRTC2 mRNA was twofold increased in CC mice versus the control animals (Figure 3.2d).

Hepatic CRTC2AA increases gluconeogenesis in vivo

Previously, we have used Xenon Live Imaging as a way to determine CREB and CRTC2 activity in mice using an adeno-CRE-Luciferase virus as a reporter, linked to the gluconeogenic program (Dentin et al., 2008; 2007). A transgenic mouse model expressing this construct was recently developed (Song et al., 2010). CRE-Luciferase mice infected with AAV.CRTC2AA had two to four thousand higher fold luciferase activity under fasted as well as refed and ad libitum conditions (Figure 3.3a and data not shown).

Given the induction of CRE-Luciferase activity in the livers of CC mice, we wondered whether hepatic gluconeogenesis rates would also be high. To this end, we tested the fasting blood glucose values of mice injected with both viruses and found that beginning at about day six post injection, CC animals had elevated blood glucose concentrations, supporting hepatic

gluconeogenesis upregulation (Figure 3.3b). Gluconeogenic capacity, measured by pyruvate tolerance testing (Figure 3.3c), was also increased in CC mice; whereas insulin sensitivity was decreased (Supplementary Figure S3.1b). Additionally, mRNA levels of *PCK1*, *G6Pase*, and *NR4A1* were increased in CC animals over control animals (Figure 3.3d). Taken together, this data indicates that the AAV.CRTC2AA causes increased gluconeogenesis rates.

Chronic hepatic expression of CRTC2AA changes metabolism and phenotypic appearance of peripheral tissues

Having seen the changes in fasting blood glucose, response to insulin, and gluconeogenesis in CC mice, we wondered whether other metabolic tissues are also affected in this setting. We performed histological studies of liver, epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) (Figure 3.4a). Livers from CC mice showed steatosis; BAT and WAT tissues accumulated more triglyceride stores than control mice. Hepatic triglycerides and glycogen (Figure 3.4b) are also 1.3 and 3 fold increased respectively. This indicates that chronic expression of an active form of CRTC2 in the liver is sufficient to change the physiology of other tissues.

Many studies link the development of hepatic insulin resistance, which can impact islet size, with the increase of lipid accumulation (Farese et al., 2012). We examined the morphology of islets of the CC and control mice

(Figure 3.4c). We found a high variability in islet size, with CC animals being more extreme in having more many more very small and very large islets, and a much smaller percentage of medium sized islets (5.4% to 28.5%) than the control mice. Interestingly given the number of larger islets, the serum insulin levels of the CC mice control mice were decreased (Figure 3.4d).

Long-term chronic CRTC2AA in the liver causes development of insulin resistance with partial phenotypic compensation

Because of the islet size variability in the CC mice, we wondered if this islet phenotype was reflecting an early stage alteration of the pancreas. In order to determine the onset of time of the islet change, we decided to study CC mice that had experienced long-term viral expression. After a prolonged viral exposure, we found that there was a partial compensation for the early increase in gluconeogenesis. In the short term of viral infection (within 7 days) there is a difference in fasting blood glucose levels of the two groups of mice, and after 40 of infection days there is a “plateauing” of fasting glucose levels (Figure 3.5a). Not only did the difference in the basal fasting blood glucose levels disappear but also the glucose clearance capability as measured by a GTT was the same between the two groups of mice (Figure 3.5b) compared to an earlier impaired clearance (Supplementary Figure 3.1a) of the CC mice. Similarly, while we observed early differences in insulin sensitivity between the

two groups, but in the long-term exposure experiment, the differences were less pronounced (Supplementary Figure 3.1b).

Based on earlier islet data at 35 days post infection, and supported by the fact that blood glucose levels seemed to stabilize after day 40, we decided to look at islet morphology in long-term exposed mice. Indeed, we found that islets in CC mice were increased in size (Figure 3.5c) and that the circulating insulin levels had stabilized at day 70, the day the islets were prepared for fixation (Figure 3.5d).

We thought that improved insulin signaling might explain the late change in phenotype in the CC mice. Previous research had indicated that CRTC2 induces expression of *IRS2* in liver, enhancing insulin signaling (Canettieri et al., 2005). This prompted us to look at the mRNA levels of *IRS2* in the long-term exposed mice (135 days). We found that while expression of *IRS2* is elevated two-fold (Figure 3.5e), *IRS1* does not significantly change, indicating specificity for *IRS2*. Additionally, there is a greater amount of IRS2 protein in the CC livers, as confirmed by western blot (Figure 3.5e). This finding raised the question why this improved insulin signaling was not observed immediately. By employing qPCR to the different earlier-term exposure samples (day 35), a lag in the elevation of *IRS2* was found (Supplementary Figure S3.2). Supporting this finding, the expression of IRS2 in hepatocytes is also equal (Supplementary Figure S3.2). Therefore we

hypothesize that the compensation of insulin signaling does not occur until a certain threshold for the chronic CRTC2$S_{171,275}$A in the liver has been reached. Our experiments indicate that this may occur between the window of 50 and 75 days after exposure to the virus.

3.4 Discussion

Energy balance in mammals has become an important aspect of study due to the link to obesity and type II diabetes. The metabolic balance of a system relies greatly on the composition of our molecular food sources. During the waking hours, we are able to consume food, and thus use glucose as an energy source, and during sleeping hours; we switch to fat burning processes, known as fatty-acid oxidation. Additionally, especially after the fat stores have been depleted, the liver maintains normal circulating blood glucose levels by de novo synthesis of glucose, known as gluconeogenesis. This process fuels tissues that cannot burn fatty acids, such as the brain and red blood cells (Wang and Sadée, 2006). The ability for the liver to produce glucose and breakdown long-chain fatty acids is controlled by circulating hormone levels, especially insulin and glucagon, which further act through a series of transcriptional factors (Spiegelman and Heinrich, 2004). As noted in the previous chapter of this thesis, one particular transcriptional co-activator, CRTC2, plays a major role in inducing gluconeogenic gene expression, with an additional induction roles for the CRTC3:CREB interaction. We wondered what, if any role, chronic upregulation of hepatic gluconeogenesis would have on the development of insulin resistance.

Using a liver specific adeno-associated virus that expresses a phosphorylation defective, constitutively active CRTC2, we found that

gluconeogenic gene expression was upregulated in hepatocytes cultured from and in CC mice (mice injected with AAV.CRTC2AA). These results indicate that CRTC2 was sufficient to drive the transcription of the rate limiting enzymes in gluconeogenesis, and in the end increase hepatic glucose production. Interestingly, the basal transcription rates of these enzymes were also upregulated in CC hepatocytes compared to the control hepatocytes. These results indicate that even under non-fasting conditions, gluconeogenesis was elevated in hepatocytes, and by extension, the liver itself. This would, as we observed, increase hepatic gluconeogenesis and gluconeogenic abilities, resulting in increased fasting blood glucose levels.

By using this chronic expression of an active form of CRTC2, we were able to study the effects of chronically increased fasting blood glucose levels. This allowed us to ask whether a constant “reset” of fasting blood glucose levels would result in a shift of insulin homeostasis. Indeed, we found evidence of insulin resistance in CC mice versus control animals: Decreased insulin sensitivity, decreased glucose clearance, increased steatosis and lipid accumulation. In conclusion, all offering evidence of whole body insulin resistance due to increased hepatic gluconeogenesis. This result would be the first documented case that increased hepatic glucose output is linked to whole body insulin resistance.

Intriguingly, in no animal a complete transformation into a diabetic state was observed. We hypothesize that the increased activity of CRTC2 induces the levels of *IRS2* expression in the CC mice. The greater amount of IRS2 would lead to greater insulin sensitivity (Awazawa et al., 2011), which could potentially slow or reverse the metabolic phenotype caused by increased hepatic gluconeogenesis. Indeed, differences in glucose and insulin sensitivity between the two groups of infected mice were dependent on the exposure time post-infection, with the long-term animals having a non-significant insulin phenotype.

Interestingly, although we observed a change in islet morphology fairly quickly after observing increased hepatic gluconeogenesis and after the late-stage compensation by IRS2, serum insulin levels of CC mice are not elevated anymore. Despite showing blunted insulin sensitivity and glucose uptake into muscles, both signs of insulin resistance, no increase in insulin production itself in a compensatory mechanism for the loss of insulin sensitivity was observed. Additionally, liver samples from CC mice with a long infection show unaffected pAKT signaling; perhaps hinting towards the initial insulin resistance phenotype has been reversed or at least moderated (data not shown).

While this study presents a link of a short-term increase in hepatic gluconeogenesis to insulin resistance, the long-term compensatory finding

points towards a more complex picture for the regulation of glucose homeostasis. This thesis still supports the generally accepting findings that with increasing the activity of CRTC2, we see an increase in CREB activity, which increases gluconeogenesis, but the finding that a long-term exposure to active CRTC2 increases IRS2 expression to compensate with increased insulin sensitivity is a new idea. This compensation by IRS2 leads to an increase in PI3Kinase activity, which in turn leads to increased pAKT signaling. This mechanism might compensate the initial insulin resistance, reducing the impact of the phenotype.

One question that this study presents is the temporal resolution of the observed changes. How high does the expression and activity of CRTC2 need to be in order to induce IRS2 expression? Is there a specific time in which increased IRS2 cannot resolve the effects the continued increases in hepatic gluconeogenesis bring, and therefore the protective effect from IRS2 is lost? The changes in the animals caused by expression of a constitutively active CRTC2 again reinforce the idea that CREB and CRTC2 interaction should serve as a target for drug discovery, with the aim to prevent the onset of insulin resistance through increased hepatic gluconeogenesis.

3.5 Materials and Methods

Adeno-associated viruses (AAV) strains and propagation

pENN AAV TBG PI (an adeno-associated virus, serotype 8 with a TBG promoter) was a generous gift from Morris Birnbaum at the University of Pennsylvania from which the viruses were derived. Briefly, pENN AAV TBG PI was digested with enzymes Acc65I and SalI. Meanwhile a pcDNA3 construct expressing a FLAG-tagged mouse CRTC2 with mutations S171A and S275A was amplified by PCR (primers: GCCGGTACCATGACCATGGATTACAAGGAT, and GCCGTCGACTAGGTGACACTATAGAATAGG) and then digested with Acc65I and SalI (NEB). The digested pENN AAV TBG PI was ligated with the FLAG.CRTC2AA PCR fragment and transformed in recombination deficient *E. coli* SURE cells (Agilent #200238).

The resulting vectors (pENN AAV TBG PI and pENN AAV TBG PI CRTC2AA) were amplified in the Salk Institute Gene Transfer Targeting and Therapeutics Core. Throughout this thesis, the amplified viruses are referred to as AAV.Empty and AAV.CRTC2AA.

Animals

Mice were housed in colony cages in a temperature controlled environment under a 12 hour light/dark cycle with free access to water and a standard Chow diet (Lab Diet 5001). Under certain experimental settings, mice

were fed a high-fat diet (60% kcal%, Research Diets Incorporated, D12492). Unless noted, studies were performed using 8-12 week old B6(Cg)-Tyr^{c-2J}/J mice from Jackson Laboratories (Bar Harbor, ME; <000058>). CRE-Luciferase mice were described previously (Song et al., 2010).

Mice were injected retroorbitally at 8-10 weeks with 10^{11} genomic copies (gc) of AAV.Empty or AAV.CRTC2AA virus, according to a protocol previously described (Miller et al., 2011). For all mice experiments performed in this thesis, we obtained approval for mouse studies from the Salk Institute Institutional Animal Care and Use Committee.

Mouse genotyping

Mouse genotypes for CRE-Luciferase were determined using tail biopsies using real-time PCR with specific probes designed for luciferase (Transnetyx, Cordova, TN).

***In vivo* analysis**

Pyruvate tolerance was tested by an intraperitoneal injection of sodium pyruvate 16 hour fasted mice with 2g sodium pyruvate / kg body weight followed by blood glucose measurements every 15-30 minutes for two hours (Wang et al., 2010). Insulin tolerance was tested by an intraperitoneal injection of 1 Unit (Humulin-R from Eli Lilly) / kg body weight followed by blood glucose measurements every 15 minutes for one hour. Glucose tolerance was tested by an intraperitoneal injection of 1g glucose / kg mouse body weight

followed by blood glucose measurements every 30 minutes for two hours. For determination of fasting blood glucose levels, mice were fasted overnight for 16 hours with free access to water. Refed blood glucose values were determined after 5 hours of free access to food returned to cage. Blood glucose values were determined using a LifeScan One Touch Ultra glucometer. For *in vivo* imaging, CRE-luciferase mice underwent conditions noted and were imaged as previously described (Song et al., 2010). Serum insulin levels were measured using the Crystal Chem Ultra Sensitive Mouse Insulin ELISA (#90080). Liver triglyceride levels were measured using LabAssy Triglyceride Kit from Wako (#290-63701), and liver glycogen levels using Sigma Glycogen Assay Kit (#MAK016). In all cases a standardization to the protein concentration of each sample took place using the Bio-Rad Bradford Protein Assay Kit.

Cell culture and *in vitro* analysis

Primary hepatocytes were derived from B6(Cg)-$Tyr^{c\text{-}2J}$/J or CRE-Luc mice as indicated (Dentin et al., 2007; Miller et al., 2011). Hepatocytes noted as AAV.Empty or AAV.CRTC2AA were harvested from 8-10 week old mice infected 3 days previously with AAV, and maintained in serum free Medium 199 (Corning #10-060). Overexpression studies in hepatocytes were done by infecting cells at 2-5 pfu/cell with previously described adenoviruses (Dentin et al., 2008). Hepatocytes were either stimulated with glucagon (100 nmol/L,

Sigma-Aldrich #G2044) or PBS as a negative control. Luciferase and β-galactosidase activities were determined as previously reported (Conkright et al., 2003).

Gene expression analysis

Total cellular RNA from whole liver or from primary hepatocytes were extracted using the RNeasy kit (Qiagen #74104) and cDNA was generated using the iScript select system (BioRad #170-8840). The cDNA was quantified via the LightCycler 480 instrument and 2x SYBR Green (Roche). Gene expression data is presented relative to parallel measured expression of housekeeping cDNA, for example *RPL32* or *Rn18S*, see individual figures for specific gene.

Protein analysis

Total protein from whole liver or primary hepatocytes was extracted in RIPA buffer (50 mM TRIS pH 7.5; 150 mM sodium chloride; 1mM EDTA; 50 mM sodium fluoride; 5 mM sodium pyrophosphate; 10 mM b-glycerophosphate; 1% NP-40) containing 1 mM sodium orthovanadate; 1mM dithiothreitol; 2 mM phenylmethanesulfonyl fluoride; 0.25% DOC (sodium deoxycholate); and 0.1% sodium dodecyl sulfate from Sigma. Proteins were quantified using the Bio-Rad Bio-Rad Protein Assay Kit and separated using SDS-PAGE.

Immunoprecipitation

For tissues, 40 µL of FLAG-M2 monoclonal antibody beads (Sigma #F3165) were pre-cleared and 1mg of lysates were added with lysis buffer to a concentration of 1µg/µL, and binding occurred under rotation overnight at 4°C. The supernatant was removed, and beads were washed five times with lysis buffer, eluted with 2x SDS loading buffer and analyzed via western blot.

Antibodies and western blots

Antibodies used for immunoblotting and immunofluorescence are in alphabetical order: CRTC3 (1:1000, CST #2720), DAPI (1:500, Invitrogen #D3571), Glucagon (1:500, Sigma #G2645), Hsp90 (1:3000, SCBt #33755), Insulin (1:500, Xymed #180067), IRS2 (1:1000, Upstate #06-506), pLKB1 (1:1000, CST #C6783). For CRTC2 (1:5000), CREB (1:5000), and pCREB (Ser133, 1:5000), rabbit polyclonal antibodies were raised against respective antigens (Salk Institute). For western blotting, all were probed by the secondary antibody goat anti rabbit HRP (1:5000, BioRad #170-6515). Proteins were transferred to PVDF membranes (Millipore #IPVH00010), developed on HyBlot film (Denville #E3108) with HyGlo ECL (Denville #E2500).

Immunofluorescence

Live hepatocytes were plated in glass chamber slides (BD #354114), and treated as indicated. Slides were then fixed for 10 minutes in 4% PFA in PBS, and then blocked for 30 minutes in Blocking Buffer [PBS with 1% donkey serum (JAX #017-000-001) and 1% BSA (Millipore #81-066-4)]. Primary antibodies were incubated overnight in PBS, and secondary antibodies linked with Alexa-Fluor-488 (donkey anti goat) or -568 (goat anti guinea pig and donkey anti rabbit) (Life Technologies) and DAPI were incubated for 2 hours. Slides were then mounted with 70% glycerol and PBS, and images taken using a Confocal Zeiss 780. Pancreases were dissected from mice and fixed in Z-Fix for 4 hours, and then sectioned in 5-10 micron slices. Slides were routinely deparafinized and rehydrated, and antigen unmasked via citrate unmasking. Slides were blocked fin Blocking Buffer, and then incubated overnight with primary antibodies, followed by fluorescent secondary antibodies.

Statistical Analysis

Data are presented as means ± s.e.m. Statistical differences for one factor between two groups were determined by use of an unpaired Student's *t*-test. For one factor between more than two groups, a one-way analysis of variance (ANOVA) test was performed. We considered values of $p<0.05$ to be statistically significant.

3.6 Figures

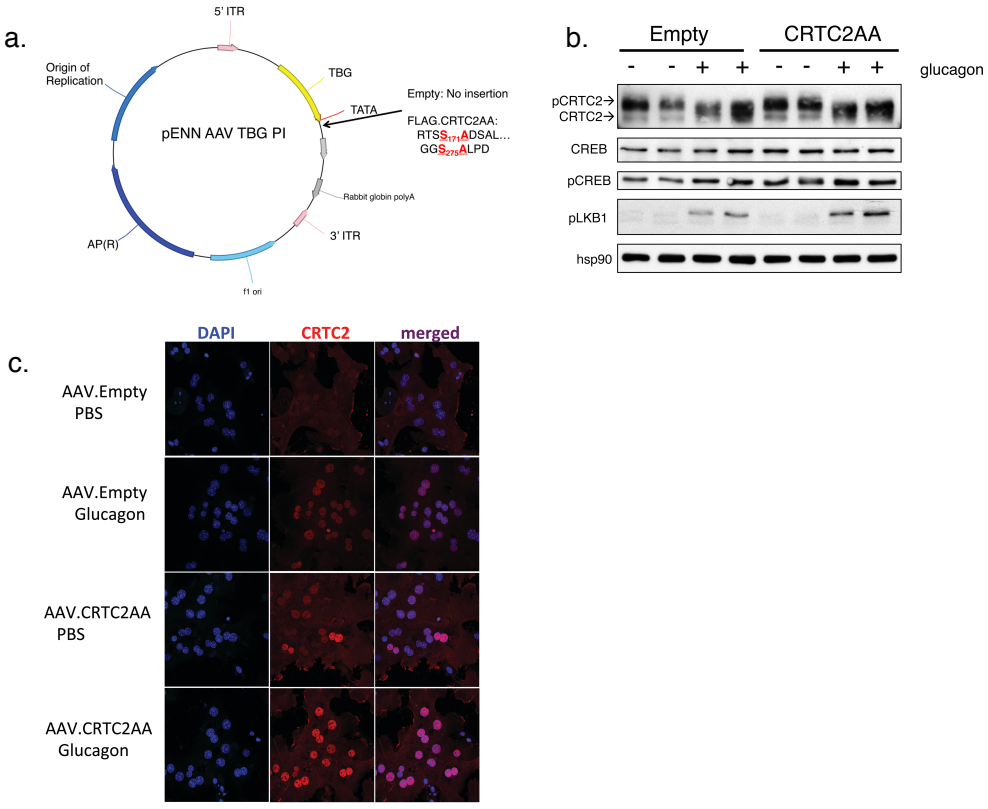


Figure 3.1 AAV.CRTC2S$_{171,275}$A (AAV.CRTC2AA) expresses the active form of CRTC2

a. Schematic representative of the plasmid pENN AAV TBG PI and pENN AAV TBG PI CRTC2AA . The AAVs are based upon this viral vector that carries the TBG (Thyroxine Binding Globulin) promoter. In the case of the AAV.CRTC2AA, serines 171 and 275 are mutated to an alanine. Moreover, it encodes the sequence of a 5' FLAG tag. FLAG.CRTC2AA is inserted into the vector after the TATA box in the multiple cloning site. **b.** Protein expression and phosphorylation data from primary hepatocytes prepared from mice injected with AAV.Empty or AAV.CRTC2AA in the absence or presence of 100nmol/L glucagon. **c.** Immunofluorescence images of primary hepatocytes from mice infected with AAV.Empty or AAV.CRTC2AA, stimulated with glucagon or PBS. DAPI is used as a nuclear marker.

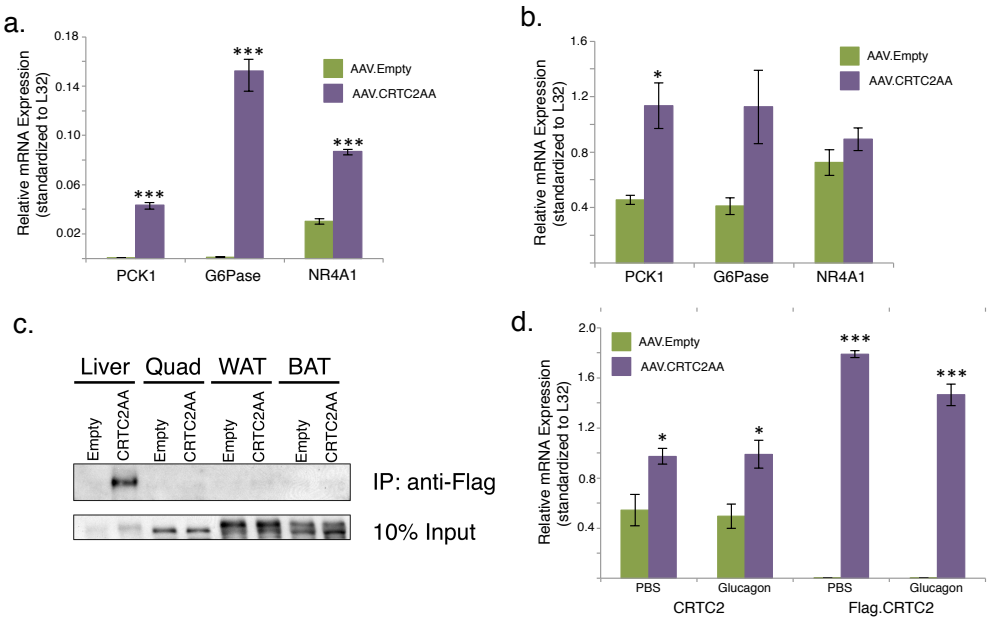


Figure 3.2 AAV.CRTC2AA expresses the active form of CRTC2 *in vitro* and *in vivo*

a and b. Relative mRNA expression of *PCK1*, *G6Pase*, and *NR4A1* of hepatocytes from mice injected with AAV.Empty or AAV.CRTC2AA under basal (**a**) and stimulated (**b**) conditions. **c.** Immunoprecipitation of FLAG from tissue lysates from mice infected 20 weeks previously with AAV.Empty or AAV.CRTC2AA and probed with CRTC2 antibody. **d.** Relative mRNA expression levels of wild type CRTC2 and Flag.CRTC2 from liver samples of mice infected with AAV.Empty or AAV.CRTC2AA, n=5.

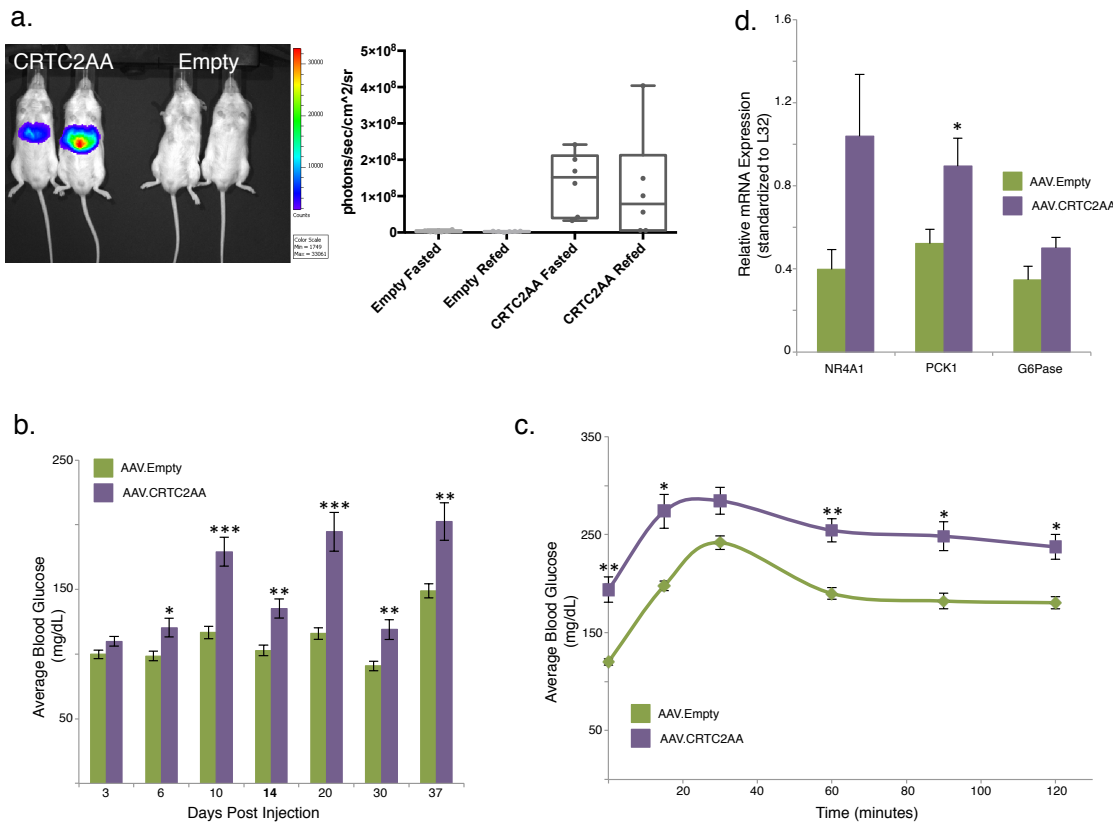


Figure 3.3 Hepatic CRTC2AA increases gluconeogenesis rate *in vivo*
a. Live Imaging performed on CRE-Luciferase mice injected with adeno-associated viruses. Left, representative image of mice under refed conditions. Right, quantification of living image values for mice under fasting and refed conditions using LivingImage Software by Xenon. **b.** Average overnight fasting blood glucose values of mice injected with AAV.Empty or AAV.CRTC2AA, n=10. **c.** Pyruvate Tolerance Test (2g/kg sodium pyruvate) of mice infected with adeno-associated viruses on day 20 post injection, n=10 per group. **d.** Relative liver mRNA expression levels of fasting mice, standardized to L32, n=6 per group.

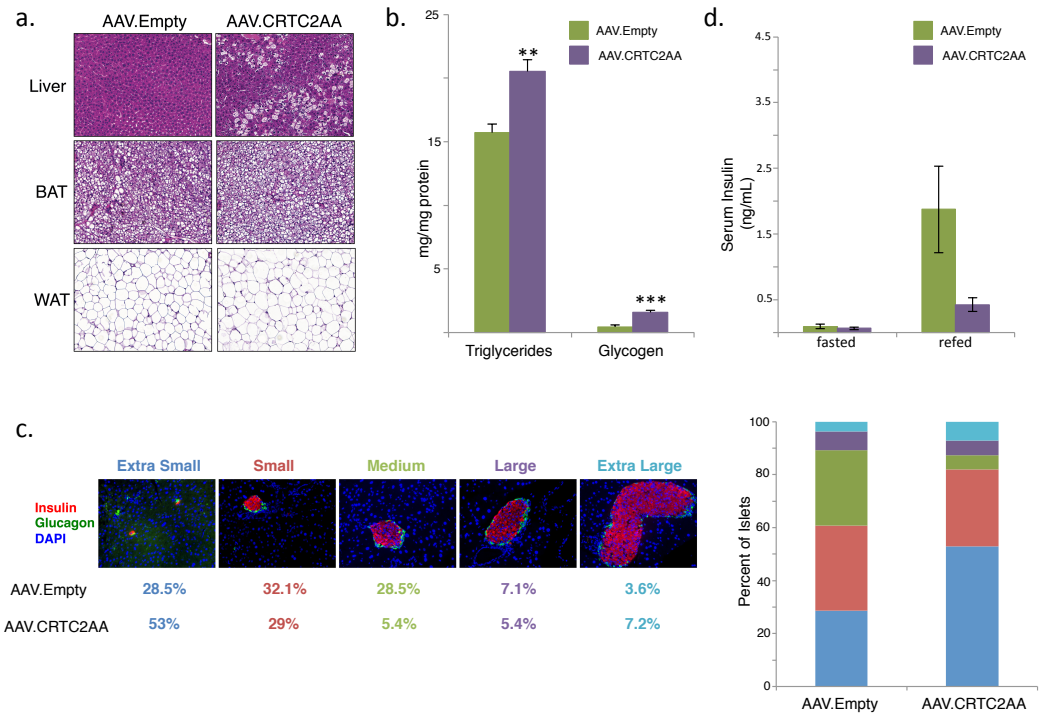


Figure 3.4 Chronic hepatic expression of CRTC2AA causes changes in metabolic and peripheral tissues.

a. Mice were infected with indicated AAV and were fasted overnight. Sections from the liver, brown adipose tissue (BAT), and epididymal white adipose tissue (WAT) were processed for histology and stained with hematoxilin and eosin (H&E) on day 35 post infection. Images were taken at 40x. **b.** Liver homogenates were assessed for triglyceride and glycogen content and standardized to protein concentration, n=5 per group. **c.** Immunofluorescence staining of AAV.Empty and AAV.CRTC2AA pancreases (day 35) for insulin and glucagon (top) and islet size is quantified (n=5 per group). **d.** Serum insulin was quantified for fasting and refed mice at day 30 post injection of adeno-associated viruses, n=7.

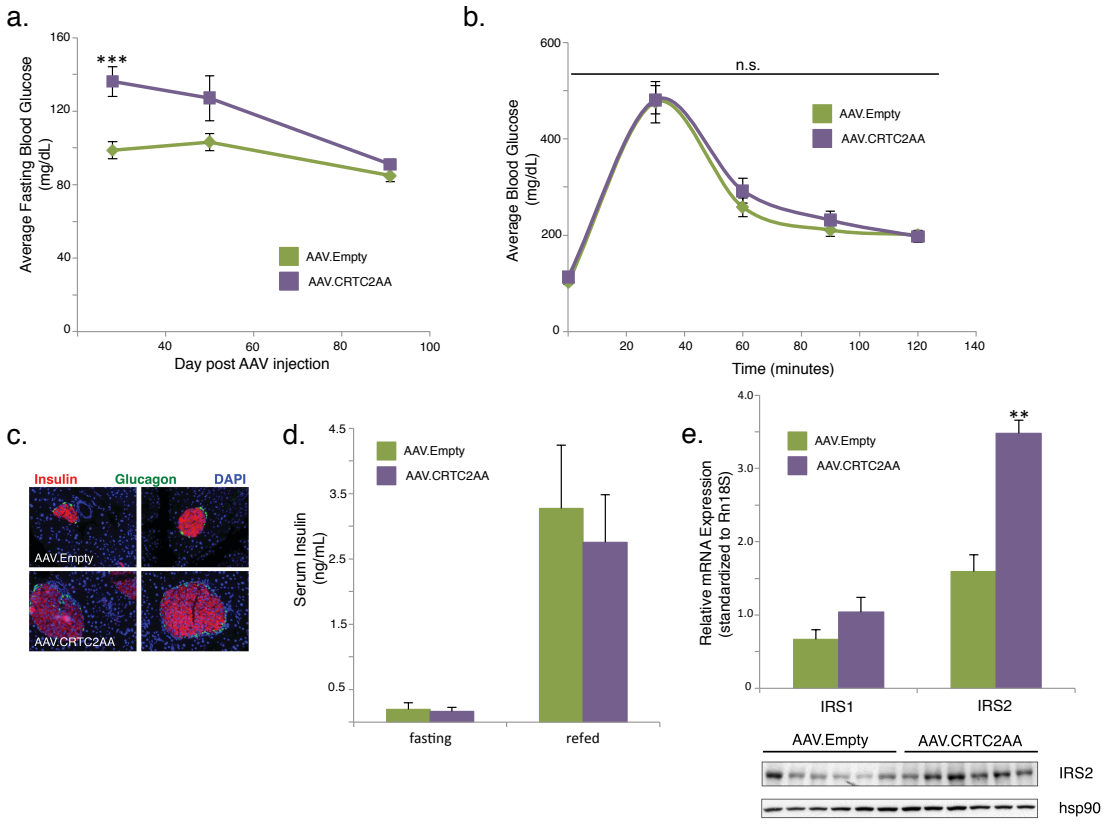
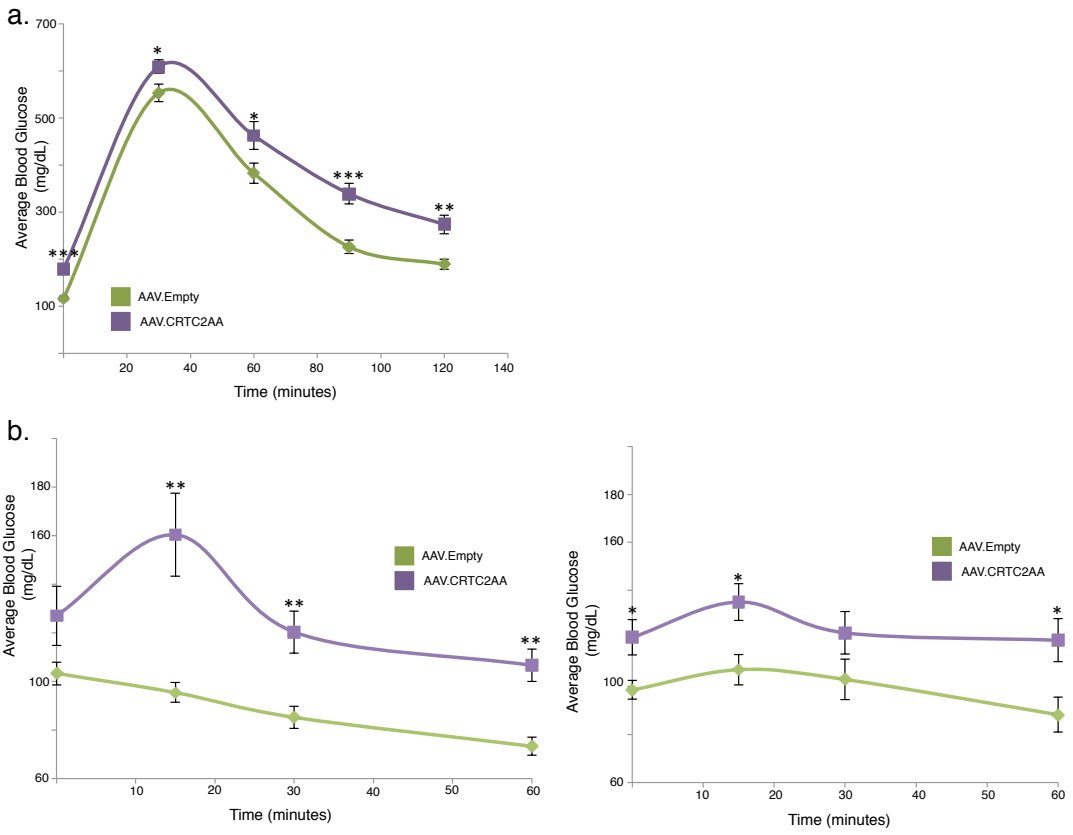
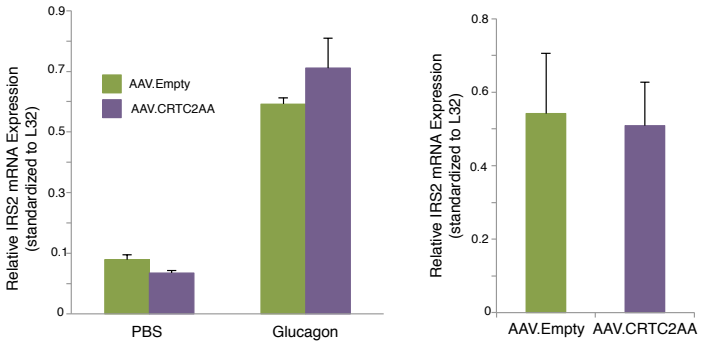


Figure 3.5 Effects caused by long-term chronic CRTC2AA in the liver are partially compensated

a. Average fasting blood glucoses over a long-term adeno-associated viral infection, n=12 per group. **b.** Glucose Tolerance Test on day 95 post injection, n=7 per group. **c.** Representative islet images from mice infected 60 days previously. **d.** Serum insulin levels were quantified from fasted and refed mice at day 70 post infection. **e.** Relative mRNA expression of IRS1 and IRS2 in livers (top), and protein expression levels of IRS2 and hsp90 from livers (bottom) from mice 136 days post injection.



Supplementary Figure 3.1. **a.** Glucose Tolerance Test (1g glucose/kg mouse) performed on day 10 post infection. Mice fasted overnight. **b.** Insulin Tolerance Tests (0.75 units/kg) on day 50 (left) and day 87 (right). n=6-10. Mice fasted for 6 hours.



Supplementary Figure 3.2. Relative *IRS2* expression in cultured hepatocytes (left) and livers of mice infected with AAV.Empty or AAV.CRTC2AA 35 days previously (right). *IRS2* standardized to *L32* expression.

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3.8 Acknowledgements

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Chapter 4 Discussion

4.1 Introduction

In the past two decades, obesity, insulin resistance, and type II diabetes are becoming more and more common worldwide. Now classified as a trend of epidemic proportions, it is becoming clear that there are many biological mechanisms contributing to the development of these metabolic disorders concerning the energy balance of the body.

One of the first proteins to be recognized as a regulator of energy balance was the adipose derived leptin. Leptin contributes to the control of appetite and hunger, promotes energy expenditure by increasing sympathetic tone, stimulating lipolysis and releasing fatty acids from white adipose tissue stores. Mice lacking the leptin gene are obese and hyperphagic, with increased serum insulin levels, islet volume, and hyperglycemia (Bock et al., 2003).

Under lean conditions, leptin enhances catecholamine signaling in adipose tissue through increases in sympathetic nerve activity (Bartness and Song, 2007; Lafontan and Langin, 2009). The triggering of β-adregeneric receptors is important for increases in lipolysis and fatty acid oxidation (Bachman, 2002), and also stimulates cAMP activity in the adipose (Hagiwara et al., 1992; Michael et al., 2000).

Meanwhile, in the liver, cAMP induces the expression of a variety of genes, especially those which promote the translation of enzymes important for gluconeogenesis through phosphorylation of CREB (cAMP Response Element Binding Protein) (Herzig et al., 2001). Simultaneously, cAMP signaling dephosphorylates members of the CREB cofactor family, the CRTCs, which are then released by 14-3-3 proteins in the cytoplasm to translocate to the nucleus and bind with CREB on a CRE site (Dentin et al., 2008; Koo et al., 2005; Luo et al., 2012; Screaton et al., 2004; Wang et al., 2010; 2009).

Hepatic gluconeogenesis plays an important role for the body under stress conditions (fasting), but during obesity, it can lead to disordered energy balance. It is thought that as a system moves towards insulin resistance, insulin signaling in the hepatocytes to turn off gluconeogenesis is less effective. This leads to an increase in hepatic gluconeogenesis that is manifested as hyperglycemia, especially under fasting conditions. This makes hepatic gluconeogenesis an attractive target for diabetes or pre-diabetes treatments (Matsumoto and Accili, 2006).

4.2 Role of CRTCs in Gluconeogenesis

Previous research completed by our lab and other groups indicate that CRTC2 contributes to the gluconeogenic program in mice. The most highly

expressed CRTC family member in liver, much of the research completed on the CRTCs concerns CRTC2 and its contribution to transcription of gluconeogenic genes including *PCK1*, *G6Pase*, and *PGC1α*, a known coactivator of glucose production (Koo et al., 2005). While CRTC2 appears to play a role leading to a straightforward induction of gluconeogenesis, it is in fact a small part of a very complex web controlling glucose production. In addition to responding to the CREB:CRTC2 induced transcription, PGC1α acts in parallel with the forkhead protein, FoxO1, also stimulating gluconeogenic enzyme expression under fasting conditions (Puigserver et al., 2003). Unlike CREB and CRTC2, FoxO1 is regulated by phosphorylation by AKT (which is activated by insulin signaling), by which FoxO1 inhibits glucose production, especially through *G6Pase* induction (Nakae et al., 2001).

It is into this complex web that we look for ways to mediate hepatic gluconeogenesis without destroying the protection it provides under fasting. CRTC2 has provided a promising target, shown by multiple groups that *in vitro*, CRTC2 has an important role in regulating glucose production (Bittinger et al., 2004; Dentin et al., 2007; Koo et al., 2005; Screaton et al., 2004). Additionally, several studies have shown the importance of CRTC2 through knockdown of the cofactor *in vivo* (Dentin et al., 2008; Saberi et al., 2009). With a knockdown of CRTC2, mice are more insulin sensitive and have lower blood glucose levels, showing the importance of CRTC2 in euglycemic control.

Surprisingly, the two different CRTC2$^{-/-}$ mice models show very modest phenotypes. The CRTC2$^{-/-}$ model generated by the Kaestner group lacks exons 4-11, and the group observed minimal effects on glucose concentration under normal chow (Le Lay et al., 2009). It is possible that this deletion of exons 4-11 creates an in-frame peptide containing the CREB binding domain (CBD) fused to the transactivation domain, which has the potential to act at wild type CRTC2 levels on CRE-Luciferase activity (Wang et al., 2010), indicating that there may not be a difference between the knockout and wild type mice. On the other hand, the CRTC2$^{-/-}$ mouse model generated in our lab (lacking the CBD) also shows a moderate phenotype: lower blood glucose levels and increased insulin sensitivity observable under high fat diet conditions (Wang et al., 2010). While CRTC2 is clearly important to glucose production *in vitro*, it is unclear exactly how much of a role CRTC2 plays in hepatic gluconeogenesis.

At this time, our lab was also investigating the role of CRTC3 by generating a CRTC3 knockout mouse. These mice appeared to have a primarily adipose driven phenotype, which linked CRTC3 to energy balance. CRTC3$^{-/-}$ mice had increased catecholamine signaling in the adipose tissue, which led to decreased adipocyte size in WAT, and increased adipocyte number in BAT, with increased amounts of Ucp1 (Uncoupling protein 1) explaining higher core body temperatures. Additionally, the null mice were

protected from obesity under high fat diet conditions, were more insulin sensitive, and had lower blood glucose levels than their wild type littermate controls (Song et al., 2010).

Other studies have recently implicated CRTC3 in a variety of other roles: Using liver cells, Kaplowitz's group found that CRTC3, but not CRTC2, was important for mitochondrial biogenesis in response to stress with induction of *PGC1α* (Than et al., 2011). In macrophages the Cohen group found that CRTC3 regulation by SIKs reprograms macrophages to an anti-inflammatory phenotype (Clark et al., 2012). These studies show the non-metabolic, additional roles that CRTC3 may play in the body.

As the CRTC family shares significant sequence homology and show similar regulation by 14-3-3 proteins (Bittinger et al., 2004; Conkright et al., 2003; Iourgenko et al., 2003; Screaton et al., 2004), we wondered whether there is overlap in function, especially between CRTC2 and CRTC3 which are expressed ubiquitously (CRTC1 being primarily localized to the brain and hypothalamus). I hypothesized that the two cofactors have overlapping roles, especially in gluconeogenesis, and this would explain the modest and conflicting phenotypes of the CRTC2$^{-/-}$ mice, but also help explain the role of CRTC3 *in vivo*.

In this work, we have demonstrated that CRTC3 is active in the liver, in much the same way that CRTC2 is, with nuclear localization and activation

under fasting conditions in hepatocytes. We observed that the knockdown of CRTC3 reduces expression of gluconeogenic genes, indicating that CRTC3 does play a role in the induction of those enzymes. Interestingly, the combined knockdown of CRTC2 and CRTC3 is slightly more potent on gluconeogenic gene expression than either alone. This may indicate that the two family members, with very similar sequences, structure, and binding patterns may have compensatory biological roles in the liver.

Finally, we see that CRTC3 is active *in vivo* during fasting, and that CRTC3 conditional knockout mice specific for the liver have lower fasting blood glucoses and greater glucose clearance than their littermate controls. This indicates to us that at least part of the phenotype we observed in the whole body knockout mice was due in part to CRTC3's role in the liver. Supporting the idea again that CRTC2 and CRTC3 have overlapping roles, the phenotype of these mice is only present after exposure to high fat diet conditions. The requirement for an environmental challenge suggests that CRTC2 may be compensating for the lack of CRTC3.

This work sheds light on the conflicting results that our group and others have seen regarding CRTC2's role in hepatic gluconeogenesis with the overlapping role of CRTC3. As mentioned previously, our work now needs to utilize conditional knockout mice for both CRTC2 and CRTC3 to ensure that effects are specific to liver, and that both family members are knocked out. It

will be interesting to determine the effects of the double knockout on specific gluconeogenic genes and especially to determine if there are particular genes that one cofactor modulates more than its family member. It is foreseeable that as CRTC3 appears to modulate mitochondrial biogenesis through association with the promoter for PGC1α under rotenone stress (Than et al., 2011), that CRTC3 regulates gluconeogenesis more through the transcription of PGC1α which can in turn upregulate other gluconeogenic enzymes.

4.3 Role of CRTC2 in Insulin Resistance

As previously noted, the occurrence of obesity, insulin resistance, hyperglycemia and diabetes worldwide is exploding, and it is essential for scientists to look at biological mechanisms which are related to these issues to identify potential drug targets and ways of preventing the diseases. These issues are often related and are often seen as a steady progression from obesity to type II diabetes. This march is often characterized by the development of either hepatic or whole body insulin resistance and closely followed by increases in fasting blood glucose levels, leading to the diagnosis of diabetes (Barthel and Schmoll, 2003; Kahn et al., 2006; Saltiel, 2012; Saltiel and Kahn, 2001).

However, the reverse progression has not to our knowledge been tested: Is increased hepatic gluconeogenesis sufficient to lead to insulin

resistance and eventually type II diabetes? Because we knew that CRTC2 (and to a lesser degree CRTC3) play important roles in the induction of gluconeogenic enzymes such as PCK1 and G6Pase, we wondered whether a chronically expressed CRTC2 in the liver would increase gluconeogenesis to the point where it caused insulin resistance.

By using an adeno-associated virus with the constitutively active CRTC2 under the control of a promoter specific to liver, we successfully expressed an increased, constitutively active CRTC2 only in the liver. As expected, we observed increased transcription of important gluconeogenic enzymes, in both cultured hepatocytes and mice infected with the constitutively active virus as compared to the control. Helping to explain the increased transcription of these enzymes, we observed more constitutively active, translated CRTC2 expressed through the viral promoter (mimicking dephosphorylated CRTC2) through western blotting. Importantly, in using transgenic mice with a CREB-luciferase activity reporter we were able to detect increased luciferase activity in the livers of the mice injected with AAV.CRTC2AA. This indicated that the expressed CRTC2AA was binding with CREB on the CRE sites in the promoters of genes (specifically the luciferase gene in this instance), and that even under refed or ad libitum conditions CREB target genes were being transcribed.

Surprisingly, given the extremely high luciferase activity, we did not observe extreme differences in fasting or ad libitum blood glucose levels in the two sets of mice. While the mice with AAV.CRTC2AA did have increased fasting blood glucose levels compared to the control mice, they were only slightly elevated. Based on previous experiences with the CRE-Luciferase transgenic mice, and mice injected with an adeno-CRE-Luciferase virus, when the luciferase activity is high in liver, blood glucose levels are also elevated in comparison to their control animal counterparts (Dentin et al., 2008; Liu et al., 2008; Ravnskjaer et al., 2013; Wang et al., 2009; 2010; 2012).

The results from this experiment showed extremely high liver luciferase activity, and yet blood glucose levels do not reflect this activity. One possible answer is that because the luciferase reporter is linked to 8 full CRE sites throughout the tissues of the mouse, that in areas of highest CREB activity, CREB and CRTC2 would bind preferentially to these full CRE sites than half CRE sites in various gluconeogenic genes. It may be that for whatever reason, the active version of CRTC2 stabilizes the binding of CREB to these repeated CRE sites, but it seems odd that the active version would not then stabilize the binding of CREB to other CRE sites as well, increasing blood glucose levels even more than we observed.

For several of the experiments performed, CRE-luciferase transgenic mice were not used, and the wild-type albino C57/BL6 mice were used

instead. We observed similarly increased fasting blood glucose levels in the wild type mice infected with the AAV.CRTC2AA as compared to the CRE-Luciferase mice infected with the same virus. These similar results argue against the idea that CREB binds more effectively to the luciferase linked 8xCRE sites. If this were the case, I would expect the difference in fasting blood glucose levels to be more exaggerated in the non-transgenic mice. Presumably there would be unbound CREB to bind to CRE sites in other genes' promoter regions instead of the luciferase CRE sites.

Obviously, while importantly demonstrating the hypothesis that elevated hepatic gluconeogenesis is able to lead to insulin resistance, this is a complicated *in vivo* system. We observed effects in the mice that correlate with the increased gluconeogenesis, but are also observing phenotypic changes that are not linked to hepatic gluconeogenesis. We have observed that a great number of theses changes can be explained by the increase in hepatic IRS2 expression. Unfortunately, it has been beyond the scope of this thesis to parse apart the timing and mechanisms of these opposing effects. At what point does IRS2 reach sufficient levels to lead to increases in PI3Kinase to phosphorylate AKT? At what levels are these factors and signaling pathways enhanced enough to begin changing the initial insulin resistant phenotype?

Compounding the issue is the central idea that IRS2 acts as a mediator between insulin signaling and gluconeogenesis by its signaling cascade through AKT that then phosphorylates FoxO1. Under basal (fasting) conditions, FoxO1 is localized in the nucleus, where it binds to the promoter for the gluconeogenic enzymes *PGC1α*, *G6Pase* and *PCK1* as well as amino acid catabolism (Haeusler and Accili, 2008; Zhang et al., 2006). Once phosphorylated FoxO1 then translocates to the cytoplasm where it is ubiquinated and degraded, providing a rapid response to hormone-mediated changes (Matsumoto and Accili, 2006). Interestingly, it was also found that nuclear FoxO1 is able to increase IRS2 gene expression, but protein levels are controlled by the mTOR modulation of the pathway (Guo et al., 2006). As the constitutively active CRTC2 is increasing IRS2, we then see effects of this expression throughout the feedback loop with FoxO1 contributing to hepatic gluconeogenesis, glycolytic, and lipogenic gene expression.

Unexpectedly, we measured three-fold induction of triglycerides in the liver without corresponding elevation in blood glucoses. While the exact link between nonalcholoic steatohepatitis (NASH) and insulin resistance is currently under debate, it is clear that the conditions often co-express (Cohen et al., 2011; Farese et al., 2012; Samuel, 2004), eventually leading to hyperglycemia. It is understood that glucose is converted to free fatty acids through lipogenesis during times of nutritional excess, which are eventually

stored as triglycerides, the primary sources of energy storage (Browning and Horton, 2004). It is possible that the observed triglyceride phenotype is due to hepatocytes of the AAV.CRTC2AA mice always producing some glucose via the constitutively active CRTC2, ensuring that lipogenesis is up (as demonstrated by two fold increase in SREBP1c transcriptional expression). This would lead to increased triglyceride storage, but also perhaps to decreased fatty acid oxidation, allowing us to observe hepatic steatosis. It would be interesting in the future to determine if tissues in the body correspond with oxidative or glycolytic reactions. By testing liver, WAT, muscles and muscle fibers, and the heart, we may be able to determine if constitutively active CRTC2 changes the oxidative metabolism of the animal's system (Blanchet et al., 2011).

This raises the question if the increase in IRS2 is sufficient to amplify insulin signaling to maintain fair glycemic control. In a recent study linking VEGF inhibitors with induced hypoxia-inducible factor-2α stability to increasing insulin sensitivity, researchers found that liver IRS2 was necessary and sufficient to mediate Hif-2α effects on glucose and insulin sensitivity (Wei et al., 2013). The mice with constitutively active HIF-2α adenoviral infection have very similar phenotypes to those with Ad.IRS2 and have much greater glucose uptake than control counterparts—lowering glucose levels in *db/db* mice to

almost normal euglycemic levels. This argues that an overexpression of IRS2 is sufficient to lower blood glucoses and increase glucose uptake.

One indication that IRS2 may be sufficient to maintain normoglycemic control is the preliminary results from a cohort of AAV.CRTC2AA and control mice that had been subjected to a western style diet. After 18 days of viral exposure, the mice were placed on to the 60% HFD and monitored. Surprisingly, these mice did not have significant differences in blood glucose levels and in fact appeared to have a trend towards lower body weights. Luciferase activity was still 30 fold greater in the AAV.CRTC2AA mice under ad libitum conditions compared to control mice, supporting the conclusion that AAV.CRTC2AA was still active. Could IRS2 expression be providing a protective phenotype to these mice?

Unfortunately the preliminary results are from a very small cohort of mice, and to begin to answer this question, several additional experiments should be performed in addition to replication with a larger cohort of mice. First, there could be several cohorts that are introduced to a HFD at different time points. If IRS2 is truly protective, mice with a longer exposure to the virus, and later western diet start date should have similar blood glucoses as compared to their controls, and those who begin earlier should have exaggerated differences from their controls. Secondly, while it may be difficult due to the use of the adeno-associated virus, it would be interesting to look at

phenotypes of mice with knockdown of IRS2. I think that the most efficient way to deal with the difficulty of already having a virus active in the system would be to use anti-sense oligo technology, which knocks down specific proteins in liver and WAT (Erion et al., 2009). If successful at knocking down but still leaving some IRS2 active in the mice, we would be able to determine if the compensation we have observed is due to IRS2, or if there is another factor which the activation of CRTC2 in the liver induces.

On the other hand, while IRS2 is well known to act through the AKT/FoxO1 insulin signaling pathway, a recent paper found that a knockout of both AKT and FoxO1 in the liver do not impact hepatic metabolic signaling through insulin or nutrient levels (Lu et al., 2012). The authors found that when Akt1 and Akt2 were deleted in the liver, mice were glucose intolerant, insulin resistant, and did not respond normally to fed conditions. Importantly, these effects normalized when FoxO1 was also deleted from the liver, suggesting that the major role of hepatic Akt is to modify FoxO1 activity. The authors suggest that insulin's regulation in hepatic gluconeogenesis is not cell-autonomous, and there is a parallel pathway that insulin signals to the liver through a second tissue, such as the CNS (Lu et al., 2012). If this suggestion is correct, some of the conflicting data we are observing may be due to increased IRS2 and insulin's parallel pathway through a secondary tissue.

One of the clearest effects of the constitutively active CRTC2 in the liver was the increase in islet size in the pancreas. This is possibly due to hepatic gluconeogenic upregulation in blood glucose levels causing islets to expand to compensate for the hyperglycemia, but serum insulin of the animals infected with AAV.CRTC2AA was never found to be higher than the control mice. Again, determining the timing at which changes are occurring in the islets was not possible for this thesis, but determining β-cell mass is essential to confirm the appearance of larger islets. It would be interesting to determine if and when the β-cells are undergoing proliferation by quantifying incorporated BrdU in β-cells. Similarly, determining rates of apoptosis, using Tunel or Caspase-3 staining, would be useful in identifying if there is a mix of proliferating and apoptotic β-cells, or solely proliferation.

There have recently been several studies identifying factors from livers that promote β-cell proliferation. Ouaamari et al demonstrate that there is a factor from the liver in liver-specific IRS knockout mice that induces β-cell proliferation unconnected to glucose and insulin levels (Ouaamari et al., 2013). Other groups have specifically identified a factor from the gene Angiopoietin-like protein *AngptL8* (also known as TD26, RIFL, Lipasin, and Betatrophin) that in mice is expressed in hepatocytes and adipose tissue, and appears to have an important role in lipid metabolism by increasing serum triglycerides and at the same time reducing clearance by LPL inhibition (Quagliarini et al.,

2012; Ren et al., 2012; Zhang, 2012). Interestingly, this protein is elevated when mice are treated with a peptide that binds to IRS and antagonizes insulin signaling (inducing insulin resistance) and correlates with a dramatic proliferation in β-cells (Yi et al., 2013). Further studies on knockout mice of *AngptL8* do not show impaired glucose metabolism or insulin sensitivity, but rather only as a mediator of trafficking fatty acids to the adipose tissue (Wang et al., 2013). Additionally, analysis through the UCSC genome browser does not indicate that *AngptL8* contains a CRE site in its promoter, or that there is strong binding of CRTC2 under glucagon stimulation. This would indicate that AngptL8 is not responsible for the increased islet sizes in this experiment, but leaves an interesting question about what might be the factor enhancing β-cell growth. By performing a RNA-Seq experiment on samples from livers from mice infected with AAV.CRTC2AA and the AAV.Empty virus, we may be able to identify some secretory proteins that are upregulated by the constitutively active CRTC2 that in turn cause β-cell proliferation.

4.4 Closing Remarks

Diabetes and its associated issues are health concerns that contribute to further complications such as hypertension, cardiovascular disease, kidney disease, neuropathy, Alzheimer's disease, and cancer, resulting in loss of productivity and life throughout the world (Folch et al., 2013; Thorpe and

Philyaw, 2012). As type II diabetes is being diagnosed in younger populations, the risk of these complications will only increase in the years to come (Sherwin and Jastreboff, 2012). It is essential that we identify the molecular mechanisms that are leading to the development of insulin resistance, hyperglycemia, and diabetes, and one such area of study is that of hepatic gluconeogenesis and its relationship with the cAMP/CREB pathway. It is my hope that the research presented here implicating an additional factor in hepatic gluconeogenesis (CRTC3) and the idea that increased hepatic gluconeogenesis itself may lead to insulin resistance will lead to other discoveries to help treat and prevent type II diabetes.

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