THE ROLE OF CREB IN THE LIVER AND ADIPOSE TISSUE

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DEDICATION

In loving memory of my mom,

Who always had answers to all my questions, for teaching me ‘joie de vivre’

To my dad,

A true family man, to whom I’ll be forever grateful in his unwavering love & support

To Yunna,

Sister, best friend, and my partner in crime, since 1987

To Gayeon

My baby sister for always bringing out the best in me

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ABSTRACT

THE ROLE OF CREB IN THE LIVER AND ADIPOSE TISSUE

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Excessive hepatic glucose production is a hallmark of insulin resistance in type 2 diabetes. The cAMP-responsive transcription factor cAMP-responsive element binding protein (CREB), thought to be a key activator of the hepatic gluconeogenic gene regulation program, has been suggested as therapeutic target to reduce glucose output by the liver. Here, I test directly the requirement for hepatocytic CREB for the maintenance of glucose homeostasis utilizing Cre-loxP for conditional, cell-type specific gene ablation. Strikingly, I find no difference in fed and fasted glucose levels, or glucose, insulin, and glucagon tolerance in mice fed normal chow or a high-fat diet in hepatocyte *Creb*-null mice compared to controls. In addition, mRNA levels of liver specific genes, including several CREB target genes involved in gluconeogenesis, were not affected by CREB deficiency in the liver. In conclusion, my data indicate that CREB has no non-redundant functions in hepatic glucose metabolism, and is therefore not likely to be a useful target for the development of anti-diabetic drugs.

Furthermore, lipolysis and thermogenesis are regulated by catecholamines signaling via β-adrenergic receptors in adipose tissue and a role for the CREB in

adipocyte function had been suggest by previous studies. However the role of CREB in adipose tissue had never been evaluated *in vivo*. Here, I test directly the requirement for adipocyte CREB for lipolysis and thermogenesis using the Cre-*loxP* system for adipocyte-specific ablation of *Creb* in mice. Loss of adipocytic *Creb* lead to a moderate decrease in fasting-induced lipolysis in adipose tissue. Strikingly, the adipocytic transcriptome was not globally affected by ablation of *Creb*. In addition, cold temperature and cAMP signaling-induced thermogenesis and white-to-beige adipocyte conversion were not changed in adipocyte specific *Creb*-null mice compared to controls. In conclusion, my data indicate that CREB has no non-redundant functions in thermogenesis, but contributes to the regulation of fasting-induced lipolysis. The underlying mechanism of reduced fasting-induced lipolysis in adipocytic CREB-deficient mice will need to be explored further.

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CHAPTER 1: General Introduction

1.1 Fuel Metabolism and Metabolic Organs

Keeping a tight balance between energy intake and expenditure is critical for all living organisms. Disturbances in energy homeostasis lead to metabolic disorders such as obesity, metabolic syndrome, and diabetes. Increase in the prevalence of obesity and diabetes is a significant public health concern, and its patient population is projected to grow faster than ever. In the U.S., currently more than two-thirds of adults are considered obese or overweight (NIDDK), and one out of every 11 people have diabetes (CDC).

Mammals have evolved to efficiently convert food into energy that can be utilized by individual cells, and to store excess energy for future use. In a healthy individual, each digestive, endocrine, and exocrine organ serves a unique role in metabolism, and the finely tuned feedback pathways between the organs ensures that blood glucose levels are maintained near 4.5mM, despite constant fluctuations in dietary intake and energy expenditure.

1.1.1 Small Intestine

During a meal, dietary nutrients are enzymatically digested and absorbed in the small intestine. Some of digestive enzymes, such as disaccharidases, are secreted from intestinal epithelial cells, while others, such as trypsin, elastase and lipases, are produced in other organs, such as the pancreas, and transported to the small intestine. The liver also plays an important role in the digestive process by producing bile, which solubilizes

triglycerides in the diet. The lumen of the small intestine is lined with small finger-like projections called villi, which increase the surface area available for absorption. Villi are covered with a single layer of epithelial cells; most of which are absorptive cells, with some goblet cells and enteroendocrine cells secreting mucus and gastrointestinal hormones, respectively. At the base of each villi, there are glandular crypts that contain replicating stem cells and paneth cells.

Dietary proteins are broken down into small peptides and amino acids by proteolytic enzymes for absorption. Since only carbohydrate monomers can be absorbed by the intestinal epithelium, complex carbohydrates are broken down into simple sugars, such as glucose. Dietary lipids are degraded into fatty acids and glycerol by lipase, and subsequently are absorbed by villi.

1.1.2 The Pancreas

The pancreas serves as both an endocrine and exocrine organ. The islets of Langerhans are the endocrine part of the pancreas that secretes hormones, but make up only 1-2% of pancreas mass. There are four types of cells in the islets of an adult pancreas: α cells, β cells, δ cells, and polypeptide cells. During fasting, glucagon secreted from α cells binds glucagon receptors on the surface of hepatocytes and promote hepatic glucose production. In addition, glucagon increases triglyceride mobilization by stimulating lipolysis in adipose tissue. Glucagon signaling also increases fatty acid oxidation, allowing the use of lipid as an energy source, in all tissues except in the central

nervous system. When blood glucose levels rise after a meal, β cells secrete insulin, which promotes uptake and storage of glucose by the liver, skeletal muscles, and adipose tissue. Also insulin increases fatty acid and triglyceride synthesis in the liver, and they are packaged into very low-density lipoproteins and transported to adipose tissue. In the adipose tissue very low-density lipoproteins are metabolized, and lipids are stored in adipocytes. Opposing actions of glucagon and insulin keep blood glucose levels within a constant range. Somatostatin secreted from δ cells is responsible for fine-tuning of hormone secretion by α and β cells. Polypeptide cells secrete pancreatic polypeptides that regulate pancreatic endocrine and exocrine secretions.

The exocrine pancreas makes up ≥90% of the total pancreas. It is composed of acinar cells connected to small ducts that drain into the pancreatic ductal tree, which in turn drain into the main pancreatic duct. Cells in the exocrine pancreas produce digestive enzymes, enzyme precursors (zymogens), and bicarbonate, which make up pancreatic juice. Pancreatic juice collected in pancreatic duct is released into the duodenum, the first section of the small intestine.

1.1.3 The Liver

The liver is a central metabolic organ, and is responsible for processing and distributing nutrients to extrahepatic tissues, including the brain. It also is important in detoxification and synthesis of serum proteins. The liver is the first organ that receives the nutrients absorbed by small intestine during a meal. Hepatocytes make up about 80%

of the total liver mass, and are involved in carbohydrate metabolism, protein metabolism and synthesis, and lipid processing and distribution.

The liver adapts to changes in blood glucose levels to maintain glucose homeostasis as depicted in Figure 1.1. Upon receiving the insulin signal, excess glucose received during a meal is converted into glycogen via glycogenesis, and stored for later use. When blood glucose levels drop, i.e. during fasting, glucagon activates glycogenolysis in hepatocytes, and glucose is released into bloodstream to be utilized by other cells in the body. When the glycogen stores are depleted, the liver, and also skeletal muscles, activates gluconeogenesis to produce glucose from non-carbohydrate precursors, such as lactate, glycerol, alanine and glutamate, to meet glucose demand.

Diet-derived lipids are packaged into chylomicrons by small intestinal epithelial cells and transported to the liver. The liver processes fatty acids into phospholipids and triacylglycerol to form very low-density lipoproteins, to be transported to adipose tissue for storage. Within hepatocytes, fatty acids can also serve as substrate for oxidation to produce energy, cholesterol, and ketone bodies. Furthermore, when excess carbohydrate is available, the liver converts carbohydrate into fat by de novo lipogenesis.

Amino acids taken up by the liver can serve as precursors for proteins, hormone and nucleotide synthesis. During fasting, muscle protein is broken down and amino acids are released into circulation, and then taken up by the liver. Some of these amino acids are used as substrates to produce precursors for gluconeogenesis, while others are use in ketogenesis.

1.1.4 Adipose Tissue

White adipose tissue is mainly composed of white adipocytes whose primary role is to store lipids. During a meal, glucose can be converted into fatty acids, and then to triacylglycerides in adipose tissue for storage. Free fatty acids enter adipocytes, and with glycerol, are assembled into triacylglycerides. When there is a demand for more energy, epinephrine stimulates triacylglyceride hydrolysis and fatty acids are released into the blood stream.

In rodents and human infants, although to a lesser extent in human adults, non-shivering thermogenesis occurs in specialized adipose tissue called 'brown adipose tissue'. Brown adipocytes have numerous mitochondria and smaller lipid droplets compared to the large, single lipid droplet as in white adipocytes. Heat is generated via uncoupling the mitochondrial respiratory chain through expression of high levels of uncoupling proteins.

1.2 cAMP Response Element-Binding Protein (CREB)

1.2.1 cAMP Signaling Pathway

cAMP was the first "second messenger" discovered as a mediator of hormone induced glucose production in the liver (Sutherland and Robison 1969; Montminy and Bilezikjian 1987) . When ligands bind to cell-surface receptors that are coupled to

heterotrimeric GTP binding proteins (G-proteins), the Gα subunit is activated and dissociates from GβGγ. There are several different isoforms of the Gα subunit that can interact with different effector molecules to carry out biological response to specific ligand binding. For instance, upon epinephrine or glucagon signaling, activated $G\alpha_s$ can stimulate adenylyl cyclase, an enzyme that converts ATP into cAMP.

Protein kinase A (PKA) is a cAMP-dependent protein kinase, whose activation is regulated by intracellular cAMP levels. In the basal state, PKA is composed of two regulatory subunits and two catalytic subunits. Binding of cAMP to regulatory subunits causes release of catalytic subunits, allowing their translocation into the nucleus. Activated catalytic subunits of PKA can regulate activity of downstream proteins by phosphorylating Ser or Thr residues.

1.2.2 CREB mediates cAMP effects on Transcriptional Regulation

Nearly three decades ago, CREB was discovered as a nuclear protein that binds to the cAMP response element (CRE), a conserved promoter element, located in the somatostatin gene, and activates its expression in response to increased intracellular cAMP levels (Montminy and Bilezikjian 1987). CREs can be either full palindromic (TGACGTCA) or half-site (TGACG or CGTCA) sequences, and are often located in proximal promoter regions (Zhang et al. 2005; Everett et al. 2013). CREB is a ubiquitously expressed DNA binding transcription factor that forms a dimer, and structurally belongs to the basic leucine zipper protein family (Hoeffler et al. 1988).

There are other family members of CREB, which in addition to CREB are the activating transcription factor 1 (ATF1) and cAMP- responsive element modulator (CREM), and these two proteins can also mediate transcriptional response to cAMP signaling. CREB family members share a conserved dimerization domain and kinase-inducible domain, and some functional redundancy exists among them (Hummler et al. 1994). The G protein-coupled receptor (GPCR) signaling pathway leading to activation of CREB is depicted in Figure 1.2. Upon glucagon or epinephrine signaling, activated PKA translocates into the nucleus, and phosphorylates CREB at Ser133 (Gonzalez and Montminy 1989). This leads to activation of CREB, and promotes target gene expression by recruitment of transcriptional co-activators, such as CREB-binding protein (CBP) and p300, and RNA polymerase II complexes to promoters containing CREs. CBP and p300 have intrinsic histone acetyltransferase activity, which leads to loosening of chromatin structure, thus increasing access for RNA polymerase, and results in increased expression of downstream genes (Ogryzko et al. 1996).

More recently, the CREB-regulated transcription coactivator (CRTC), also known as transducer of regulated CREB activity (TORC), family of cofactors was discovered (Iourgenko et al. 2003). Although DNA binding by CREB does not depend on recruitment of CRTC, presence of CRTC has been shown to enhance RNA polymerase II interaction (Conkright et al. 2003). In the absence of a cAMP signal, cytoplasmic CRTC is sequestered by 14-3-3 proteins, due to phosphorylation at Ser171 by Ser/Thr kinase 2 (SIK2) (Screaton et al. 2004). Elevation of cytoplasmic cAMP levels leads to inhibition of SIK2 and dephosphorylation of CRTC, ultimately resulting in its dissociation from 14-

3-3 proteins and translocation into the nucleus for interaction with CREB (Screaton et al. 2004).

1.3 CREB in Glucose Homeostasis

1.3.1 Hepatocytic CREB as the Central Regulator of Gluconeogenesis

When blood glucose levels are low, glucagon secretion leads to inhibition of glycogen producing glycogen synthase and activation of glycogen phosphorylase, a rate-limiting enzyme involved in breakdown of glycogen into glucose. During prolonged fasting, starvation, or following intense exercise, glucose is also generated via gluconeogenesis. Gluconeogenesis is essentially a reversal of glycolysis (Figure 1.3). Three endergonic steps in glycolysis are catalyzed by three different key enzymes in gluconeogenesis: phosphoenolpyruvate carboxykinase (PEPCK), fructose 1, 6-bisphosphatase (FBPase), and glucose 6-phosphatase (G6Pase) (Figure 1.3).

Lactate is a major source of carbon atoms for gluconeogenesis. Lactate produced by glycolysis in non-hepatic tissues is transported to the liver, and lactate dehydrogenase converts lactate into pyruvate, the first designated substrate of gluconeogenesis (Figure 1.3). All amino acids, except leucine and lysine, can be converted into glucose. During hypoglycemia, muscle proteins are broken down into amino acids and transported to the liver. Some amino acids are catabolized into pyruvate, oxaloacetate or precursors of these.

Glycerol from the breakdown of triacylglycerol in adipose also can be used for gluconeogenesis.

Previous studies suggested CREB as the master regulator of gluconeogenesis in the liver. CREs are located in proximal promoter regions of key gluconeogenic genes, suggesting direct transcriptional regulation by CREB (Zhang et al. 2005; Everett et al. 2013). The model suggests that during short-term fasting, glucagon binds to GPCR on the surface of hepatocytes, and activated CREB can induce transcription of gluconeogenic genes, such as *Pepck* and *G6pase* (Montoliu et al. 1994; Herzig et al. 2001; Thiel et al. 2005) (Figure 1.4). CREB also increases expression of peroxisome proliferator-activated receptor- γ (PPAR-γ) coactivator-1α (*Pgc-1α*), which can also interact with other co-activators at the transcription start site located in key gluconeogenic genes, further activating expression of the gluconeogenic programing during prolonged fasting (Herzig et al. 2001) (Figure 1.4).

1.3.2 CREB as a Potential Therapeutic Target for Type 2 Diabetes Mellitus (T2DM)

Type 2 diabetes mellitus is a chronic metabolic disorder marked by hyperglycemia, endocrine pancreatic dysfunction, and insulin resistance. T2DM accounts for about 90% of all diagnosed cases of diabetes and is a major public health concern as the fastest growing disease in the world. According to the CDC, there are currently 25.8 million Americans with diabetes, comprising 8.3% of the U.S. population. In 2010, diabetes was the 7th leading cause of death in the U.S. (CDC). This is likely to be an

underestimate, since failure to maintain glucose homeostasis contributes to other detrimental complications such as cardiovascular disease, blindness, hepatic steatosis, and kidney failure. Elevated hepatic gluconeogenesis, caused by increased glucagon levels, decreased insulin secretion, and insulin resistance, is the major contributor to fasting hyperglycemia in type 2 diabetic patients (Magnusson et al. 1992; Gastaldelli et al. 2000). There are three different types of drugs for T2DM: ones that help pancreatic insulin secretion, ones enhance insulin sensitivity in peripheral tissue, and others that limit excess glucose output from the liver. Although many advances have been made in type 2 diabetes treatments, there are still safety and tolerability issues. With the expected increase in the diabetic patient population and cost associated with the disease, there is a strong need for development of a new class of drugs.

As the major mediator of cAMP effects on transcription of gluconeogenic genes in the liver, CREB has been suggested as a potential drug target to reduce hyperglycemia and hepatic insulin resistance in T2DM patients. To understand the effect of CREB inhibition on glucose homeostasis, a dominant-negative inhibitor of CREB, A-CREB, was utilized in many studies. A-CREB contains an acidic amphipathic protein sequence that replaces the CREB basic region connected to the N-terminus of the dimerization domain (Ahn et al. 1998). A-CREB forms a heterodimer with endogenous CREB protein and prevents CREB from binding DNA. Expression of A-CREB via systemic injection of adenovirus expressing A-CREB leads to a decrease in fasting glucose levels compared to that of control, accompanied by a 4-fold decrease in expression levels of *Pepck* and *G6pase* (Herzig et al. 2001). Also, overexpression of A-CREB was able to lower blood

glucose levels of *db*/*db* mice to a normal range by lowering *Pepck* and *Pgc1α* mRNA levels (Herzig et al. 2001). In a separate study, in order to induce tissue-specific reduction of *Creb* expression, antisense oligonucleotide (ASO) was also utilized. Similar to the A-CREB study, improvements in fasting glucose levels and lower gluconeogenic gene expression levels were observed when hepatic and adipocytic *Creb* expression levels were reduced by ASO in the rodent models of insulin resistance and obesity (Erion et al. 2009). Together, these results suggest CREB as a novel therapeutic target to lowering blood glucose levels in T2DM patients by inhibiting excess gluconeogenesis in the liver.

However, despite both approaches leading to reduction of blood glucose levels, they exhibited a contradicting hepatic lipid phenotype. Inhibition of CREB by A-CREB lead to increased expression levels of lipogenic genes, such as *Cd36*, *Fas*, *Acl*, and *Srebp*, with development of a fatty liver phenotype (Herzig et al. 2003). On the other hand, Erion and colleagues found that CREB ASO alleviated fatty liver by reducing hepatic triacylglycerol content and lowering lipogenic gene expression levels (Erion et al. 2009). Fatty liver is commonly observed in type 2 diabetes patients, and although conditions itself can be reversible; it can develop into detrimental conditions such as liver cirrhosis and liver cancer, if left untreated. Thus this disagreement in the fatty liver phenotype poses a serious threat in developing drugs against CREB.

Contradicting phenotypes from these studies could be explained in part by the limitations of approaches taken. In the A-CREB study, expression of A-CREB was under the control of the cytomegalovirus (CMV) promoter. Thus it is difficult to determine if the lower blood glucose levels seen in A-CREB expressing mice were exclusively due to

the inhibition of CREB activity in the liver. Furthermore, there is a possibility that some observed phenotypes resulted from A-CREB interacting with other basic leucine zipper proteins, including other CREB family members (Ahn et al. 1998). Similarly, CREB-ASO treatment affected *Creb* mRNA levels in both liver and white adipose tissue, which makes it hard to determine if inhibition of hepatic, adipocytic CREB or combination of both is required for the improvement of insulin sensitivity. In addition, there are potential off-target effects by ASO. Therefore, there is a need for proper reevaluation of the current understanding of CREB's role in glucose homeostasis, to determine whether it could be the next therapeutic target for T2DM.

1.4 Adipose Tissue Biology and Function

1.4.1 White and Brown Adipocytes

Despite their initial origin from pluripotent mesenchymal stem cells (MSCs), white and brown adipocytes come from different precursor cells. Indeed, brown adipocytes originate from myogenic factor 5 (myf5) and paired-box 7 (pax7) positive precursors, which also give rise to myocytes (Hasty et al. 1993; Seale et al. 2008). The differences between white and brown adipocytes are highlighted further by functions they serve. As mentioned earlier, the main role of white adipose tissue (WAT) is to store excess energy in a form of lipid droplets. Storage and utilization of lipids allows adaptation to different energy needs throughout the day. Furthermore, WAT acts as an

endocrine organ, producing and secreting protein hormones. These adipose-derived hormones, adipokines, can influence inflammation (Bruun et al. 2001) and energy metabolism (Hu et al. 1996; Halaas et al. 1997; Steppan et al. 2001). Unlike WAT, brown adipose tissue (BAT) mainly functions in thermogenesis. White and brown adipocytes have distinctive morphology that best serves their function. White adipocytes are mostly made of a large single lipid droplet with its nucleus pushed to periphery. In contrast, brown adipocytes contains multiple, small lipid droplets and numerous mitochondria, which gives it a brown hue.

1.4.2 Lipolysis

Upon increased energy demand, triacylglycerol is catabolized to generate non-esterified fatty acids (NEFAs) via lipolysis from adipocytes. Lipolysis can be induced by several hormones; such as glucagon (Blecher et al. 1969; Exton et al. 1972), epinephrine (Blecher et al. 1969; Exton et al. 1972), norepinephrine, growth hormone (Dietz and Schwartz 1991), adrenocorticotropic hormone (Blecher et al. 1969), and thyroid-stimulating hormone (Goodman and Bray 1966; Gagnon et al. 2010). Binding of these hormones to GPCRs leads to activation of the cAMP signaling pathway, and activation of lipolytic enzymes. During lipolysis, triacylglycerol is initially hydrolyzed into diacylglycerols and NEFAs by adipose triglyceride lipase (ATGL) (Zimmermann et al. 2004). Hormone-sensitive lipase (HSL) is the rate-limiting enzyme of diacylglycerol hydrolysis that produces monoacylglycerol and NEFAs (Osuga et al. 2000; Haemmerle et al. 2002). HSL is also capable of hydrolyzing other acylesters like triacylglycerol,

monoacylglycerol, cholesteryl esters, and retinyl esters. As the name suggests, lipolytic hormone signaling leads to phosphorylation of HSL by PKA, and activated HSL translocates to the lipid droplet (Clifford et al. 2000). Lastly, monoglyceride lipase (MGL) breaks down monoacylglycerol into glycerol and NEFAs (Karlsson et al. 1997). NEFAs and glycerol produced during lipolysis are delivered to peripheral tissues for energy production.

1.4.3 Thermogenesis and Energy Expenditure

BAT is a major site of non-shivering thermogenesis in many mammals. Uncoupling protein-1 (UCP1) in the inner membrane of brown adipocyte mitochondria decreases the proton gradient, and uncouples oxidative phosphorylation, leading to heat generation. Thermogenesis is regulated mainly by the sympathetic nervous system through norepinephrine signaling (Landsberg et al. 1984). B_3-adrenergic receptors are mainly found in white and brown adipose tissue and regulate lipolysis and thermogenesis. Norepinephrine binds B_3-adrenergic receptors on brown adipocytes, and the cAMP signaling is induced downstream (Cannon et al. 1996). A rise in intracellular levels activates PKA, which phosphorylates a series of target enzymes that stimulates expression and activation of UCP1. PGC1α is coactivator of adaptive thermogenesis, and enhance transcription of *Ucp1* (Puigserver et al. 1998; Wu et al. 1999). Also, lipolysis is induced by activation of HSL and deactivation of perillipin that releases glycerol and free fatty acids from lipid droplets. Fatty acids serve as both activators of UCP1 and substrates for oxidative phosphorylation (Figure 1.5).

It has been demonstrated that thermogenesis can be also activated by food intake as well as cold exposure, linking energy expenditure and thermogenesis (Rothwell and Stock 1979; Rothwell et al. 1982). Thus, there is interest in BAT as a potential anti-obesity target, since thermogenesis burns and 'wastes' large amounts of lipids and glucose (Lockie et al. 2013; Mund and Frishman 2013; Chechi et al. 2014). Defective thermogenesis is shown to be associated with obesity in rodent models of genetic obesity and T2DM, due to deficits in energy expenditure (Trayhurn and James 1978; Trayhurn 1979; Hogan and Himms-Hagen 1980; Trayhurn and Fuller 1980). In humans, presence of BAT in adults has only been recognized recently (Hany et al. 2002; Yeung et al. 2003). Some studies have indicated an inverse correlation between body mass index and BAT activity (Cypess et al. 2009; Saito et al. 2009; van Marken Lichtenbelt et al. 2009). Therefore BAT activity can potentially contribute to energy expenditure in adult humans.

1.4.4 Beige Adipocytes

More recently, another class of adipocytes called beige (brite) adipocyte was discovered. Beige adipocytes are subset of traditional white adipocytes transformed to express UCP1 after cold stimulation or chronic treatment with agonists that elevate intracellular cAMP levels (Young et al. 1984; Cousin et al. 1992; Ghorbani et al. 1997; Ghorbani and Himms-Hagen 1997; Petrovic et al. 2010). In rodents, beige adipocytes mostly arise from the inguinal WAT (Vitali et al. 2012). Although these beige adipocytes are similar to white adipocytes in the basal state with low expression of thermogenic gene program, cAMP stimulation increases expression of thermogenic genes including *Ucp1* at

levels similar to those in brown adipocytes, suggesting thermogenic ability (Wu et al. 2012). Molecular characteristics of human brown adipocytes are similar to that of murine beige adipocytes rather than brown adipocytes (Wu et al. 2006). Thus simulants that induce 'browning' of white adipose tissue may be useful in treating obesity and diabetes in humans.

1.4.5 CREB in Adipose Tissue

In previous study, obese mice expressing a dominant-negative CREB in adipocytes displayed improved insulin sensitivity and were protected from adipose tissue inflammation (Qi et al. 2009). In addition, CREB-regulated transcription coactivator 3 (CRTC3) was shown to promote obesity by attenuating β-adrenergic receptor signaling in adipose tissue (Song et al. 2010). These studies suggest that adipocytic CREB plays an important role in energy homeostasis.

In brown adipocytes, CREB can be activated by both classical β_3- adrenergic/PKA and α_1-adrenergic/protein kinase C pathway (Chaudhry and Granneman 1999; Thonberg et al. 2002). Thermogenesis is under positive adrenergic control and it is possible for CREB to mediate expression of key genes involved in thermogenesis, including *Ucp1*. Proximal promoter regions of the mouse *Ucp1* gene contain half-CREs along with other cis-regulatory elements such as peroxisome proliferator-activated receptor response elements (PPREs) and thyroid response elements (TREs) (Kozak et al. 1994; Rim and Kozak 2002). Cold-stimulated UCP1 levels in beige adipocytes were higher in the A/J

strain of mice compared to that of the obesity-prone C57BL/6J strain, and coincided with higher activation of CREB (Xue et al. 2005). Furthermore, CREB was found to form a heterodimer with CCAAT/enhancer-binding protein beta (C/EBPβ), and regulate cAMP-induced expression of *Pgc1α* in adipocytes which also plays a role in cold-induced thermogenesis by regulating *Ucp1* expression (Karamanlidis et al. 2007; Karamitri et al. 2009). Despite these studies implying potential regulation of thermogenesis by CREB, the role of CREB in adipocytes has never been investigated directly.

1.5 Hypothesis and Objectives

The liver is the central regulator of glucose homeostasis. It quickly adapts to changes in energy demand of the body, storing excess glucose during feeding and producing glucose via glycogenolysis and gluconeogenesis during fasting and intense physical activity. In T2DM patients, increased hepatic glucose output is the major cause of hyperglycemia. CREB has been established as the central regulator of gluconeogenesis (Herzig et al. 2001; Erion et al. 2009), implying it as an attractive therapeutic target to reduce blood glucose levels. Based on previous studies, loss of hepatic CREB is expected to reduce circulating glucose levels due to decreased gluconeogenesis. To address this hypothesis, in Chapter 3, I evaluate glucose homeostasis and insulin sensitivity in mice with hepatocytic ablation of CREB, and reveal that CREB has no non-redundant function in hepatic regulation of glucose homeostasis, which disproves conventional understanding of CREB's role as the master regulator gluconeogenesis (Herzig et al.

2001; Erion et al. 2009). This work provides definitive understanding of the role of CREB in the liver, and helps determining whether CREB is a useful therapeutic target.

Adipose tissue is a dynamic organ actively involved in regulation of energy expenditure. Lipolysis and thermogenesis are under the control of adrenergic signaling coupled to the cAMP-signaling pathway. As one of the major effectors of cAMP on transcriptional regulation, I hypothesize that CREB is involved in activation of thermogenesis and white-to-beige adipocyte transformation via regulation of *Ucp1* expression. In Chapter 4, I investigate the role of CREB in adipose tissue in regulation of thermogenesis and beige adipocyte formation. This work investigates CREB in thermogenesis, which has been stipulated but never tested before.

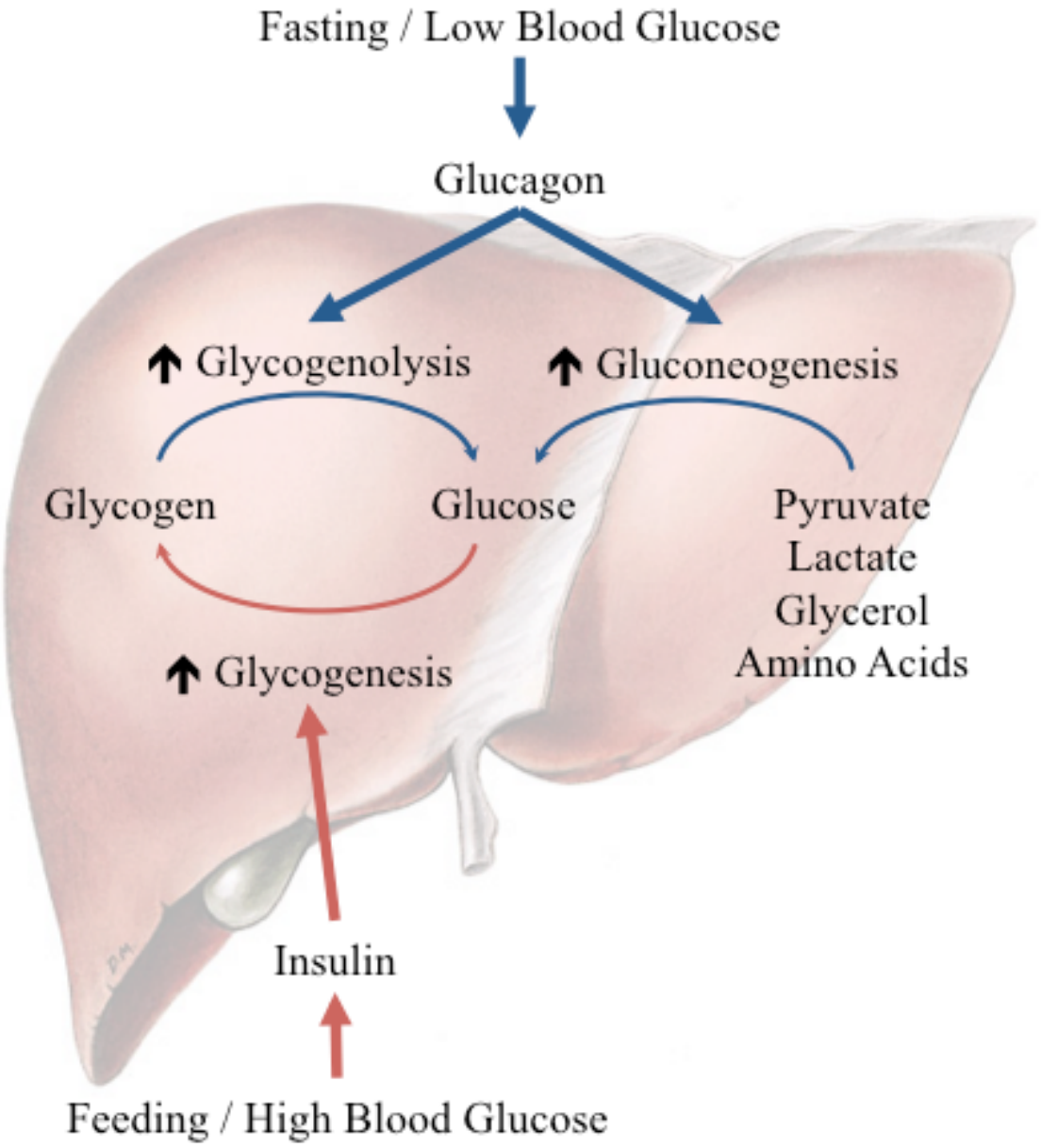

Figure 1.1: Hepatic Glucose Metabolism

The liver responds to glucagon and insulin signaling and adapts to different blood glucose levels. Excess glucose is stored in the liver in the form of glycogen. When needed, glucose can be released from glycogen stores or produced from non-carbohydrate precursor.

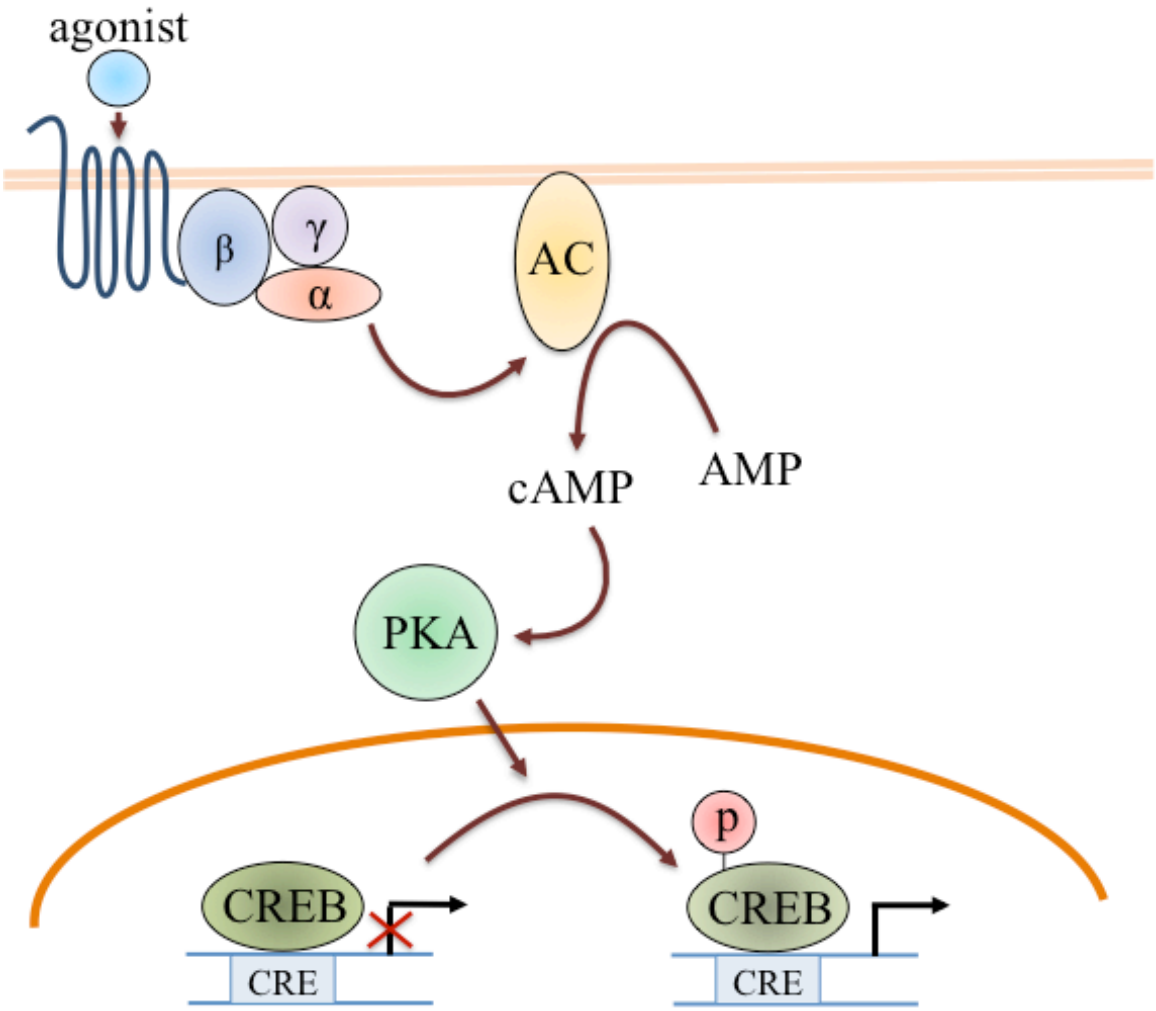

Figure 1.2: Activation of CREB via cAMP signaling

In a basal state, inactive CREB is bound to CREs near the transcription start site of target genes. Binding of agonist to GPCRs lead to increase in intracellular cAMP levels. cAMPs can activate PKA, and its catalytic subunits translocate to the nucleus. CREB is activated when PKA phosphorylates Ser133 on CREB, inducing transcription.

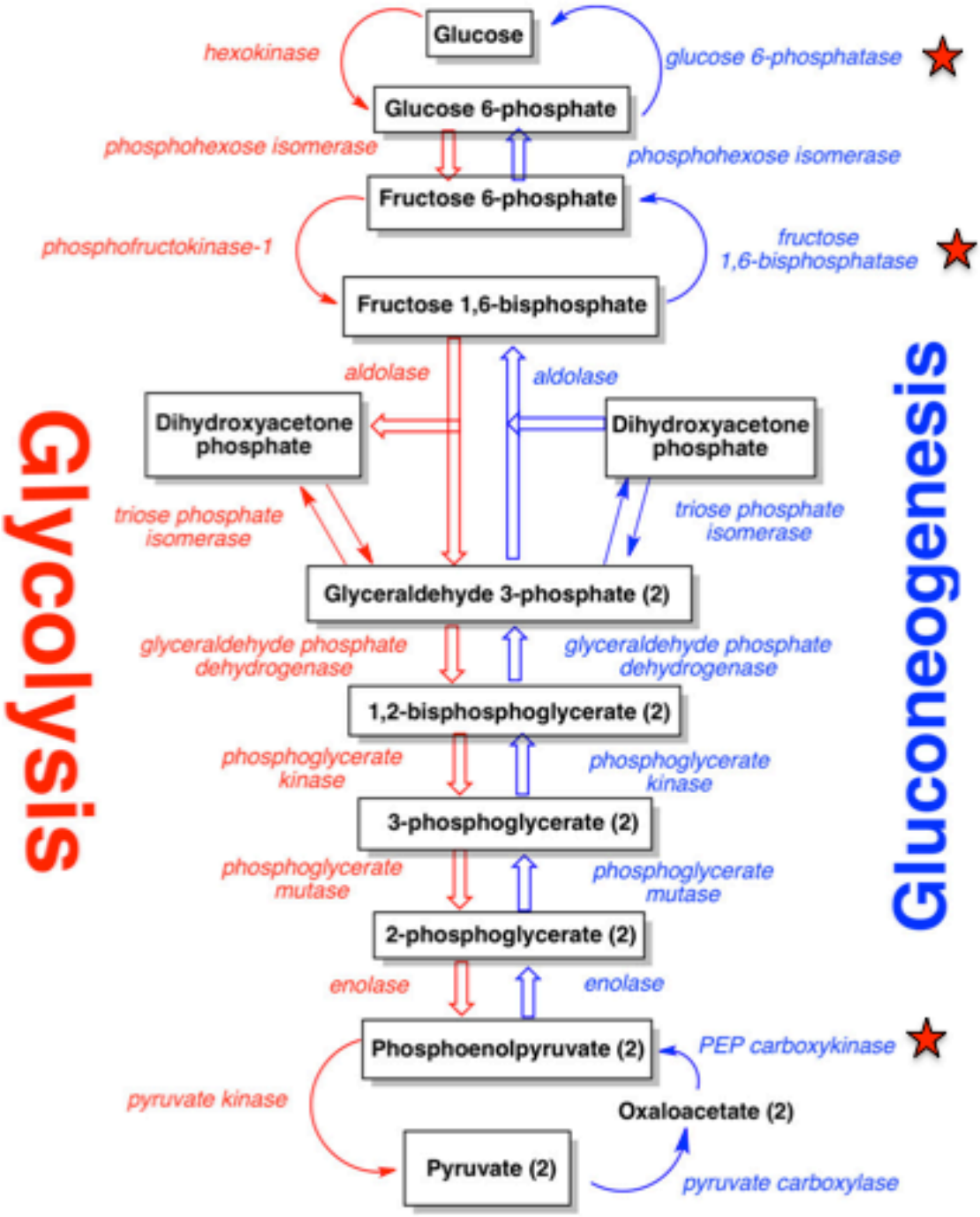

Figure 1.3: Glycolysis and Gluconeogenesis

Gluconeogenesis is essentially a reversal of glycolysis. Three key gluconeogenic enzymes that bypass endergonic reactions are highlighted with stars.

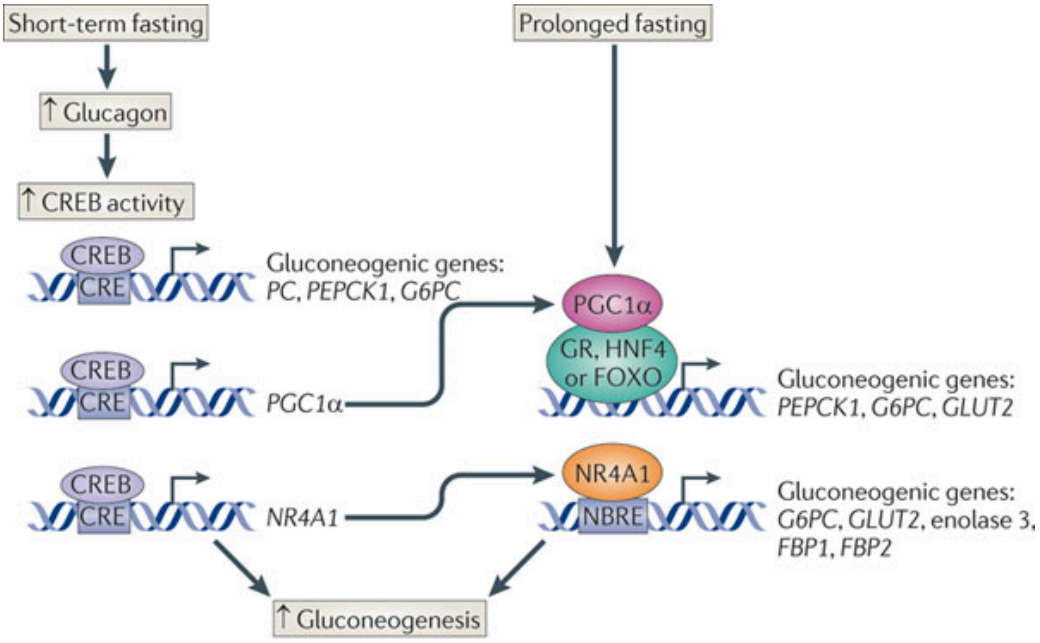

Figure 1.4: Model of proposed transcriptional regulation of gluconeogenic genes by CREB

from (Altarejos and Montminy 2011). During short-term fasting, CREB stimulates transcription of *Pgc1α* and members of the nuclear receptor subfamily 4 group A (*Nr4a*) family of orphan nuclear receptors, in addition to key gluconeogenic genes. During prolonged fasting, PGC1α and NR4A1 can further induce gluconeogenesis by interacting with other transcription factors or directly binding to cis-regulatory regions of gluconeogenic genes.

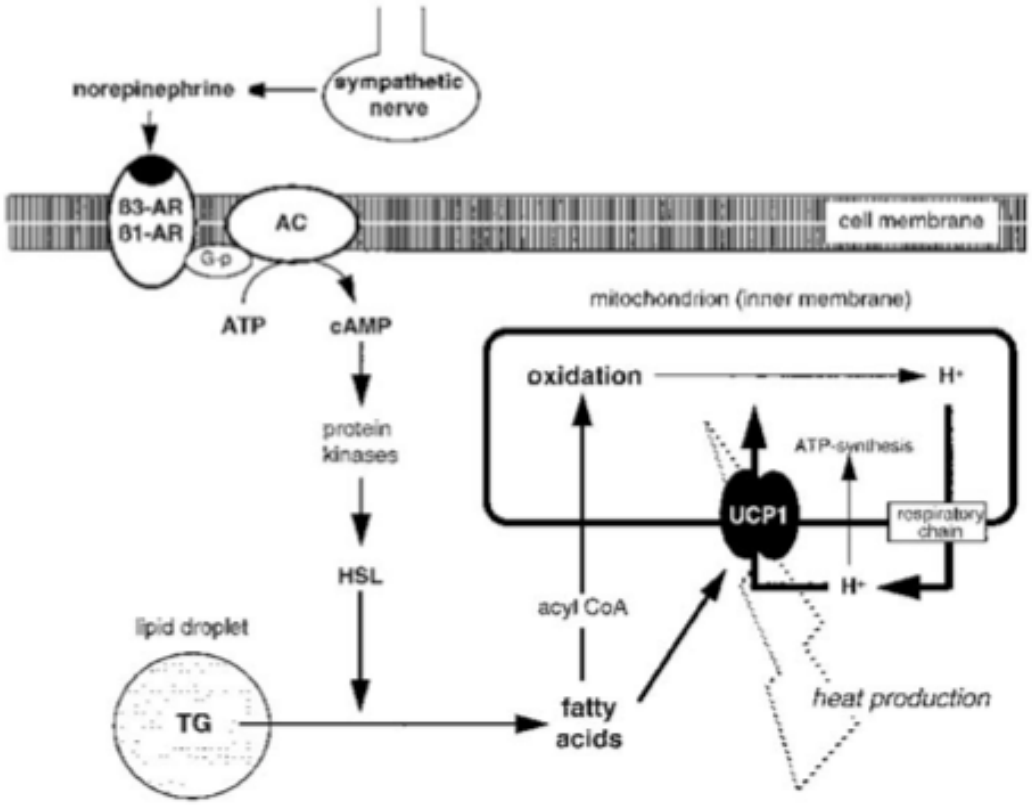

Figure 1.5: Thermogenesis

from (Klaus 2001). Norepinephrine activates cAMP signaling via β-adrenergic receptors. PKA activates HSL via phosphorylation, and triacylglycerols are hydrolyzed to produce glycerol and fatty acids. Fatty acids are substrates for oxidative phosphorylation. In addition, fatty acids can also activate UCP1, which mediates heat generation by uncoupling respiratory chain.

CHAPTER 2: Materials and Methods

Animals

For the derivation of *Creb*^{loxP} mice, a 12kb DNA fragment containing exon 11 of the *Creb* gene was retrieved from C57BL/6J mouse BAC clone RP23-31C24 via bacterial recombination. One *loxP* site was inserted upstream of exon 11, while a pair of *loxP* sites flanking a neomycin selection cassette was placed downstream of exon 11. The linearized targeting vector was electroporated into B6 ES cells (Chemicon) and clones surviving selection were screened for homologous recombination by Southern blot analysis. Targeted clones were injected into C57BL/6J-derived blastocysts that were then transferred to pseudo-pregnant females. Male offspring were mated to C57BL/6J females and ES cell-derived offspring were identified by PCR-based genotyping. Mice harboring the targeted insertion of the three *loxP* sites in the *Creb* locus were crossed to the EIIa-Cre line to achieve mosaic germ line deletion of *loxP*-flanked sequences. The derivation of the *Creb*^{loxP} was performed by Dr. John Le Lay.

For liver-specific deletion of the *Creb*^{loxP} alleles, *Creb*$^{loxP/loxP}$ mice were anaesthetized with isoflurane and injected with adeno-associated virus expressing Cre-recombinase under the control of the thyroxine-binding globulin (*Tbg*) promoter (AAV-Cre). Homozygous mice injected with virus expressing GFP (AAV-GFP) were used as controls. Adeno-associated virus are generated by the Penn Vector Core. The expression of GFP or Cre in wild-type livers using this viral delivery system did not result in any detectable changes in carbohydrate or lipid metabolism. Normal mouse-chow diet (5015; Purina Mills) fed mice were injected with adeno-associated virus at 8 to 10 weeks of age. A cohort of mice was started on high fat diet (60% kcal from fat; D12492; Research

Diets) upon weaning. Mice fed a high fat diet were injected with adeno-associated virus at 12 to 14 weeks of age. All mice are given virus injection of 2 x 10^{11} genomic copies per mouse. Experiments were performed two weeks after virus injection to ensure complete deletion and decay of residual proteins.

For adipocyte-specific deletion of *Creb*^{loxP} alleles, *Creb*$^{loxP/loxP}$ mice were crossed with *Adipoq-Cre* mice (Eguchi et al. 2011; Lee et al. 2013). *Creb*$^{loxP/loxP}$ mice were used as controls. Mice were analyzed between 3 to 5 months of age.

All the animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Whole Cell Lysate Preparation and Western Blot Analysis

Tissue fragments were homogenized by hand in 200μL of RIPA buffer containing 20mmol/l Tris (pH 8.0), 5mmol/l EDTA, 150mmol/l NaCl, 1% TritonX-100, 1% SDS and 0.5% deoxycholic acid, supplemented with a protease inhibitor cocktail (Roche), then sonicated using a Bioruptor (Diagenode). Whole-cell lysates were centrifuged at maximum speed for 15 min to sediment cellular debris, and the supernatant was collected. Protein concentrations were measured via Bradford assay using the "Protein Assay Reagent" (Bio-Rad).

Approximately 50μg of whole-cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked in buffer

containing 5% milk and 0.1% Tween-20 in 1X PBS (milk-PBST) for 30 min, then incubated overnight at 4°C with primary antibodies diluted in milk-PBST. After washing, membranes were incubated with HRP-conjugated secondary antibodies in milk-PBST for 1hr at room temperature. Proteins were visualized using the ECL-prime Western Blotting Detection System (Amersham GE).

Immunohistochemistry

Following tissue harvest, tissues were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde overnight, then embedded in paraffin, or cryoprotected in 30% sucrose/PBS overnight at 4°C and frozen embedded in OCT and frozen. Tissue blocks were sectioned by the CHOP Pathology Core. Sections were blocked with normal donkey serum in PBS and incubated with primary antibody at 4°C overnight. Fluorophore-conjugated secondary antibodies were applied for 2 h at room temperature, and nuclei were stained with DAPI. Hepatocytes were counted using an Olympus BG-2 microscope connected to a video camera with a color monitor.

Antibodies

CREB antibody was purchased from Cell Signaling, USA (#9197). HNF4α antibody was purchased from R&D Systems (2ZH1415H). Cyclophilin B was obtained from Pierce (PA1-027A). Donkey-anti-rabbit IgG and donkey anti-mouse IgG were

obtained from Amersham GE. Cy3-conjucated donkey anti-rabbit secondary antibody and Cy5-conjugated donkey anti-mouse secondary antibody were from Jackson ImmunoResearch Laboratories)

Glucose Tolerance Test, Insulin Tolerance Test, and Glucagon Stimulation Test

Glucose tolerance tests were performed on mice fasted for 16hrs; while insulin tolerance test and glucagon stimulation test were performed on mice fasted for 3hrs. Animals on regular chow were administered 2g/kg body weight glucose, while those on the high fat diet were administered 1.5g/kg body weight glucose to prevent glucose excursions outside the measurable range. Animals were administered 0.75U/kg body weight human insulin (Novo Nordisk), or 16μg/kg body weight glucagon by intra-peritoneal injection. Blood was sampled from the tail vein at multiple time points and glucose levels were measured with a Breeze 2 Glucose Meter (Bayer).

Metabolic Cage Study with Norepinephrine and CL-316,243 Treatment

During comprehensive metabolic monitoring, animals were kept at 30°C. 75mg/kg body weight nembutol was administered for anesthesia. Once mice are sedated, animals were given subcutaneous injection of 1mg/kg body weight norepinephrine (Sigma Aldrich), or CL-316,243 (Sigma Aldrich). O_2 consumption and CO_2 production were measured, and body heat produced was calculated by indirect calorimetry.

Metabolic cage experiments were performed by the Mouse Phenotyping, Physiology and Metabolism Core.

CL-316,243 Treatment

Animals were moved to 30°C one-week prior to the start of injection, and kept at 30°C until tissue harvest. Once acclimated to thermoneutrality, animals were injected 1mg/kg body weight CL-316,243 (Sigma Aldrich) subcutaneously, once a day for 3 days, and their tissues were collected 24hrs after the last injection.

Hepatic Lipids, Plasma Lipid and Plasma Adiponectin Measurement

Liver fragments were homogenized in PBS. Blood was collected from the tail vein after 16hr fasting and 2hr refeeding with lithium-heparin coated microvette (Sarstedt). Blood samples were centrifuged for 5 min at 4°C to separate plasma. Hepatic triacylglycerol and cholesterol levels were measured via colorimetric assay with Infinity triglyceride reagents (Thermo Scientific) and Infinity cholesterol reagents (Thermo Scientific). Plasma NEFA, triacylglycerol and β-hydroxybutyrate were measured via colorimetric assay with HR Series NEFA-HR (2) kit (Wako Diagnostics), Infinity triglyceride reagents (Thermo Scientific) and β-hydroxybutyrate liquicolor kit (Stanbio), respectively. Plasma adiponectin levels were measured using an adiponectin ELISA kit (Linco).

RNA Isolation and Quantitative RT-PCR Analysis

Total cellular RNA was extracted from tissue fragments using TRIzol (Invitrogen) and the RNeasy Kit (Qiagen), according to the manufacturer's protocol. Approximately 1 μg of total RNA was reverse transcribed using oligo(dT) and Superscript II Reverse Transcriptase (Invitrogen). The resulting cDNA samples were employed as template for quantitative RT-PCR experiments performed with Brilliant III Ultra-Fast SYBR QPCR Mastermix (Agilent) and the SYBR Green (with dissociation curve) program on the Mx300 Multiplex Quantitative PCR System (Stratagene). Reactions were performed in triplicate and normalized relative to the ROX reference dye. Median cycle threshold values (Ct) were determined and used for analyses. Expression levels were normalized to those of hypoxanthine-guanine phosphoribosyltransferase (HPRT) as the internal control. Primers are listed in Table 2.1.

mRNA Expression Profiling via Microarray

RNA isolated from the liver and adipose tissue of overnight-fasted four control and four *Creb* mutant mice, were amplified and labeled using Low-Input Quick Amp labeling kits (Agilent). Labeled samples were hybridized overnight to a 4×44 whole mouse genome array (Agilent). Arrays were washed and then scanned with the G2565B DNA microarray scanner (Agilent). Genes displaying a fold-change over 1.5-fold between mutants and controls and a false discovery rate <10%, calculated using

significance analysis of microarray analysis (Tusher et al. 2001), were determined as significantly changed. Data analysis was prformed by the Functional Genomics Core.

	Primer Sequence ('5→3')	
mRNA	**Fwd**	**Rev**
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG
Creb	AAGCAGCACGGAAGAGAGAG	TTTTCAAGCACTGCCACTCTG
Crem	TGCCTGGTATTCCCAAGATT	TTGTATTGCCCCGTGCTAGT
Dio2	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG
Fbpase	GCATCGCACAGCTCTATGGT	ACAGGTAGCGTAGGACGACT
G6pase	GCAAGGAGACCCAGGATTCTT	TGGGCTAGGGAAAGGAGTCAT
Pepck	TGCCCAAGGCAACTTAAGGG	CAGTAAACACCCCCATCGCT
Pgc1α	GGTCGAACGAAACTGACTTCG	GCAGGGTCAAAATCGTCTGAG
Pparα	GCGTACGGCAATGGCTTTAT	GAACGGCTTCCTCAGGTTCTT
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG

Table 2.1: List of quantitative RT-PCR primers

CHAPTER 3: The Transcription Factor CREB has No Non-Redundant Function in Hepatic Glucose Metabolism

As Published in *Diabetologia,* June 2014

3.1 Abstract

Excessive hepatic glucose production is a hallmark of insulin resistance in type 2 diabetes. The cAMP-responsive transcription factor cAMP-responsive element binding protein (CREB), thought to be a key activator of the hepatic gluconeogenic gene regulation program, has been suggested as therapeutic target to reduce glucose output by the liver. Here, I test directly the requirement for hepatocytic CREB for the maintenance of glucose homeostasis. I derived mice with a *Creb* loxP allele for conditional, cell-type specific gene ablation. Hepatocyte-specific deletion of *Creb* was induced by injecting $Creb^{loxP/loxP}$ mice with Cre-expressing adeno-associated virus (AAV-Cre). Strikingly, I find no difference in fed and fasted glucose levels, or glucose, insulin, and glucagon tolerance in mice fed normal chow or a high-fat diet. In addition, mRNA levels of liver specific genes, including several CREB target genes involved in gluconeogenesis, were not affected by CREB deficiency in the liver. In conclusion, my data indicate that CREB has no non-redundant functions in hepatic glucose metabolism, and is therefore not likely to be a useful target for the development of anti-diabetic drugs.

3.2 Introduction

The cAMP response element binding protein (CREB) is a ubiquitous transcription factor that belongs to the basic leucine zipper class (Dwarki et al. 1990). CREB contributes to the regulation of genes containing cAMP-responsive elements (CREs) in response to changes in cellular cAMP levels. In the liver, key gluconeogenic genes contain CREs in their proximal promoters (Zhang et al. 2005; Everett et al. 2013). Previous studies suggested that CREB is required for the regulation of gluconeogenesis during fasting via recruitment of transcriptional co-activators to cis-regulatory elements of relevant genes upon glucagon signaling (Herzig et al. 2001; Zhou et al. 2004; Koo et al. 2005; Le Lay et al. 2009).

Elevated hepatic gluconeogenesis, caused by increased glucagon levels, insufficient insulin secretion, and insulin resistance, is the major contributor of fasting hyperglycemia in type 2 diabetic patients (DeFronzo et al. 1989; Magnusson et al. 1992; Gastaldelli et al. 2000). Despite previous reports implicating inhibition of CREB as a means to reduce blood glucose in the liver, the specific requirement for CREB in hepatic glucose metabolism has never been addressed, and prior studies on CREB function reported conflicting results (Herzig et al. 2001; Herzig et al. 2003; Erion et al. 2009). Specifically, there was a significant increase in hepatic triacylglycerol levels in the livers of mice expressing acidic-CREB (A-CREB), a dominant-negative inhibitor of CREB function, while mice treated with antisense oligonucleotides (ASO) were protected from

hepatic steatosis (Herzig et al. 2003; Erion et al. 2009), suggestive of non-specific effects of one or both approaches.

Therefore, to assess the specific role of CREB in the liver in vivo, I employed a conditional gene ablation approach. By deleting *Creb* in adult hepatocytes using Cre recombinase expressing adeno-associated virus in $Creb^{loxP/loxP}$ mice, I demonstrate that CREB is not required for the regulation of hepatic glucose metabolism.

3.3 Results

3.3.1 Conditional ablation of *Creb* in hepatocytes

To assess hepatic CREB function *in vivo*, a $Creb^{loxP}$ conditional null allele was derived via homologous recombination in mouse embryonic stem cells. The targeting strategy used to construct this mutant allele is shown in Figure 3.1. *loxP* sites were positioned upstream and downstream of exon 11 to result in deletion of this critical exon encoding the DNA binding domain upon exposure to Cre recombinase. In order to obtain mice with hepatocyte-specific deletion of *Creb*, I used an AAV serotype that preferentially infects hepatocytes, and controlled expression of Cre recombinase further by placing it under the control of the hepatocyte-specific thyroxine-binding globulin (*Tbg*)-promoter (Figure 3.1). Quantitative RT-PCR indicated an 80% decrease in *Creb* mRNA expression in whole liver, consistent with the contribution of hepatocytes to liver mass (Figure 3.2a). Western blotting confirmed the effective reduction of CREB protein

levels in the livers of *Creb*$^{L/L}$, AAV-Cre mice, with the remaining CREB protein in mutant animals stemming from cells other than hepatocytes (Figure 3.2b). In order to confirm complete ablation of *Creb* specifically in hepatocytes, dual label-immunofluorescence staining with antibodies against the hepatocyte-specific marker (HNF4α) and CREB protein (Figure 3.2c, d) were performed. CREB protein was absent from all hepatocytes two weeks post AAV-Cre injection (Figure 3.2e), while *Creb* expression in cholangiocytes and non-paranchymal cells such as stellate and Kupffer cells was maintained (Figure 3.2e), indicating 100% efficiency and specificity of the AAV-Cre system.

3.3.2 No changes in blood glucose homeostasis in the absence of hepatic *Creb*

Since previous studies had suggested that CREB serves as the master regulator of gluconeogenesis via transcriptional regulation, I first examined blood glucose levels in *Creb*$^{L/L}$; AAV-Cre mice and control *Creb*$^{L/L}$; AAV-GFP mice after fasting and during refeeding. Surprisingly, there was no difference between blood glucose levels of control and *Creb*-deficient mice (Figure 3.3a). To further analyze the effect of *Creb* ablation on glucose homeostasis, glucose and insulin tolerance tests were performed. In agreement with results from fasted and refed glucose measurements, no significant differences between control and *Creb*-null mice were observed in these assays (Figure 3.3b, e). In addition, insulin levels during glucose tolerance tests were indistinguishable between the two groups (Figure 3.3c). To assess the possibility that CREB's role in regulating gluconeogenesis might be masked by glycogen breakdown during a short-term fast, blood

glucose levels were measured during a 72 hour-long starvation period, when hepatic gluconeogenesis becomes essential. Strikingly, even during the prolonged fast, hepatocyte-specific *Creb* ablation had no effect on blood glucose levels (Figure 3.3d). Thus, CREB is not required in hepatocytes to maintain blood glucose levels.

3.3.3 Ablation of hepatic *Creb* does not improve insulin sensitivity

It has been reported that inhibition of CREB activity in the liver leads to improvement of hepatic insulin sensitivity in various insulin-resistant rodent models, suggesting that CREB might be an attractive therapeutic target for the treatment of type 2 diabetic patients (Herzig et al. 2001; Erion et al. 2009). To evaluate this possibility, I assessed the effects of hepatocyte-specific deletion of *Creb* on hepatic and peripheral insulin sensitivity in the high-fat diet-feeding model. Unlike previous reports employing different modes of non-specific CREB inhibition (see discussion), I did not observe decreases in blood glucose levels after fasting and during refeeding in *Creb*-deficient mice that had been on a high-fat diet for 10 weeks relative to control mice (Figure 3.4a). Because of animals developing insulin resistance after high-fat feeding, I used a smaller glucose amount for the studies of high-fat diet fed mice to prevent glucose excursions outside the measurable range. There was no improvement in glucose or insulin tolerance in high-fat diet fed *Creb* mutant mice compared to controls (Figure 3.4b, c). To investigate the effects of hepatocytic *Creb* ablation in mediating glucagon signaling and activation of the gluconeogenic gene expression program, glucagon stimulation tests

were performed. However, loss of hepatocytic *Creb* did not change post-glucagon blood glucose levels compared to controls with intact hepatic *Creb* (Figure 3.4d).

3.3.4 Loss of *Creb* does not improve nor aggravate fatty liver condition in insulin-resistance mice

Previous studies reported opposite effect of *Creb* inhibition on fatty liver condition. Hepatic lipid accumulation was significantly increased in mice expressing A-CREB compared to controls (Herzig et al. 2003), while *Creb*-ASO treatment alleviated fatty liver condition by lowering hepatic lipid levels and lipogenic gene expression levels (Erion et al. 2009). To understand the role of *Creb* in the regulation of hepatic lipid accumulation, fatty liver condition was evaluated via histology of high-fat diet fed control and hepatocytic *Creb* mutant mice. H&E (Figure 3.5a, b) and Oil Red O (Figure 3.5c, d) staining of liver sections revealed no significant alterations in high-fat diet induced fatty liver condition in hepatocytic *Creb*-null mice compared to controls. Furthermore, hepatic triglyceride and cholesterol levels were comparable between the two groups (Figure 3.5e, f). In addition, loss of *Creb* in hepatocytes did not change circulating lipid levels and alanine transaminase (ALT) levels compared to controls (Figure 3.6).

3.3.5 Expression of gluconeogenic genes does not change in the absence of hepatocytic Creb

Although there were no physiological changes in hepatocyte-specific *Creb* null mice, I investigated the effect of hepatocytic *Creb* ablation at the transcriptional level. To this end, I harvested livers after an overnight fast to ensure full activation of the gluconeogenic gene expression program. Next, mRNA levels of key gluconeogenic genes were measured, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1α*), glucose 6-phosphatase (*G6pase*), phosphoenolpyruvate carboxykinase (*Pepck*), and fructose bisphosphatase (*Fbpase*), in regular chow fed (Figure 3.7a) or high-fat diet fed mice (Figure 3.7b) via quantitative RT-PCR. In contrast to previous studies that had reported an 80% decrease in expression levels in mice expressing a dominant-negative mutant version of CREB relative to controls (Herzig et al., Nature 2001), I did not observe any significant changes in steady-state mRNA levels of these gluconeogenic genes in hepatocyte-specific *Creb* null mice (Figure 3.7a and b). To assess effects of *Creb* on entire hepatic transcriptome, expression profiling via microarray was performed (Figure 3.7c). Only 21 genes were significantly differentially expressed in Creb-null liver compared with controls, none of which are known to function in glucose metabolism (Table 3.1). This strongly suggest other factors, including other CREB-family members which can also bind to common CRE site, may bind promoters of CREB target genes and induce gene expression in the absence of CREB. cAMP-responsive element modulator (CREM), another member of CREB family of DNA binding proteins, can also mediate transcriptional response to cAMP signaling, and there is some functional redundancy between CREB and CREB (Hummler et al. 1994). To determine potential functional

compensation by CREM in the absence of CREB, *Crem* mRNA levels were measured. In the livers of hepatocyte specific *Creb*-null mice, there was a 2-fold increase in *Crem* expression levels, suggesting the regulation of CREB target genes by CREM (Figure 3.8).

3.4 Discussion

CREB has been established as the master regulator of gluconeogenesis during fasting. Multiple studies were performed to determine the role of CREB in the liver by utilizing tissue specific dominant-negative (Herzig et al. 2001; Herzig et al. 2003) or antisense nucleotide approaches (Erion et al. 2009). Both approaches resulted in significant decreases in blood glucose levels, and reduced gluconeogenic gene expression in the liver. These results suggested CREB as a potential therapeutic target to improve insulin sensitivity in type 2 diabetes patients. However the two approaches reported divergent effects on hepatic triacylglycerol metabolism, strongly suggestive of non-specific effects in one or both models. The two reports also differed on the proposed mediators of hepatic CREB function. Thus, Herzig and colleagues reported that CREB regulated hepatic lipid metabolism via peroxisome proliferator-activated receptor gamma (PPARγ) (Herzig et al. 2003), while ASO treatment had no effect on PPARγ expression levels (Erion et al. 2009). In addition, these studies neither directly nor specifically addressed the role of hepatic CREB due to inherent limitations of the methodologies employed. In both studies, CREB activity was suppressed in other tissues as well as the liver. In the A-CREB study, expression of CREB was under the control of cytomegalovirus (CMV) promoter (Herzig et al. 2001; Herzig et al. 2003), hence no

tissue specificity could be achieved. Similarly, *Creb*-ASO lead to decrease in *Creb* mRNA levels in both the liver and adipose tissue (Erion et al. 2009). Multiple organs in the body control glucose metabolism, and alterations in any of its players can lead to the disruption of regulation. For instance, there are suggestions that CREB in the brain might be involved in regulation of appetite and food intake (Altarejos et al. 2008; Yadav et al. 2011; Ren et al. 2013). Therefore it is possible that the blood glucose lowering effect by A-CREB or *Creb*-ASO were due to suppression of CREB in combination of tissues. Furthermore, there are well documented off-target effects associated with dominant-negative CREB mutant proteins (Ahn et al. 1998), while the ASO approach lead to decreased *Creb* mRNA levels in adipose tissue as well as in the liver (Erion et al. 2009). In addition, it has been shown that ASO can have biological effects that are not solely resulting in specific degradation of their target mRNAs (Woolf et al. 1992; Fisher et al. 2002).

To investigate the requirement for CREB in liver metabolism, I utilized the Cre-*loxP* system for conditional ablation of *Creb* in the adult hepatocytes. In my model, I was able to confirm complete loss of CREB protein in every hepatocyte post AAV-Cre injection, while expression of CREB in non-hepatocytes was maintained. In contrast to previous studies suggesting improved insulin sensitivity through inhibition of CREB activity via dominant-negative or ASO approach, deletion of hepatocytic *Creb* had no effect on glucose homeostasis and insulin sensitivity. In addition, expression levels of key gluconeogenic genes, such as *Pgc-1α, G6pase, Pepck and FBPase*, in *Creb*-deficient liver were comparable to those of control mice, in contrast to previous studies reporting a

50-80% decrease in expression levels following A-CREB overexpression or ASO treatment (Herzig et al. 2003; Erion et al. 2009). In addition, as shown by global expression profiling, *Creb* ablation had insignificant effect on genes involved in hepatic metabolism and function, indicating that other factors may bind and regulate CREB targets in the absence of CREB. Furthermore, deletion of *Creb* did not improve insulin sensitivity of mice that were fed a high-fat diet to mimic type 2 diabetic conditions. The different outcome of CREB deficiency reported here is likely due to the specificity of my model system relative to the dominant-negative and ASO approach, which inhibits CREB in multiple organs including the liver, and may impact factors other than CREB. My results suggest functional redundancy between proteins that bind to CREs as indicated by the lack of change in expression levels of CREB target genes in the absence of hepatocytic CREB. Partial redundancy among members of mammalian transcription factor gene family has been reported previously (Hummler et al. 1994), and an increased expression levels of *Crem* in the hepatocytic *Creb*-null livers suggests potential functional compensation. Nevertheless, these findings put into question the current status of CREB as the master regulator of gluconeogenesis and the suitability of specific CREB inhibitors as anti-diabetic drugs.

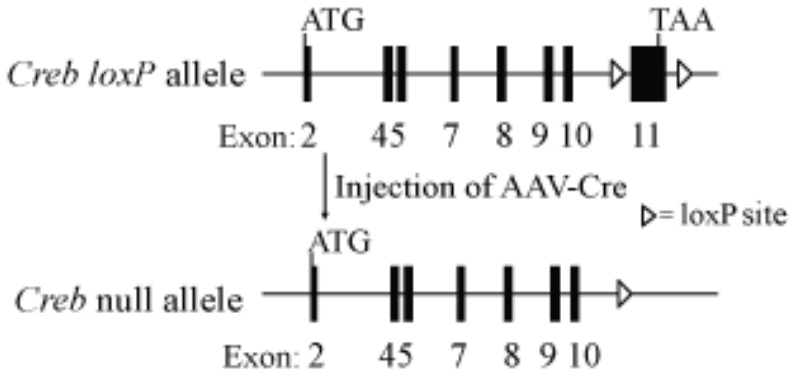

Figure 3.1: Design of *Creb*^{loxP} allele

loxP sites (white triangles) were positioned to flank the critical exon 11 of the *Creb* gene. Mice harboring the *Creb loxP* allele were injected with AAV-Cre or AAV-GFP (control). In hepatocytes, which are infected by AAV-Cre, removal of exon 11 produces a null allele.

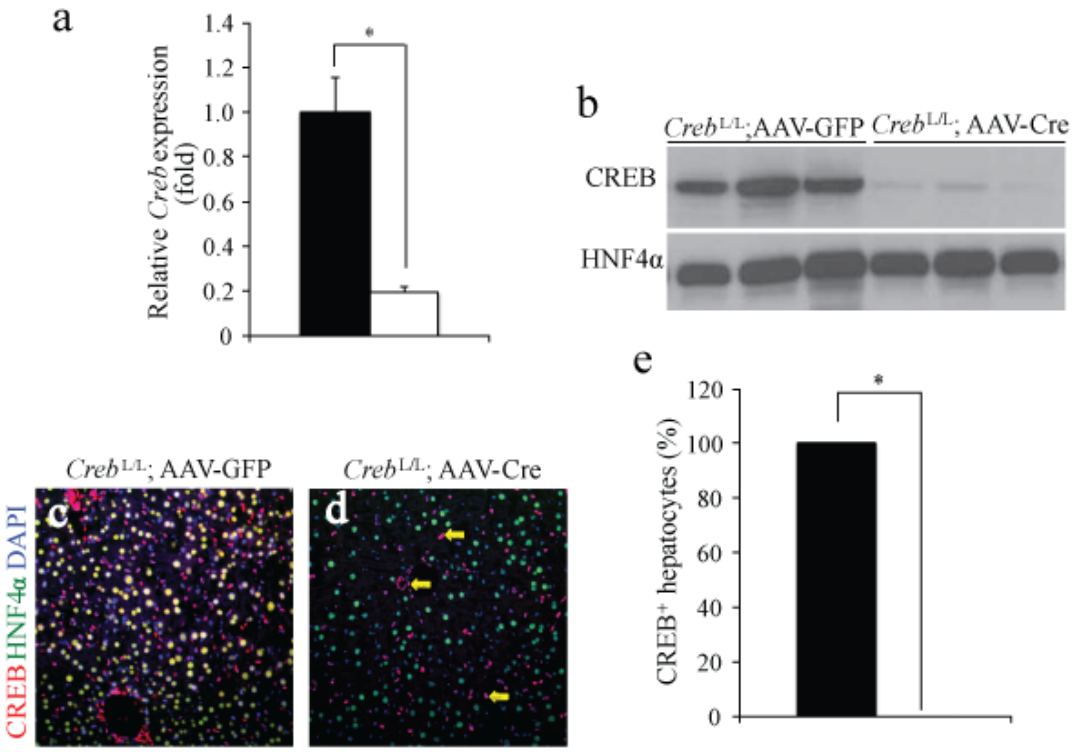

Figure 3.2: Conditional gene ablation of *Creb* in hepatocytes

(a) Quantitative RT-PCR to measure Creb mRNA levels in the liver of *Creb* $^{L/L}$ mice injected with control virus (AAV-GFP; black bar) or AAV-Cre (white bar). Transcription levels in *Creb* $^{L/L}$; AAV-GFP mice were set to 1. N = 5 for each group. Values are presented as average ± SE. *$p < 0.005$. (b) Western blot analysis of whole-cell liver lysates taken from livers of Creb L/L mice injected with AAV-GFP or AAV-Cre as indicated. HNF4α was used as loading control. (c and d) Liver sections from mice of indicated genotypes were stained with antibody against CREB (red) and HNF4α (green). Only HNF4α− non-parenchymal cells maintain CREB expression (yellow arrowheads) in *Creb* $^{L/L}$; AAV-Cre livers. (e) Percentage of CREB+ hepatocytes (HNF4α+ cells) in control (AAV-GFP injected; black bar) or mutant (AAV-Cre injected; white bar) liver. n = 3 for each group. *$p < 0.005$.

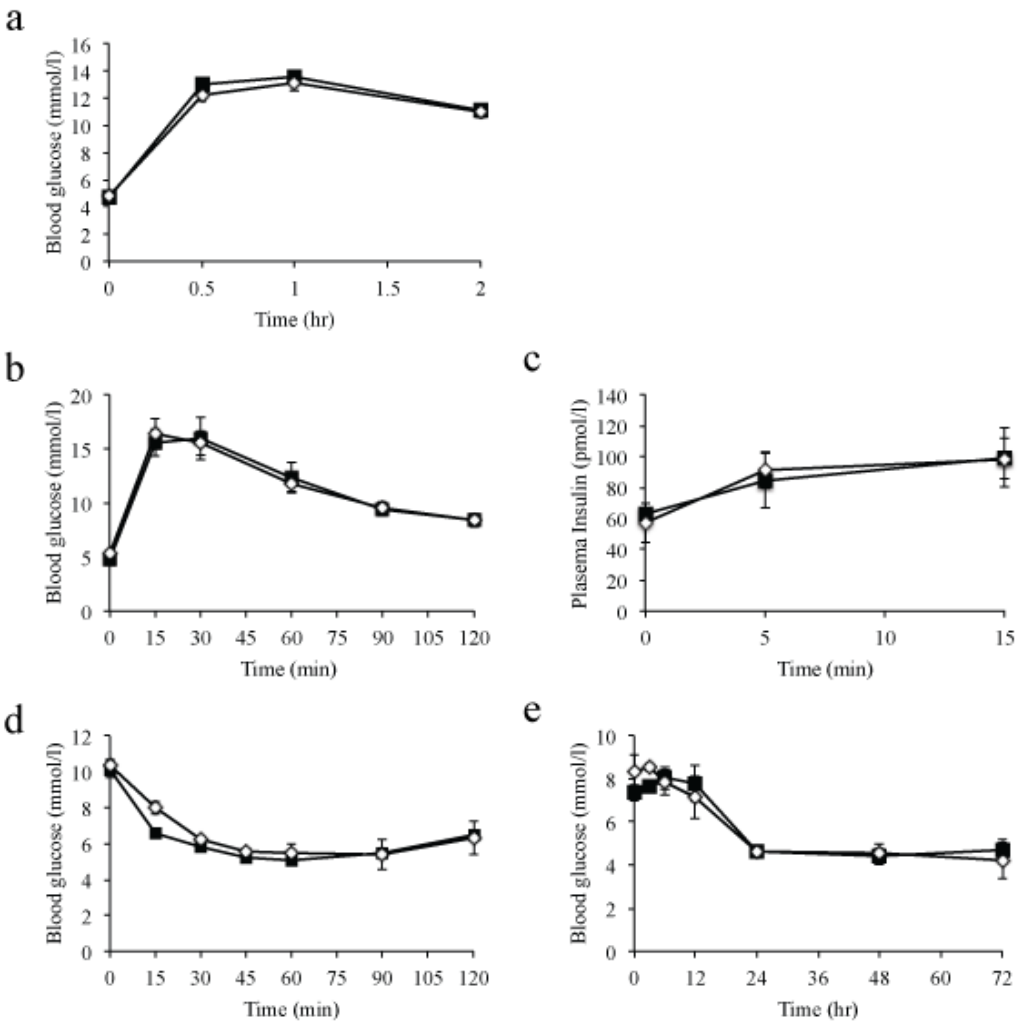

Figure 3.3: Mice lacking *Creb* in hepatocytes maintain normal glucose homeostasis

(a) Blood glucose levels of control (*Creb* $^{L/L}$; AAV-GFP) and hepatocyte-specific Creb mutant mice (*Creb* $^{L/L}$; AAV-Cre) after 24 h fasting (0 time point) and during re-feeding at the times indicated. N = 5–6 for each group. (b) Blood glucose levels and (c) insulin levels of control and Creb-deficient mice after overnight fasting (0 min) and following injection of a glucose bolus (2 g/kg body weight) at the times indicated. N = 5-6 for each group. (d) Blood glucose levels of control and *Creb*-deficient mice after 3 h fasting (0 min) and following an insulin injection at the times indicated. n = 5 for each group. (e) Blood glucose levels of control and *Creb*-deficient mice during the course of 72 h fast. N = 4 for each group. Black squares, *Creb* $^{L/L}$; AAV-GFP. White diamonds, *Creb* $^{L/L}$; AAV-Cre.

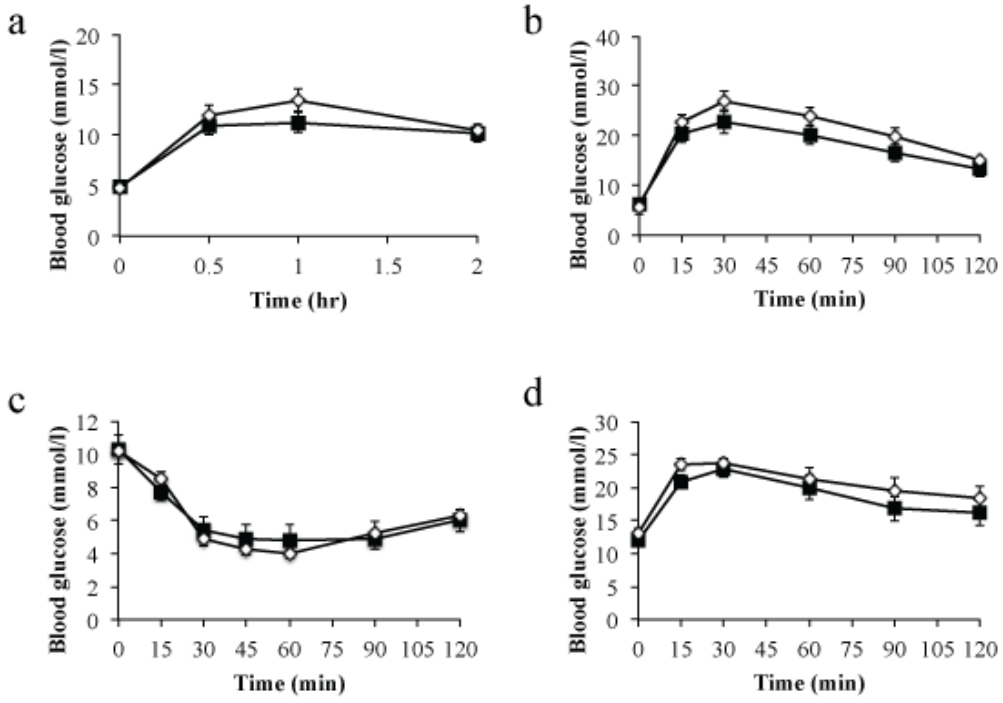

Figure 3.4: Deletion of hepatocytic *Creb* does not improve glucose homeostasis of high-fat diet fed mice

(a) Blood glucose levels of high-fat diet fed control (*Creb* $^{L/L}$; AAV-GFP) and hepatocyte-specific *Creb*-null mice (*Creb* $^{L/L}$; AAV-Cre) after 24 h fasting (0 time point) and during re-feeding at the times indicated. N = 9 for each group. (b) Blood glucose levels of high-fat diet fed control and *Creb*-deficient mice after overnight fast (0 time point) and following injection of a glucose bolus (1.5 g/kg body weight) at the times indicated. N = 15 for each group. (c) Blood glucose levels of high-fat diet fed control and *Creb* mutant mice after 3 h fasting (0 time point) and following an insulin injection at the times indicated. N = 6–7 for each group. (d) Blood glucose levels of high-fat diet control and *Creb*-deficient mice after 3 h fasting (0 time point) and following a glucagon injection at the times indicated. n = 5 for each group. Black squares, *Creb* $^{L/L}$; AAV-GFP. White diamonds, *Creb* $^{L/L}$; AAV-Cre.

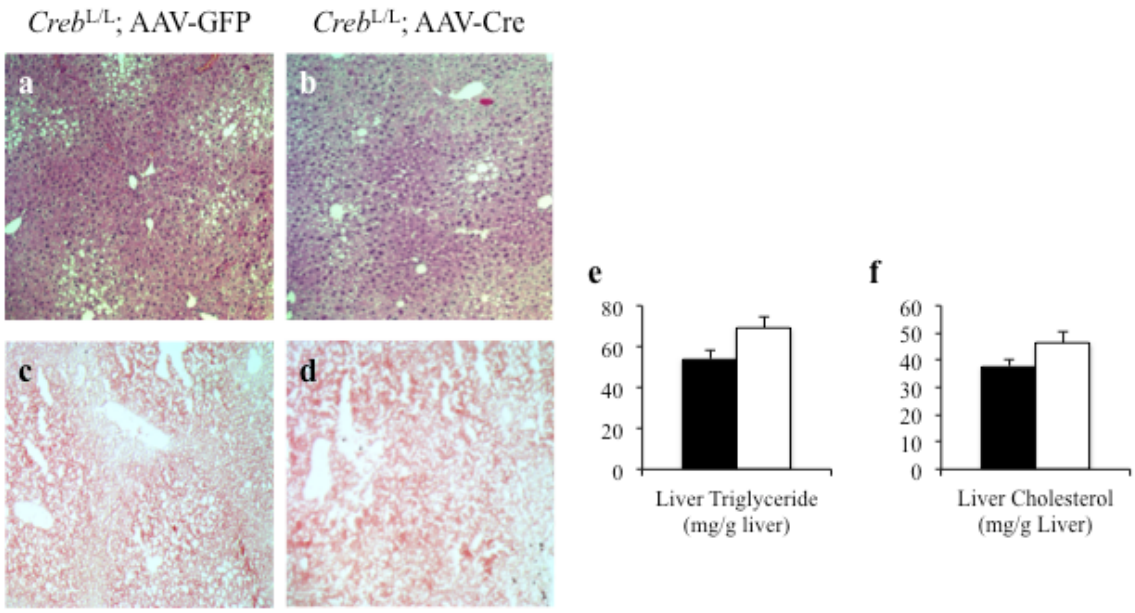

Figure 3.5: Deletion of hepatocytic *Creb* does not improve fatty liver of high-fat diet fed mice

(a and b) H&E staining of liver sections from mice of indicated genotypes (c and d) Liver sections from mice of indicated genotypes were stained with Oil Red O visualization of neutral triglycerides and lipids. Quantitative measurement of triglyceride (e) and Cholesterol (f) levels in high-fat diet fed control (*Creb* $^{L/L}$; AAV-GFP; black bar) or mutant (*Creb* $^{L/L}$; AAV-Cre; white bar) livers. Values are presented as average ± SE. n=11 for each group.

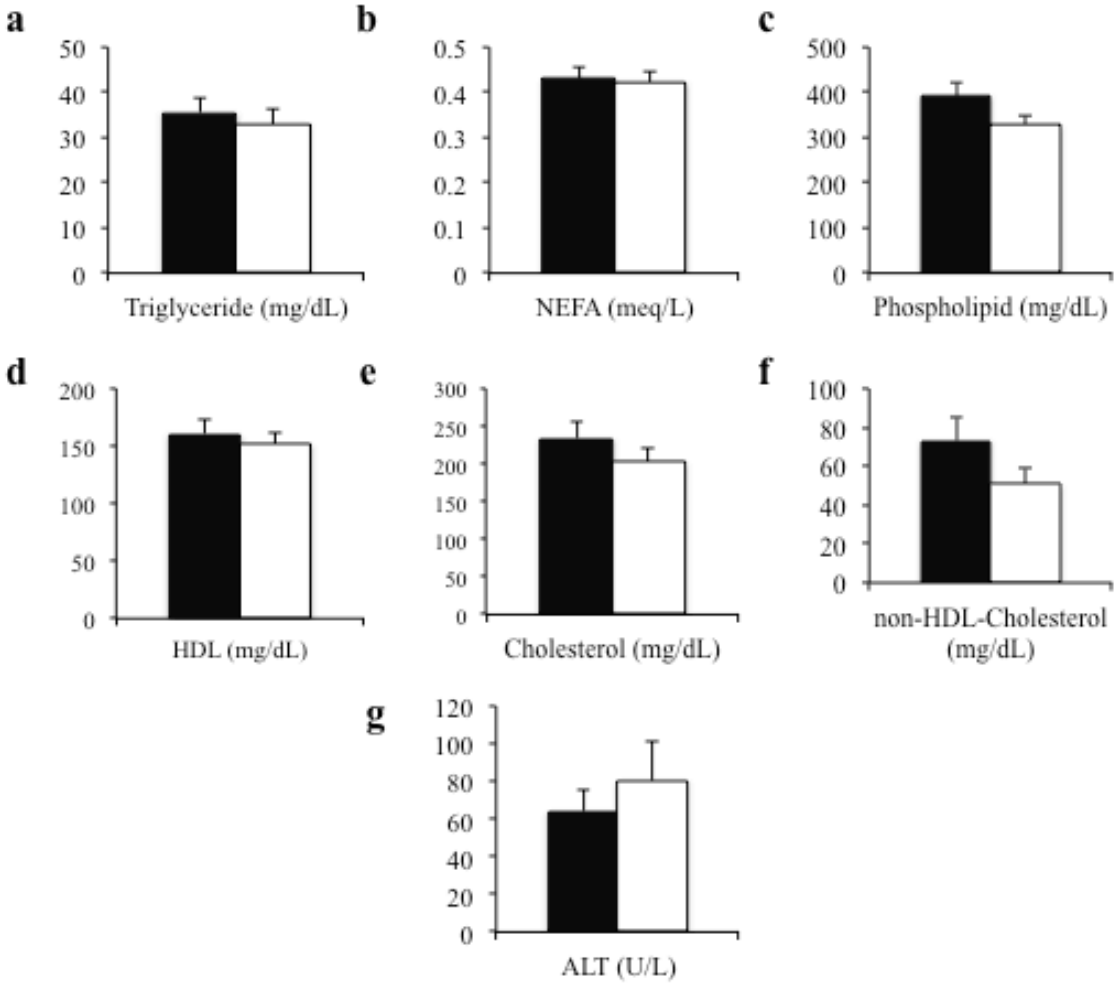

Figure 3.6: Loss of CREB in hepatocytes does not change plasma lipid profiles

To determine lipid profiles in circulations, plasma triglyceride (a), non-esterified fatty acids (NEFA; b), phospholipid (c), high-density lipoprotein (HDL; d), cholesterol (e), and non-HDL-cholesterol (f) levels were measured in high-fat diet fed control (AAV-GFP injected; black bar) or mutant (AAV-Cre injected; white bar) mice. In addition, Plasma alanine transaminase (ALT; g) levels were measured to detect liver injury. Values are presented as average ± SE. n=7 for each group.

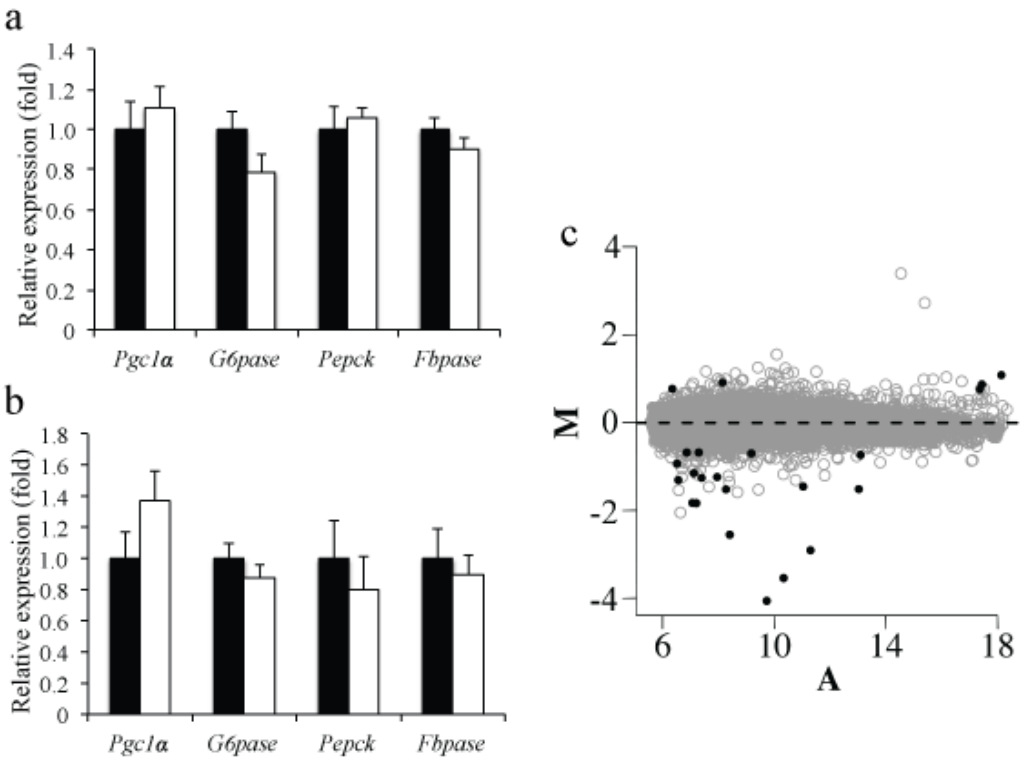

Figure 3.7: Loss of CREB in hepatocytes does not change gluconeogenic gene expression

(a) Quantitative RT-PCR to measure mRNA levels of *Pgc1α*, *G6pase*, *Pepck* and *Fbpase* in the liver of control and liver-specific Creb mutant mice after an overnight fast. n = 5–6 for each group. (b) Quantitative RT-PCR to measure relative mRNA levels of *Pgc1α*, *G6pase*, *Pepck* and *Fbpase* in the liver of high-fat fed control and hepatocyte-specific Creb-deficient mice after an overnight fast. n = 5–6 for each group. Transcription levels of control mice were set to 1. Values are presented as average ± SE. Black bar, *Creb* $^{L/L}$; AAV-GFP. White bar, *Creb* $^{L/L}$; AAV-Cre. (c) MA-plot of *Creb* $^{L/L}$; AAV-GFP liver RNA vs. *Creb* $^{L/L}$; AAV-Cre liver RNA from regular chow diet fed mice after an overnight fast. The M-axis is the log2 fold-change between the two groups, while the A-axis denotes the log2 average intensity in the two groups. Each point represents a gene (grey), and significantly differentially expressed genes (10% false discovery rate, >1.5-fold change) are marked in black. The dashed line indicates median M value for all points.

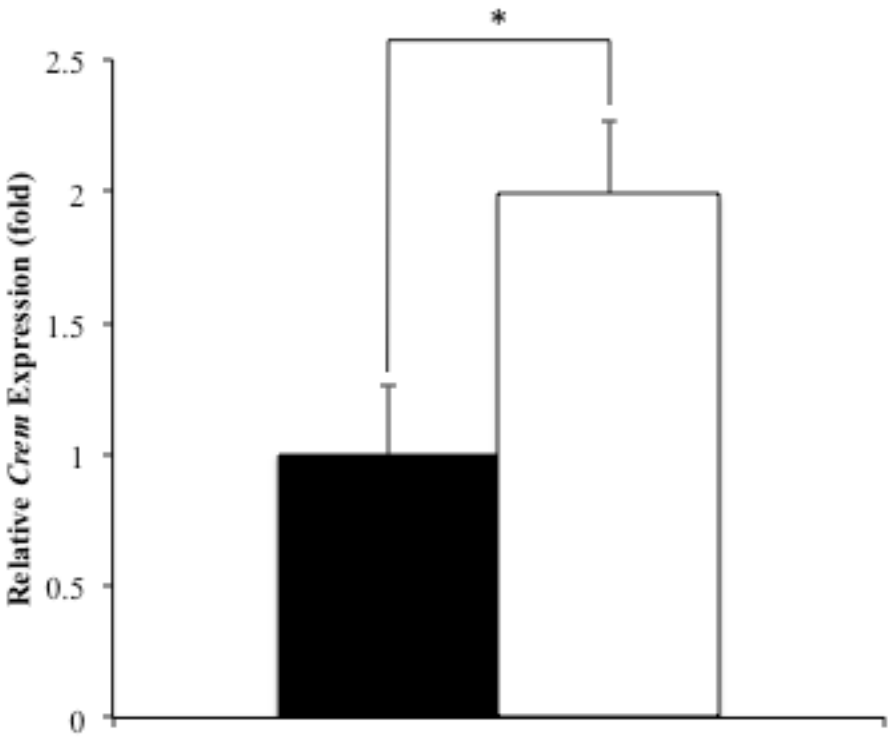

Figure 3.8: ***Crem* expression levels were increased by 2-fold in the livers of mice lacking hepatocytic *Creb***

Quantitative RT-PCR to measure mRNA levels of *Crem* in iWAT and BAT of control and adipocyte-specific *Creb*-null mice. Black bar, *Creb* $^{L/L}$; AAV-GFP. White bar, *Creb* $^{L/L}$; AAV-Cre. Transcription levels in *Creb*$^{L/L}$ mice were set to 1. Values are presented as average ± SE. *$p < 0.05$. $n = 5–6$ for each group.

Gene Name	Description	Fold Change
Mup20	major urinary protein 20	2.136
Moxd1	monooxygenase, DBH-like 1	1.915
NAP112258-1	major urinary protein, pseudogene 10	1.846
Cyp2c55	cytochrome P450, family 2, subfamily c, polypeptide 55	1.705
A_55_P2144090	major urinary protein, pseudogene 3	1.69
Tceal8	transcription elongation factor A (SII)-like 8	-1.592
Olfr1229	olfactory receptor 1229	-1.592
1700013B16Rik	RIKEN cDNA 1700013B16 gene	-1.595
Clpx	caseinolytic peptidase X (E.coli)	-1.631
Gpr137b	G protein-coupled receptor 137B	-1.88
Ttc37	tetratricopeptide repeat domain 37	-2.141
Mfsd6	major facilitator superfamily domain containing 6	-2.342
E130012A19Rik	RIKEN cDNA E130012A19 gene	-2.41
Neat1	nuclear paraspeckle assembly transcript 1 (non-protein coding)	-2.564
Camk2n2	calcium/calmodulin-dependent protein kinase II inhibitor 2	-2.674
Tmem215	transmembrane protein 215	-2.703
Kif4b	kinesin family member 4B	-3.165
Cntn4	contactin 4	-3.322
Krtap5-5	keratin associated protein 5-5	-5.348
2310065F04Rik	RIKEN cDNA 2310065F04 gene	-7.042
Ren1	renin 1 structural	-15.152

Table 3.1: Significantly differentially expressed genes in hepatocyte specific *Creb*-null liver

CHAPTER 4: Adipocytic CREB Regulates Fasting-Induced Lipolysis but Has No Non-Redundant Function in Thermogenesis

4.1 Abstract

Lipolysis and thermogenesis are regulated by catecholamines signaling via β-adrenergic receptors in adipose tissue. Previous studies suggested a role for the cAMP-responsive transcription factor cAMP-responsive element binding protein (CREB) in adipocyte function, but this hypothesis was never functionally confirmed by an adipose-specific ablation of *Creb in vivo*. Here, I test directly the requirement for adipocyte CREB for lipolysis and thermogenesis using the Cre-*loxP* system for adipocyte-specific ablation of *Creb* in mice. Loss of adipocytic *Creb* lead to a moderate decrease in fasting-induced lipolysis in adipose tissue. Strikingly, the adipocytic transcriptome was not globally affected by ablation of *Creb*. In addition, cold temperature and cAMP signaling-induced thermogenesis and white-to-beige adipocyte conversion were not changed in adipocyte specific *Creb*-null mice compared to controls. In conclusion, my data indicate that CREB has no non-redundant functions in thermogenesis, but contributes to the regulation of fasting-induced lipolysis. The underlying mechanism of reduced fasting-induced lipolysis in adipocytic CREB-deficient mice will need to be explored further.

4.2 Introduction

Adipose tissue is an active metabolic organ, in which excess energy is stored for use in times of food deprivation in the form of lipids. In addition, adipose tissue also provides protection and insulation for internal organs, generates body heat, and communicates with other organs via adipose-derived hormones. The two types of adipocytes that comprise the majority of adipose tissue are white adipocytes and brown adipocytes. Unilocular white adipocytes store energy in the form of large single lipid droplets in their cytoplasm. Unlike white adipocytes, multilocular brown adipocytes carry fat in multiple small lipid droplets, and also contain numerous mitochondria, which give brown fat its dark color. The main function of brown adipocytes is thermogenesis in response to cold exposure. (Rothwell and Stock 1979; Rothwell et al. 1982). Brown adipocytes express uncoupling protein-1 (UCP1) in the inner membranes of mitochondria, which assist heat production by uncoupling oxidative phosphorylation from electron transport. Prolonged exposure to low temperature or catecholamine signaling results in the transformation of subpopulations of white adipocytes into beige adipocytes that expresses UCP1 (Young et al. 1984; Cousin et al. 1992; Petrovic et al. 2010).

There are multiple adipose depots in the body. The major fat depots are abdominal subcutaneous white adipose tissue, visceral white adipose tissue, and subclavicular/subscapular brown adipose tissue. (Frühbeck 2008; Bjorndal et al. 2011; Cinti 2012). Both white adipose tissue (WAT) and brown adipose tissue (BAT) are innervated by sympathetic nerve endings (Cottle and Cottle 1970; Bowers et al. 2004; Bartness et al. 2010). Lipolysis and thermogenesis are regulated by catecholamine

signaling via β-adrenergic receptors (Landsberg et al. 1984; Carpéné et al. 1998). Binding of the catecholamine norepinephrine to β_3-adrenergic receptors on brown and white adipocytes leads to increased cytoplasmic cAMP levels, which activate protein kinase A (PKA). PKA phosphorylates hormone-sensitive lipase (HSL), and activated HSL hydrolyzes lipid triacylglycerols in lipid droplets, releasing glycerol and fatty acids. In brown adipocytes, released fatty acids activate UCP1, in addition to acting as substrates for oxidative phosphorylation.

Although all adipose depots play a role in buffering energy imbalance, metabolic rates can vary among the depots. For example, visceral white fat is more metabolically active than subcutaneous white fat, contributing more to circulating free fatty acid levels (Wajchenberg 2000; Hajer et al. 2008). Due to the difference in lipolytic rates, visceral fat contributes more in development of metabolic syndrome and type 2 diabetes mellitus (T2DM) relative to the other depots (Bjorndal et al. 2011). Furthermore, white adipocytes in inguinal subcutaneous WAT are more susceptible to beige transformation than those in epididymal WAT, even though all white fat depots express *Ucp1* upon cold exposure or catecholamine treatment. (Himms-Hagen et al. 2000; Barbatelli et al. 2010; Vitali et al. 2012; Wu et al. 2012; Harms and Seale 2013).

Recently, BAT has received increased attention recently due to its implications as a potential target for the treatment of obesity (Lockie et al. 2013; Mund and Frishman 2013; Chechi et al. 2014). In rodents, ablation of BAT or UCP1 leads to obesity due to loss of diet-induced thermogenesis (Lowell et al. 1993; Feldmann et al. 2009). Similarly, chronic treatment with a β_3-adrenergic receptor-specific agonist reverses obesity and insulin-resistance in rodents (Ghorbani et al. 1997; Ghorbani and Himms-Hagen 1997;

Liu et al. 1998). These data suggest that heat production from BAT is a critical regulator of whole body energy metabolism. In humans, presence of BAT in adults has only been recognized recently (Hany et al. 2002; Yeung et al. 2003). Some studies have indicated an inverse correlation between body mass index and BAT activity (Cypess et al. 2009; Saito et al. 2009; van Marken Lichtenbelt et al. 2009). Therefore, BAT activity can potentially contribute to energy expenditure in adult humans. Furthermore, molecular characteristics of human brown adipocytes are similar to that of murine beige adipocytes rather than brown adipocytes (Wu et al. 2006). Thus, stimulants that induce 'browning' of white adipose tissue may be useful in treating obesity and diabetes in humans.

cAMP response element-binding protein (CREB) is a transcription factor that binds to cAMP response elements (CREs) in *cis*-regulatory regions, and mediates cAMP effects on transcriptional regulation. In adipocytes, β_3- adrenergic signaling can activate CREB (Chaudhry and Granneman 1999; Thonberg et al. 2002). In a previous study, obese mice expressing a dominant-negative CREB specifically in brown and white adipocytes displayed improved insulin sensitivity, and were protected from adipose tissue inflammation (Qi et al. 2009). In addition, CREB-regulated transcription coactivator 3 (CRTC3) was shown to promote obesity by attenuating β-adrenergic receptor signaling in adipose tissue (Song et al. 2010). The proximal promoter region of the mouse *Ucp1* gene contains CRE half-sites along with other *cis*-regulatory elements (Kozak et al. 1994; Rim and Kozak 2002), suggesting possible direct regulation of its expression by CREB. In fact, cold-stimulated *Ucp1* levels in beige adipocytes were higher in the A/J strain of mice compared to that of the obesity-prone C57BL/6J strain, and correlated with higher

activation of CREB (Xue et al. 2005). Furthermore, adipocytic CREB has been shown to regulate cAMP-induced expression of peroxisome proliferator-activated receptor- γ coactivator-1α (*Pgc1α)* (Karamanlidis et al. 2007; Karamitri et al. 2009), which enhances *Ucp1* expression during cold-induced thermogenesis. Therefore, I investigated the role of CREB in adipose tissue, taking advantage of the Cre-*LoxP* cell type specific gene ablation approach. I hypothesized that loss of CREB in adipose tissue would lead to a decrease in lipolysis and thermogenesis. In my study, loss of adipocytic CREB leads to a moderate decrease in adipose tissue lipolysis. After subsequent evaluation of BAT activation and β_3-adrenergic receptor specific agonist-induced browning of white adipocytes, I demonstrate that CREB is not required for the regulation of thermogenesis.

4.3 Results

4.3.1 Ablation of CREB in Adipose Tissue does not affect body composition and adipocyte morphology

To assess adipocytic CREB function *in vivo*, I crossed homozygous $Creb^{loxP}$ mice (Lee et al. 2014) with *Adipoq-Cre* mice, which expresses Cre recombinase in adipocytes (Eguchi et al. 2011; Lee et al. 2013). Quantitative RT-PCR confirmed an ~80% decrease in *Creb* mRNA levels in both inguinal WAT (iWAT) and interscapular BAT of $Creb^{loxP/loxP}$*; Adipoq-Cre* mice (Figure 4.1a). Effective reduction in CREB protein levels in iWAT and BAT was confirmed by western blotting (Figure 4.1b).

Body composition was determined using nuclear magnetic resonance (NMR), and calculated as percentage lean and fat mass of total body weight for both the control and adipocyte-specific CREB-null mice. Body weights were comparable between the two groups, with similar lean to fat mass ratios, indicating that loss of adipocytic CREB did not affect overall body composition (Figure 4.2). Furthermore, there were also no significant difference in the histology of iWAT and BAT of adipocytic CREB-null mice relative to controls (Figure 4.3).

4.3.2 Loss of CREB in adipose tissue decreases fasting plasma fatty acid levels

During fasting, glucagon and catecholamine induce lipolysis in adipose tissue to release fatty acids and glycerol, which are utilized in peripheral tissues for energy production. To investigate the role of CREB in lipolysis, I measured circulating lipid levels in control and adipocyte-specific CREB-null mice during fasting and refeeding. Interestingly, while plasma triacylglycerol levels and ketone body levels were unchanged, fasting plasma non-esterified fatty acids (NEFA) levels were 40% lower in *Creb*$^{loxP/loxP}$*; Adipoq-Cre* mice compared to controls (Figure 4.4a, c, and d), indicating defects in fasting-induced lipolysis.

Adiponectin is an adipose tissue secreted hormone involved in the regulation of glucose and fatty acid metabolism. In adipose tissue, adiponectin facilitates lipid uptake and inhibits lipolysis (Kim et al. 2007; Qiao et al. 2011; Wedellová et al. 2011). In a previous study, inhibition of adipocytic CREB using a dominant-negative approach led to

an increase in circulating adiponectin levels (Qi et al. 2009). I measured circulating adiponectin levels to determine if loss of adipocytic CREB had any effect on adiponectin secretion in my model. Plasma adiponectin levels of adipocyte-specific CREB-null mice were comparable to those of control mice (Figure 4.4b).

To assess the involvement of CREB in the adipocyte transcriptome, I performed expression profiling via microarray in epididymal WAT isolated from both adipocytic *Creb*-null mice and controls that were fasted overnight. Surprisingly, loss of CREB had no effect on WAT gene expression and only a single gene, TAF-6-like RNA Polymerase II, p300/CBP-associated factor (PCAF)-associated factor (*Taf6l*), was found to be differentially expressed between $Creb^{loxP/loxP}$ and $Creb^{loxP/loxP}$; Adipoq-Cre (Table 4.1). Despite a 10-fold increase in expression levels, currently there is no known function for *taf6l* in lipid metabolism. Thus, the underlying mechanism of reduced fasting-induced lipolysis in adipocytic CREB-deficient mice it is still unclear, and further studies need to be performed to address this issue.

4.3.3 Ablation of adipocytic *Creb* does not affect BAT activity

Although loss of *Creb* in adipose tissue did not affect the morphology of BAT, I nevertheless investigated if *Creb* deficiency affected thermogenesis. To evaluate cold-induced thermogenesis, mice were kept at 4°C for 3 hours, and the core temperature was measured every 30 minutes. There were no appreciable differences in the changes of core temperature between $Creb^{loxP/loxP}$ *and* $Creb^{loxP/loxP}$*; Adipoq-Cre* mice (Figure 4.5). Because mice are able to maintain their core temperature via shivering as well as by

thermogenesis, I wanted to further determine and confirm the degree of BAT activation in adipocytic *Creb*-null mice.

To remove any shivering effects, I utilized pharmacological reagents to induce BAT activation. *In vivo*, BAT thermogenesis is activated by norepinephrine from the sympathetic nervous system (Landsberg et al. 1984). Mice were anesthetized and kept at 30°C, within the mouse thermoneutrality zone, during the course of the experiment to exclude shivering-induced thermogenesis. Norepinephrine-induced heat production was calculated by measuring CO_2 production and O_2 consumption. At the basal state, heat production was not significantly different between control mice and adipocyte-specific CREB mutants (Figure 4.6). Treatment with subcutaneous injection of norepinephrine increased body heat production by 5-fold in both control and mutant animals, and both the CO_2 production and the O_2 consumption were comparable between the two groups (Figure 4.6). These results indicate that loss of *Creb* has no effect on norepinephrine-induced thermogenesis.

In brown adipocytes, the effect of norepinephrine is carried out by β_3-adrenergic receptors coupled to the cAMP-signaling pathway (Cannon et al. 1996). However, it is important to note that norepinephrine also targets the liver and skeletal muscle as well as adipose tissue. Since β_3-adrenergic receptors are mainly present in adipose tissue, I decided to repeat the above experiment using the highly selective β_3-adrenergic receptor agonist CL-316,243, to eliminate any potential influence of other tissues on heat production. CL-316,243 was previously demonstrated to increase energy expenditure by activating thermogenesis in BAT (Grujic et al. 1997). Overall, the effects of CL-316,243

on CO_2 production and O_2 consumption were similar to those of norepinephrine, and loss of CREB had no effect on CL 316,243-induced heat production (Figure 4.7). This suggests CREB is not required for β_3-adrenergic receptor-mediated thermogenesis by BAT.

4.3.4 CREB is not required for CL-316,243-induced thermogenic gene expression.

Prolonged exposures to cold temperatures or treatment with β3-adrenergic receptor agonists, promotes the transformation of subpopulations of white adipocytes into beige adipocytes that express *Ucp1* (Young et al. 1984; Cousin et al. 1992; Petrovic et al. 2010). *Ucp1* contains CRE half-sites in its proximal promoter region (Kozak et al. 1994; Rim and Kozak 2002), and CREB can potentially regulate its expression during white-to-beige transformation at the transcriptional level. To further test the role of CREB in this process, both adipocytic CREB-null and control mice were kept within thermoneutrality and treated with CL-316,243 for 3 days. BAT and iWAT were harvested, and quantitative PR-PCR was performed to determine the expression levels of BAT identity genes and thermogenic genes. As expected, CL-316,243 treatment increased expression of thermogenic genes such as *Ucp1*, *Pgc-1α*, and type II iodothyronine deiodinase (*Dio2*) in BAT (Figure 4.8a). Loss of CREB did not have any effect on expression of BAT identity genes (Figure 4.8b). In iWAT, CL-316,243 treatment increased expression of *Ucp1*, indicating the presence of beige adipocytes (Figure 4.9a). Absence of CREB had no significant impact on CL-316,243-induced expression of thermogenic genes in iWAT (Figure 4.9a). As reported previously, expression levels of *Cidea* were increased in

iWAT after CL-316,243 (Figure 4.9b). However, the expression levels of BAT identity genes were similar between control and CREB-null cells (Figure 4.9b). These results indicate that both the transcriptional regulation of thermogenic genes by the cAMP signaling pathway and the browning of white adipocytes do not require CREB.

4.4 Discussion

Previous studies suggested a role for CREB in adipocyte function, but this hypothesis was never functionally confirmed by an adipose-specific ablation of *Creb in vivo*. Inhibition of CREB reduced inflammations in adipose tissue and increased secretion of adiponectin in mice expressing adipocytic A-CREB (Qi et al. 2009). However, there are well documented off-target effects associated with dominant-negative CREB mutant proteins (Ahn et al. 1998). Thus it is possible that the inhibition of combinations of proteins by dimerization with A-CREBs lead to the observed phenotype in the study above. Furthermore, a role for CREB in the regulation of thermogenesis and development of beige adipocytes, had been suggested based on enhanced CREB expression and activation in brown adipocytes upon rise in intracellular cAMP levels (Chaudhry and Granneman 1999; Thonberg et al. 2002), and CREB's observed ability to regulate the expression of *Pgc1α* and *Ucp1* (Kozak et al. 1994; Nedergaard et al. 2001; Rim and Kozak 2002). However, none of above studies investigated the requirement of CREB in thermogenesis.

To directly evaluate the requirement of CREB in lipolysis and thermogenesis, I utilized the Cre-*loxP* system for adipocyte specific ablation of *Creb* in mice. Loss of adipocytic CREB did not affect body weight or composition. BAT and iWAT of adipose tissue-specific CREB mutant had normal histology compared to controls. Interestingly, fasting-induced lipolysis was reduced in adipocytic CREB-null mice, but there were no alterations in circulating adiponectin levels. Expression analysis of global RNA levels in white adipose tissue did not reveal any potential mechanism underling the suppression of lipolysis in mice lacking adipocytic CREB. This might be explained in part by the fact that only a subpopulation of adipocytes in white adipose tissue undergoes lipolysis upon catecholamine signaling and that this changes in gene expression in a subset of adipocytes are masked by the bulk of unresponsive cells. To address this issue, future experiments will examine the effect of loss of CREB on lipolysis and lipolytic gene expression *in vitro* using primary adipocytes.

In regards to CREB's role in thermogenesis, $Creb^{loxP/loxP}$*; Adipoq-Cre* mice had similar cold tolerance as the control mice. BAT activation was further tested by treatment with norepinephrine and β_3-adrenergic agonist CL-316,243. Despite the suggestions from previous studies, loss of CREB had no effect on thermogenesis. Furthermore, expression levels of *Ucp1* and *Pgc1α* in adipocytic CREB-null BAT and iWAT were comparable to those of controls in both saline and CL-316,243 treated animals, suggesting proteins besides CREB can also bind to *cis*-regulatory elements in thermogenic genes containing CREs, and regulate their expression. Partial functional redundancy exists between the CREB/AFT family members that share dimerization domain and kinase-inducible

domain with CREB (Hummler et al. 1994), and they may be able to dimerize and recruit other transcription factors to promoters of target genes in the absence of CREB. This study thoroughly examined the role of CREB in thermogenesis at both the physiological and molecular levels, and I conclude that there are no non-redundant functions of CREB in the cAMP-mediated activation of thermogenesis and the white-to-beige adipocyte transformation, while it regulates fasting-induced lipolysis through an as of yet unknown mechanism.

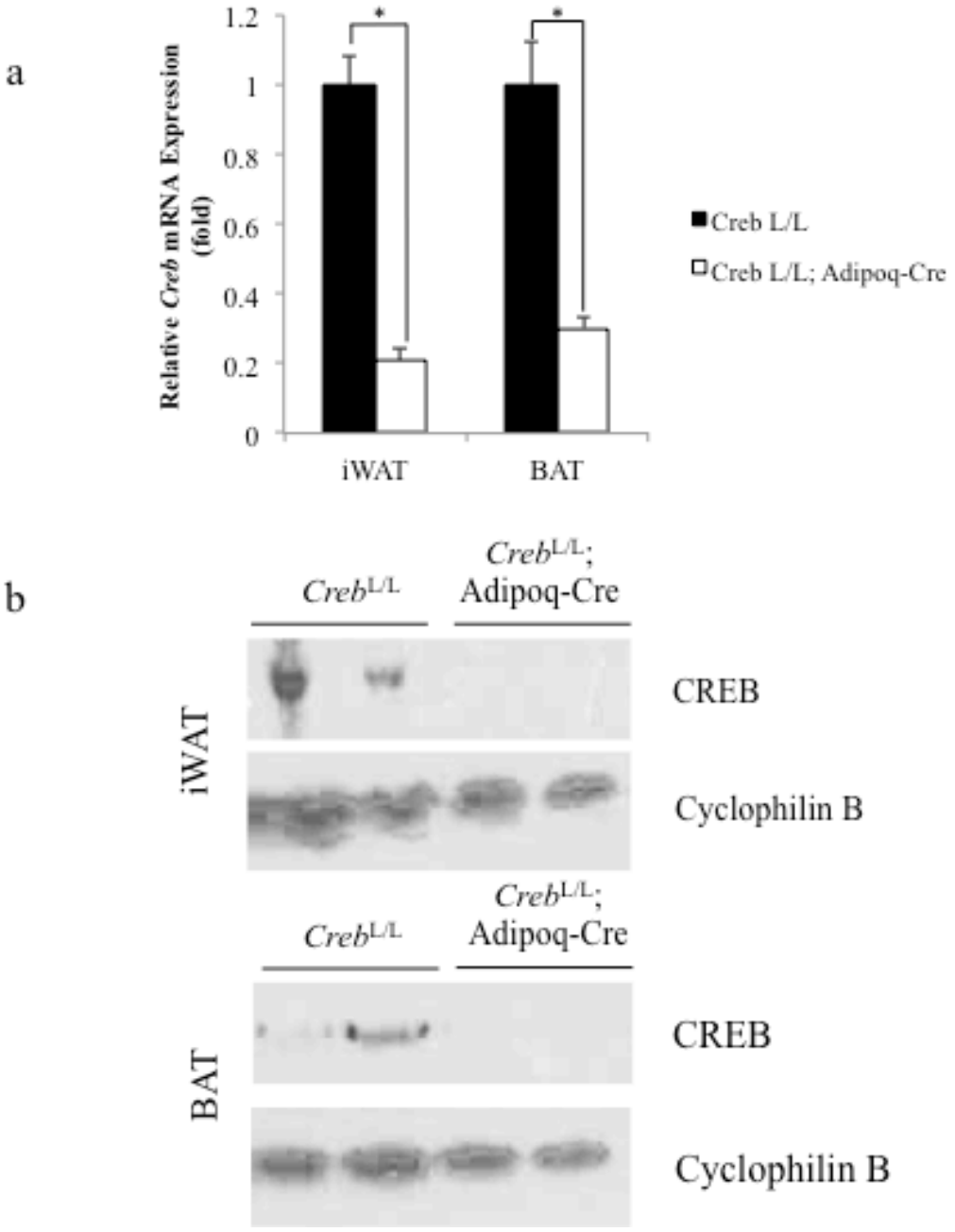

Figure 4.1: *Creb* ablation in adipocytes

(a) Quantitative RT-PCR to determine *Creb* mRNA levels in iWAT and BAT. Transcription levels in $Creb^{L/L}$ mice in each tissue were set to 1. Values are presented as the average ± SE. *$p < 0.005$. n = 7–8 for each group. (b) Western blot analysis of whole-cell lysates confirms loss of CREB in mutant $Creb^{L/L}$; *Adipoq-Cre* mice in both iWAT and BAT of control and mutant mice as indicated. Cyclophilin B was used as a loading control.

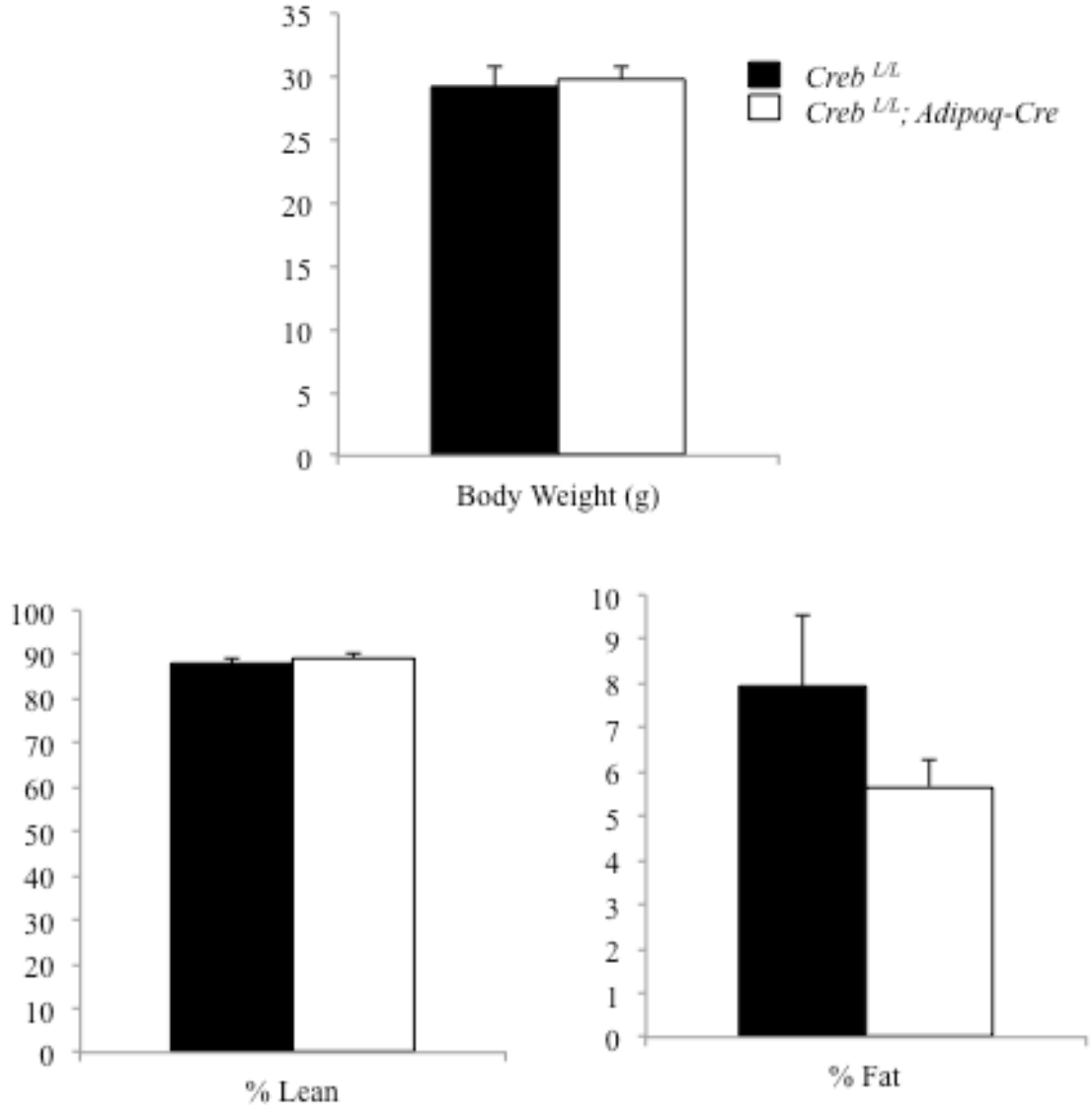

Figure 4.2: Loss of CREB does not affect body composition

Body weight and nuclear magnetic resonance (NMR) analysis to determine the percent lean mass and percent fat mass of control (*Creb*$^{L/L}$) and mutant (*Creb*$^{L/L;}$ *Adipoq-Cre*) mice. Values are presented as the average ± SE. n=7–8 in each group.

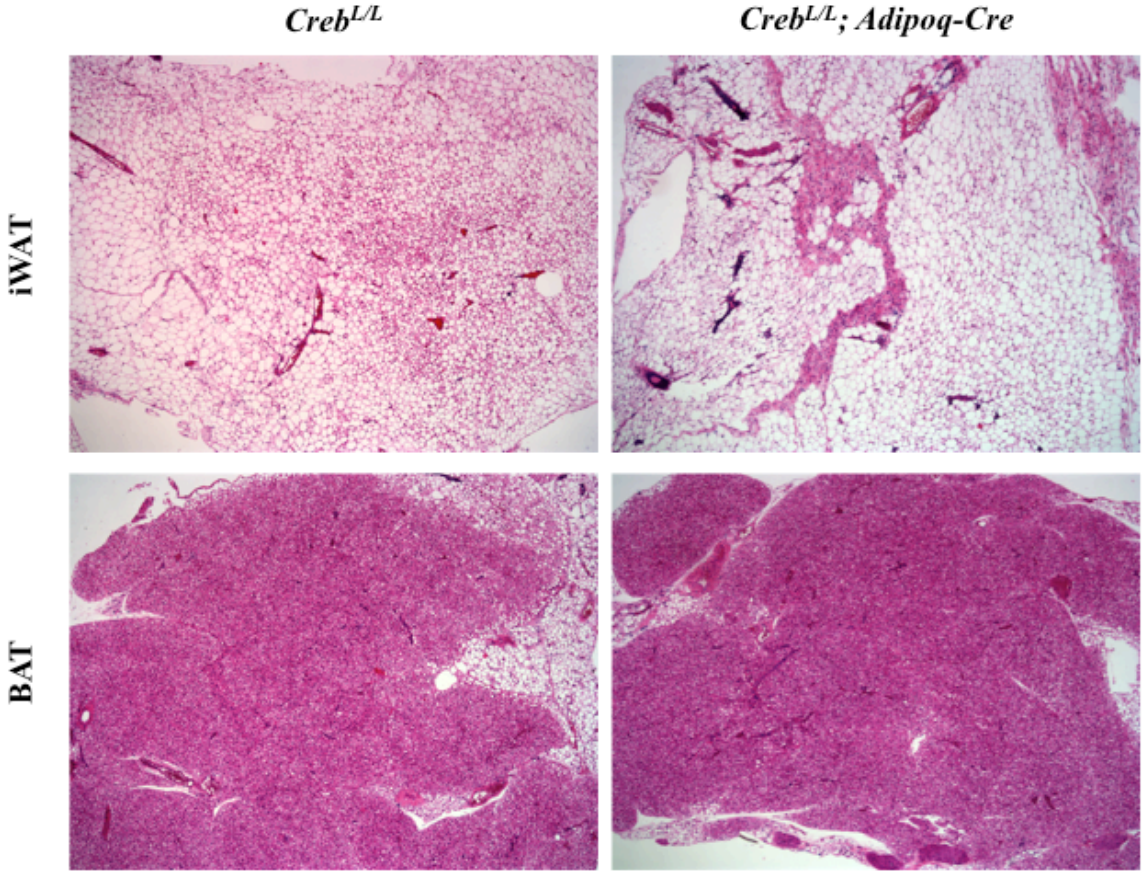

Figure 4.3: *Creb*-null iWAT and BAT displayed normal adipocyte morphology

H&E staining of iWAT and BAT isolated from both control (*Creb*$^{L/L}$) and mutant (*Creb*$^{L/L}$*; Adipoq-Cre*) mice.

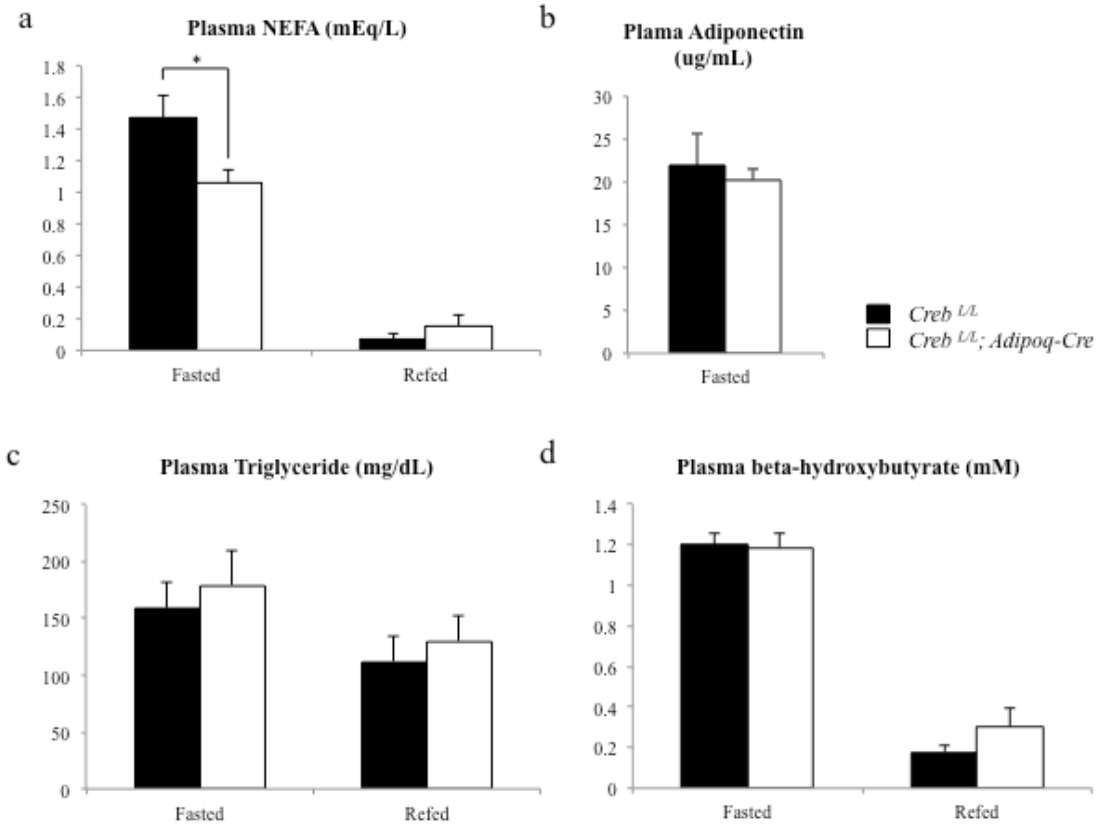

Figure 4.4: Reduction of fasting plasma non-esterified fatty acids (NEFA) levels in adipocytic CREB-null mice

Circulating NEFA (a), adiponectin (b), triacylglyceride (c), and β-hydroxybutyrate (d) levels were measured. Fasted plasma was collected after 16hr fasting, and refed plasma was collected after 2hr refeeding. Values are presented as average ± SE. n=17–18 in each group.

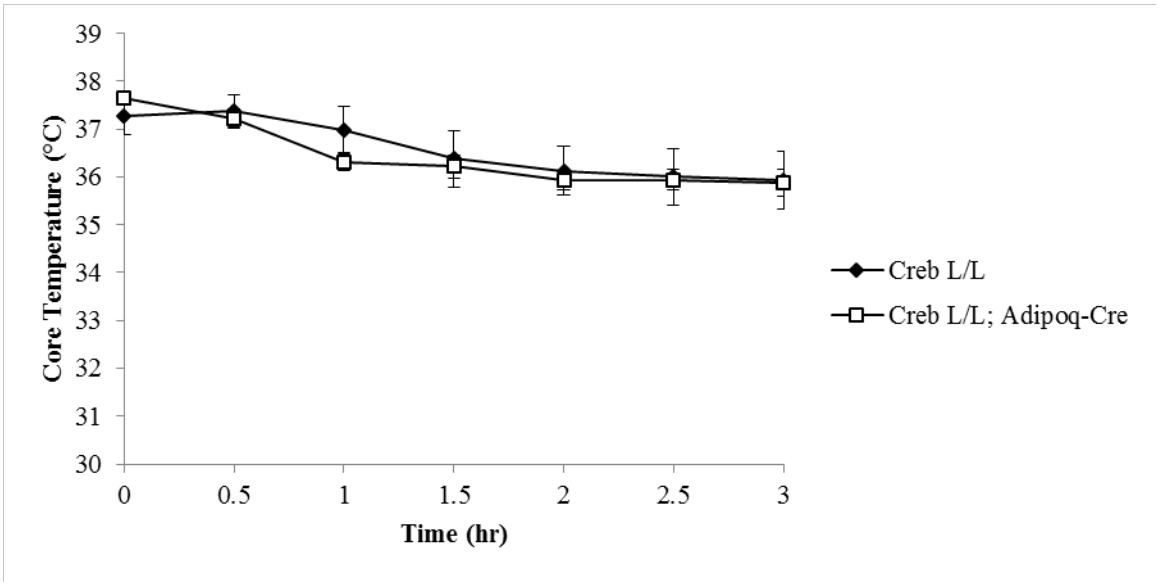

Figure 4.5: Response to cold exposure was not affected by the loss of adipocytic CREB

Core temperature measurements of *Creb*$^{loxp/loxp}$ and *Creb*$^{loxp/loxp}$*; Adipoq-Cre* mice during 3 hour exposure to 4°C. Values are presented as the average ± SE. n=4-5 for each group.

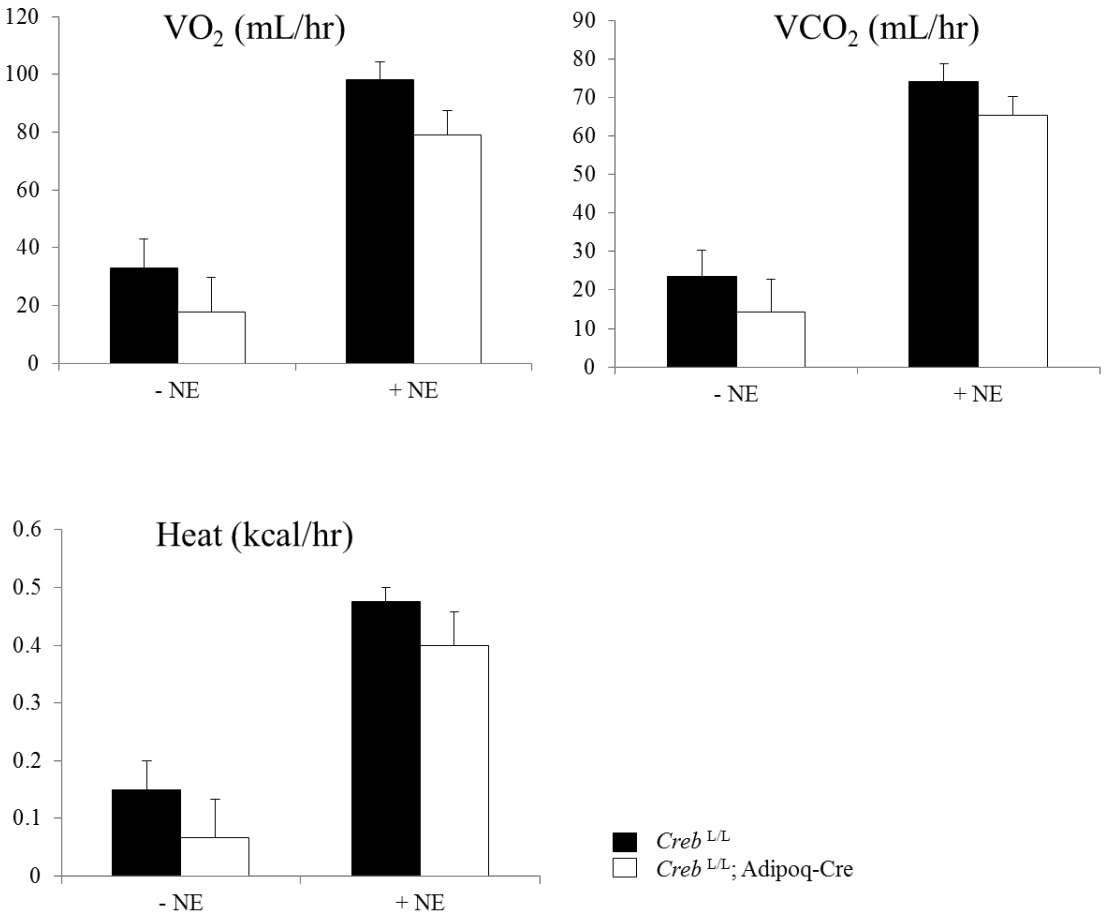

Figure 4.6: Adipocytic CREB-null mice exhibited similar norepinephrine (NE)-induced thermogenesis

O_2 consumption and CO_2 production were measured before and after NE injection. Heat production was calculated via indirect calorimetry. Mice were kept under anesthesia and at 30°C during the experiment. Values are presented as the average ± SE. n=3–4 for each group.

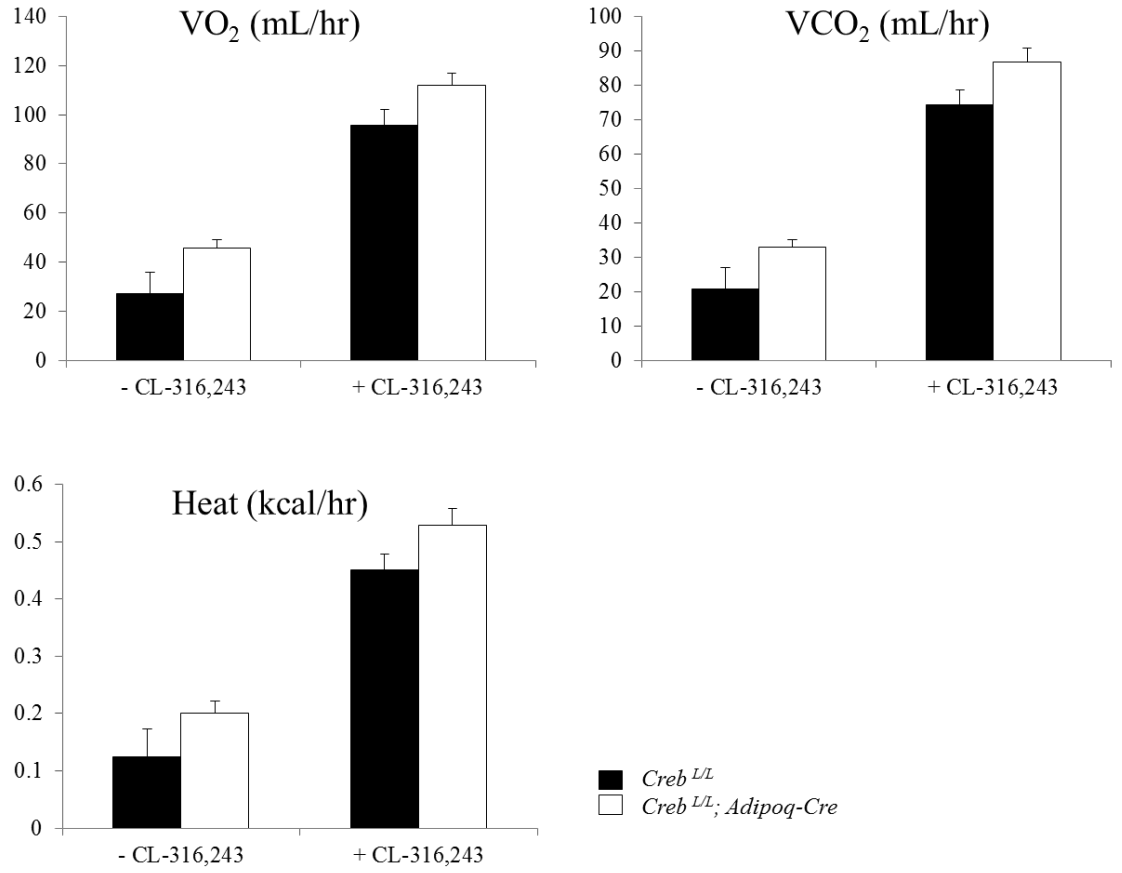

Figure 4.7: CL-316,243-induced thermogenesis of adipocyte specific CREB mutant mice was similar to controls

CL-316,243 is a β_3-adrenergic receptor agonist. O_2 consumption and CO_2 production was measured before and after CL-316,243 injection. Heat production was calculated via indirect calorimetry. Mice were kept under anesthesia and at 30°C during the experiment. Values are presented as the average ± SE. n=4–7 for each group.

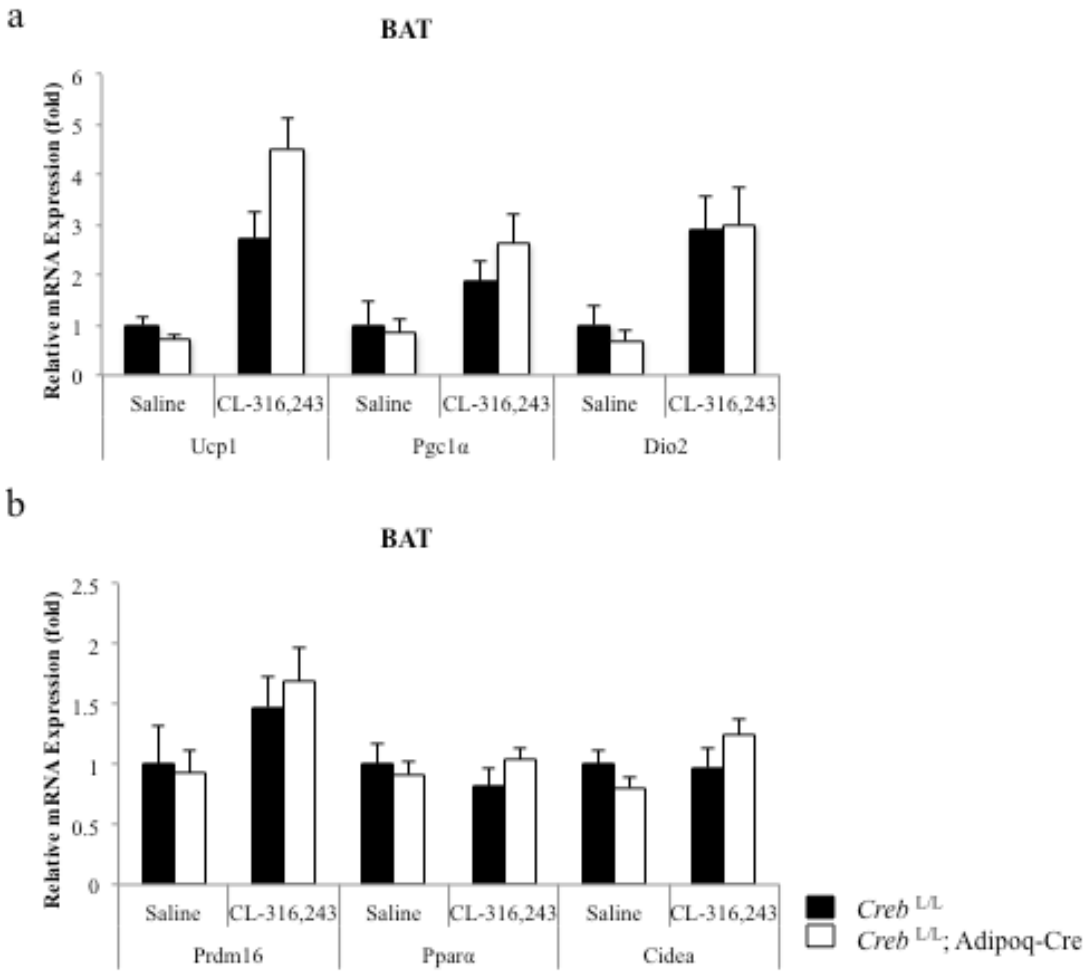

Figure 4.8: CL-316,243-induced expression levels of thermogenic genes in BAT of adipocytic CREB-null mice were similar to those of controls

(a) Quantitative RT-PCR to measure mRNA levels of *Ucp1, Pgc1α*, and *Dio2* in the BAT of control and adipocyte-specific adipocytic *Creb* mutant mice after 3-day injection with saline or CL-316,243. n = 9–12 for each group. (b) Quantitative RT-PCR to measure relative mRNA levels of *Prdm16*, *Pparα* and *Cidea* in BAT of control and adipocyte-specific Creb-deficient mice after saline or CL-316,243 treatment for 3 days. Transcription levels of control mice were set to 1. Values are presented as the average ± SE. n = 9–12 for each group.

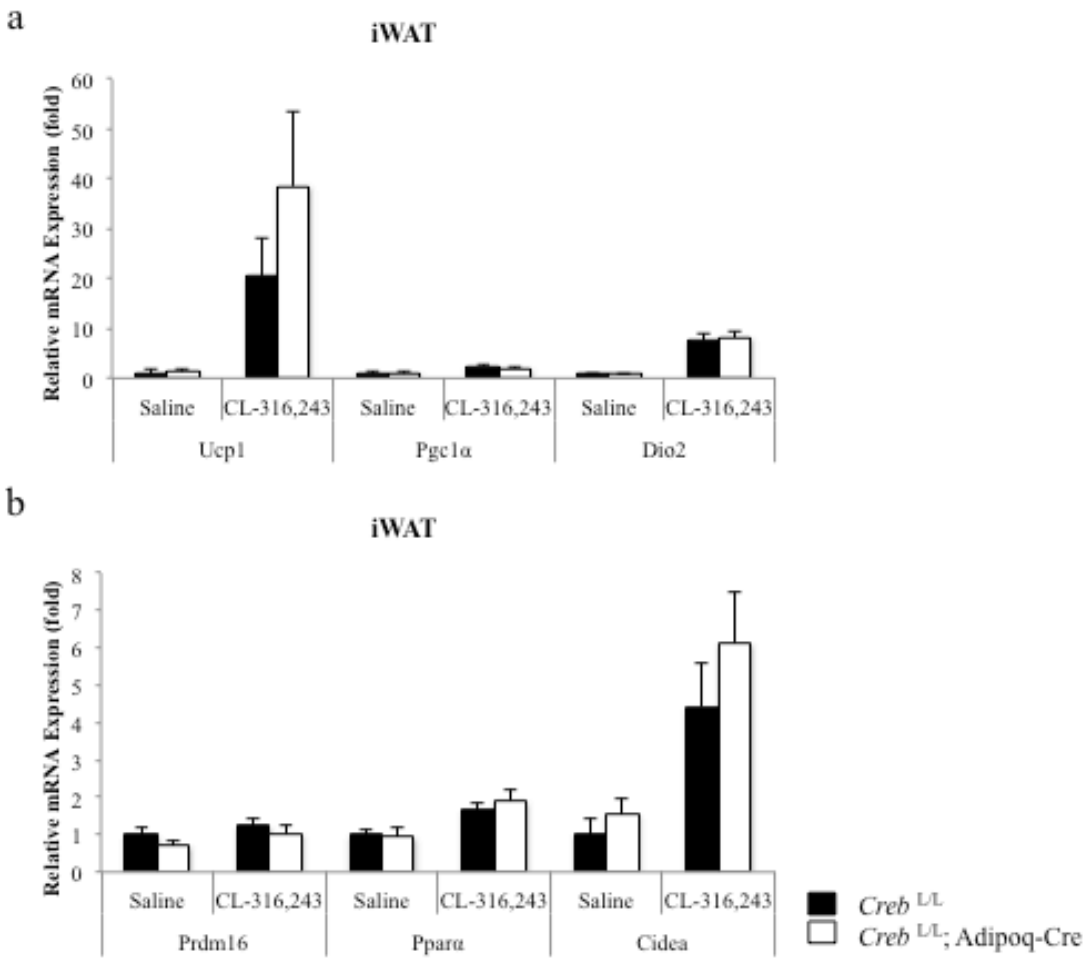

Figure 4.9: Similar expression levels of thermogenic and BAT identity genes in inguinal WAT (iWAT) of adipocytic *Creb*-null mice compared to controls

(a) Quantitative RT-PCR to measure mRNA levels of *Ucp1, Pgc1α*, and *Dio2* in the iWAT of control ($Creb^{L/L}$) and adipocyte-specific *Creb* mutant ($Creb^{L/L}$*; Adipoq-Cre)* mice after 3-day injection with saline or CL-316,243. n = 9–12 for each group. (b) Quantitative RT-PCR to measure relative mRNA levels of *Prdm16*, *Pparα* and *Cidea* in iWAT of control and adipocyte-specific Creb-deficient mice after saline or CL-316,243 treatment for 3 days. Transcription levels of control mice were set to 1. Values are presented as the average ± SE. n = 9–12 for each group.

Gene Name	Description	Fold Change
Taf6l	TAF-6-like RNA Polymerase II, p300/CBP-associated factor (PCAF)-associated factor	10.79

Table 4.1: A Single gene was significantly differentially expressed in adipocyte specific *Creb*-null eWAT

CHAPTER 5: Thesis Summary and Future Directions

5.1 Thesis Summary

In this thesis, I investigated of the role of the transcription factor CREB in the function of the liver and adipose tissue. Initially, I evaluated the role of hepatic CREB in glucose homeostasis and insulin sensitivity. Previous studies described CREB as the central regulator of the gluconeogenesis in the liver (Herzig et al. 2001; Erion et al. 2009). In those studies, inhibition of CREB activity reduced blood glucose levels and improved whole-body insulin sensitivity in rodent models of diabetes and insulin resistance, suggesting CREB as a potential therapeutic target to reduce hyperglycemia in T2DM patients. However, those studies utilized approaches that did not specifically target hepatocytic CREB. Therefore, I employed the Cre-*loxP* system to specifically determine the role of CREB in the regulation of hepatic glucose metabolism in Chapter III. Contrary to the previous understanding of CREB function, I report that glucose homeostasis was unaffected in mice conditionally deficient for *Creb* in the hepatocyte lineage (*Creb*$^{L/L}$*; AAV-Cre*). In addition, loss of hepatocytic CREB did not improve insulin sensitivity in mice fed a high fat diet. Indeed, gluconeogenic gene expression levels in hepatocytic *Creb* mutants were comparable to those of controls. Therefore, CREB has no non-redundant function in hepatic glucose metabolism. My data questions previous understanding of CREB and its suggested role in regulation of gluconeogenesis, and thus CREB is not a plausible therapeutic target to treat T2DM.

Secondly, I investigated the role of CREB in the regulation of lipolysis and thermogenesis in adipose tissue. Previous reports suggested CREB as a mediator for cAMP-effects on transcriptional regulation of lipogenic and thermogenic genes by

directly binding to their *cis*-regulatory regions (Kozak et al. 1994; Rim and Kozak 2002; Karamanlidis et al. 2007; Karamitri et al. 2009). However despite these suggestions, the function of CREB in adipocytes had never been directly evaluated. To assess adipocytic CREB function *in vivo*, I crossed homozygous $Creb^{loxP}$ mice (Lee et al. 2014) with *Adipoq-Cre* mice, which expresses Cre recombinase in adipocytes (Eguchi et al. 2011; Lee et al. 2013). In Chapter IV, I report data on the effect of *Creb* ablation in the regulation of lipolysis and thermogenesis in adipose tissue. Body composition and adipocyte morphology in adipocyte-specific *Creb*-null ($Creb^{loxP/loxP}$; *Adipoq-Cre*) mice were comparable to controls. Interestingly, fasting-induced lipolysis was moderately decreased in adipocytic *Creb*-null mice. Previous studies reported reduced catecholamine-induced lipolysis in obese subjects compared to non-obese subjects (Jensen et al. 1989; Bougneres et al. 1997; Langin et al. 2005). My data suggest CREB is required for fasting-induced lipolysis and dysfunction of CREB may contribute to the development of obesity. However, the expression profiling of epididymal WAT using microarray did not reveal potential CREB target genes that may affect lipolysis. Hence, the underlying mechanism of reduced fasting-induced lipolysis in adipocytic CREB-deficient mice it is still unclear, and further studies need to be performed to address this issue.

The understanding of CREB function in the liver and adipose tissue prior to this work was largely based upon studies that utilized a dominant-negative inhibitor of CREB (A-CREB) (Herzig et al. 2001; Herzig et al. 2003; Qi et al. 2009) or an antisense oligonucleotide (ASO) approach (Erion et al. 2009). A key shortcoming of these studies

is the lack of tissue specificity. CREB is a ubiquitously expressed transcription factor that serves various functions in different tissues. Therefore, inhibition of CREB activity in multiple metabolic tissues may lead to dysregulation of whole-body metabolism, making it harder to determine the tissue-specific role of CREB in the regulation of metabolism. Furthermore, the A-CREB dominant-negative model can inhibit other basic leucine zipper proteins in addition to CREB. In the A-CREB protein, an acidic amphipathic protein sequence replaces the CREB basic region connected to the N-terminus of the dimerization domain, allowing it to form a heterodimer with endogenous CREB protein, and prevents CREB binding to DNA (Ahn et al. 1998). Since the other members of the CREB/ATF share a conserved dimerization domain with CREB (Hummler et al. 1994), it is possible for A-CREB to inhibit these related proteins as well. Likewise, A-CREB may also bind to other basic leucine zipper proteins such as CCAAT/enhancer-binding protein beta (C/EBPβ), which dimerizes with CREB to bind CRE half-sites in *Ucp1* gene (Karamanlidis et al. 2007; Karamitri et al. 2009). The results of this thesis highlight the importance of re-evaluating previously suggested drug targets for human disease, and caution must to be taken when interpreting metabolic findings from mouse models with ill-defined molecular manipulations.

5.2 Future Directions

5.2.1 Functional redundancy among CREB, activating cAMP-responsive element modulator (CREM) and transcription factor-1 (ATF1) in the regulation of hepatic glucose metabolism

In Chapter III, I used conditional gene ablation to show that the transcription factor *Creb* has no non-redundant function in hepatic glucose metabolism. In the absence of hepatocytic CREB, there no changes in expression levels of proposed CREB target genes, which I suggest was due to functional redundancy between members of the CREB/ATF protein family. At least two additional members of the CREB/ATF family, namely CREM and ATF1, can also mediate transcriptional responses to cAMP signaling (Hurst and Jones 1987; Hai et al. 1988; Philippe and Missotten 1990; Hummler et al. 1994). These three CREB family members also share a conserved dimerization domain and kinase-inducible domain, suggesting partial functional redundancy. Indeed, CREM and ATF1 are capable of dimerizing with CREB as well as CREB, and all of which are targeted by PKA for phosphorylation (Hummler et al. 1994).

In Chapter III, I observed a two-fold increase in *Crem* mRNA and protein levels in the livers of $Creb^{loxP/loxP}$*;AAV-Cre* mice. Therefore, absence of changes in blood glucose levels and gluconeogenic gene expression in hepatocyte-specific *Creb*-null mice is likely explained by functional compensation by CREM. Similar hypothesis can be proposed with *Aft1,* if *Atf1* expression levels in the livers of hepatocytic *Creb*-deficient mice as well.

The above hypothesis could be further investigated by chromatin immunoprecipitation sequencing (ChIP-Seq) to determine if CREM and ATF1 bind to

CREB target genes in the absence of CREB. Comparison of these ChIP-Seq data to existing genome-wide CREB-binding data (Everett et al. 2013) may also reveal specific subclasses of CREB target genes are bound and regulated by CREM and ATF1. If CREM and/or ATF1 bind to gluconeogenic genes in the absence of CREB, as I hypothesize, the next step will be to generate mice with hepatocyte-specific ablation of *Creb*, *Crem* and *Atf1*. First, mice carry *loxP* flanked alleles of *Crem* and *Atf1*will be generated and these mice will be bred together with existing $Creb^{loxP/loxP}$ to generate $Creb^{loxP/loxP}$; $Crem^{loxP/loxP}$ $Atf1^{loxP/loxP}$mice. Hepatocyte-specific deletion of *loxP*-flanked alleles can be achieved by injection of AAV-Cre, as I demonstrated in Chapter III. Evaluating glucose metabolism and insulin resistance in these mice will complete the analysis of transcriptional regulation of glucose homeostasis by the cAMP-responsive transcription factors in the liver, and their impact on the regulation of blood glucose levels.

Genome-wide CREB-binding data revealed CREB binding at lipid metabolism genes (Everett et al. 2013). However, previous studies reported contradicting effects of CREB inhibition on the accumulation of lipid in the liver (Herzig et al. 2003; Erion et al. 2009). In Chapter III, I demonstrated that ablation of hepatocytic *Creb* alone does not improve nor aggravate fatty liver condition in high-fat diet fed, insulin-resistance mice, and this could be also due to potential functional compensation by CREM and ATF1. Therefore, the evaluation of hepatic and circulating lipid levels in $Creb^{loxP/loxP}$; $Crem^{loxP/loxP}$; $Atf1^{loxP/loxP}$; *AAV-Cre mice* is of particular importance, in order to understand if the CREB family of DNA binding proteins plays a role in the regulation of hepatic lipid metabolism.

5.2.2 Mechanism underlying reduced fasting-induced lipogenesis in adipocytic *Creb*-null mice

In Chapter IV, I demonstrated that CREB plays a role in the regulation of lipolysis during fasting with adipocyte-specific deletion of *Creb* using Cre-*loxP* system. However, transcriptome analysis of epididymal WAT by microarray expression profiling did not reveal any potential mechanism underlying the suppression of lipolysis in mice lacking adipocytic CREB. The absence of significantly differentially expressed genes in the above analyses can be explained in part by the fact that only a subpopulation of adipocytes in white adipose tissue undergoes lipolysis upon catecholamine signaling. It is also possible that the microarray platform was not sensitive enough to detect small changes in gene expression; this could overcome by the more sensitive RNA sequencing-based expression analysis.

To address these issues, primary preadipocytes isolated from mouse adipose tissue will be differentiated *in vitro* to determine changes in lipolytic gene expression in response to β-adrenergic signaling. Treating adipocytes with β-adrenergic agonist *in vitro* will ensure induction of lipolysis in all cells. Mammalian preadipocyte differentiation can be achieved *in vitro* with IDX cocktail, composed of insulin, dexamethasone, and isobutylmethylxanthine (IBMX) (Green and Meuth 1974; Russell and Ho 1976; Student et al. 1980). In preadipocytes, insulin signals through insulin growth factor-1 (IGF1) receptor to activate the adipogenic cascade (Rosen and MacDougald 2006). Dexamethasone treatment activates the glucocorticoid signaling

pathway which regulates adipocyte differentiation (Chapman et al. 1985), and IBMX increases intracellular cAMP levels by inhibiting soluble cyclic nucleotide (Elks and Manganiello 1985).

I previously isolated primary preadipocytes from inguinal WAT (iWAT) of *Creb*$^{loxP/loxP}$*; Adipoq-Cre* mice, and differentiated them *in vitro* by addition of the IDX cocktail. However, *Creb* was not deleted in differentiated adipocytes from *Creb*$^{loxP/loxP}$*; Adipoq-Cre* mice. Although *Adipoq* promoter should be active post-differentiation, it was identified that *Adipoq-Cre* is not efficient in deleting loxP-flanked alleles *in vitro*. Therefore, I am currently isolating primary preadipocytes from iWATs of *Creb*$^{loxP/loxP}$ mice, and they will be differentiated into mature white adipocytes in the culture using IDX cocktail. Ablation of *Creb* will be achieved by transfecting differentiated *Creb*$^{loxP/loxP}$ primary preadipocytes with *Cre*-mRNA. *Creb*$^{loxP/loxP}$ cells transfected with *eGFP*-mRNA will be used as controls. The cells will be treated with norepinephrine to induce lipolysis, relative levels of lipolysis between *Cre*-mRNA and *eGFP*-mRNA transfected adipocytes will be determined by measuring glycerol levels in collected media. Cells will also be harvested for RNA, and quantitative RT-PCR will be performed to determine and compare expression levels of lipolysis genes between *Cre*-mRNA and *eGFP*-mRNA transfected-*Creb*$^{L/L}$ primary white adipocytes. If significantly differentially expressed genes are identified, CREB binding at the promoters of these genes will be determined by cross-referencing genome-wide CREB-binding data that I obtained previously via chromatin-immunoprecipitation sequencing (ChIP-Seq) on white adipose tissue.

5.2.3 The role of adipocytic CREB in the development of insulin-resistance and obesity

In humans, fat tissues from obese individuals are resistant to catecholamine-induced lipolysis when compared to those of non-obese subjects (Jensen et al. 1989; Bougneres et al. 1997; Langin et al. 2005). In Chapter IV, I demonstrated decreased fasting-induced lipolysis in regular chow fed *Creb*-null mice. My results reinforce the importance of understanding adipocytic CREB's role in metabolic dysregulation, and suggest CREB have therapeutic potential for treatment of obesity. The diet-induced obesity model employed in Chapter III can be applied to $Creb^{loxP/loxP}$*; Adipoq-Cre* mice to study the function of *Creb* and lipolysis in the context of obesity and insulin-resistance. Body composition, energy expenditure, and circulating lipid levels will be evaluated to determine if ablation of *Creb* accelerates the development of obesity. In addition, catecholamine-induced lipolysis can be evaluated *in vitro* using fat biopsies from $Creb^{loxP/loxP}$ and $Creb^{loxP/loxP}$*; Adipoq-Cre* mice. Should the role of CREB in the development of obesity be confirmed in rodents, expression levels of *Creb* mRNA and protein should be measured in fat biopsies from and obese and non-obese human subjects to further validate CREB as a potential therapeutic target for obesity.

Although mice are commonly used in basic research to model human diseases, many pharmacological agents that work well in mice turn out to be ineffective in humans. Therefore, both similarities and differences between the mouse models of obesity and

obesity in humans must be thoroughly investigated before any serious consideration of mouse-model based targets can be used in human drug and therapeutic development.

REFERENCES

Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C. 1998. A Dominant-Negative Inhibitor of CREB Reveals that It Is a General Mediator of Stimulus-Dependent Transcription of c-fos. *Molecular and cellular biology* **18**: 967-977.

Altarejos JY, Goebel N, Conkright MD, Inoue H, Xie J, Arias CM, Sawchenko PE, Montminy M. 2008. The Creb1 coactivator Crtc1 is required for energy balance and fertility. *Nature medicine* **14**: 1112-1117.

Altarejos JY, Montminy M. 2011. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. *Nature reviews Molecular cell biology* **12**: 141-151.

Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, Giacobino JP, Matteis RD, Cinti S. 2010. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *American Journal of Physiology - Endocrinology And Metabolism* **298**: E1244-E1253.

Bartness TJ, Vaughan CH, Song CK. 2010. Sympathetic and sensory innervation of brown adipose tissue. *Int J Obes (Lond)* **34 Suppl 1**: S36-42.

Bjorndal B, Burri L, Staalesen V, Skorve J, Berge RK. 2011. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. *J Obes* **2011**: 490650.

Blecher M, Merlino NS, Ro'Ane JT, Flynn PD. 1969. Independence of the Effects of Epinephrine, Glucagon, and Adrenocorticotropin on Glucose Utilization from Those on Lipolysis in Isolated Rat Adipose Cells. *J Biol Chem* **244**: 3423-3429.

Bougneres P, Stunff CL, Pecqueur C, Pinglier E, Adnot P, Ricquier D. 1997. In vivo resistance of lipolysis to epinephrine. A new feature of childhood onset obesity. *Journal of Clinical Investigation* **99**: 2568-2573.

Bowers RR, Festuccia WTL, Song CK, Shi H, Migliorini RH, Bartness TJ. 2004. Sympathetic innervation of white adipose tissue and its regulation of fat cell number. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **286**: R1167-R1175.

Bruun JM, Pedersen SB, Richelsen B. 2001. Regulation of Interleukin 8 Production and Gene Expression in Human Adipose Tissue in Vitro. *The Journal of Clinical Endocrinology & Metabolism* **86**: 1267-1273.

Cannon B, Jacobsson A, Rehnmark S, Nedergaard J. 1996. Signal transduction in brown adipose tissue recruitment: noradrenaline and beyond. *Int J Obes Relat Metab Disord* **20 Suppl 3**: S36-42.

Carpéné C, Bousquet-Mélou A, Galitzky J, Berlan M, Lafontan M. 1998. Lipolytic Effects of β1-, β2-, and β3-Adrenergic Agonists in White Adipose Tissue of Mammals. *Annals of the New York Academy of Sciences* **839**: 186-189.

Chapman AB, Knight DM, Ringold GM. 1985. Glucocorticoid regulation of adipocyte differentiation: hormonal triggering of the developmental program and induction of a differentiation-dependent gene. *J Cell Biol* **101**: 1227-1235.

Chaudhry A, Granneman JG. 1999. Differential regulation of functional responses by β-adrenergic receptor subtypes in brown adipocytes. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **277**: R147-R153.

Chechi K, Nedergaard J, Richard D. 2014. Brown adipose tissue as an anti-obesity tissue in humans. *Obes Rev* **15**: 92-106.

Cinti S. 2012. The adipose organ at a glance. *Dis Model Mech* **5**: 588-594.

Clifford GM, Londos C, Kraemer FB, Vernon RG, Yeaman SJ. 2000. Translocation of Hormone-sensitive Lipase and Perilipin upon Lipolytic Stimulation of Rat Adipocytes. *J Biol Chem* **275**: 5011-5015.

Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M. 2003. TORCs: Transducers of Regulated CREB Activity. *Molecular Cell* **12**: 413-423.

Cottle MKW, Cottle WH. 1970. Adrenergic Fibers in Brown Fat of Cold-Acclimated Rats. *J Histochem Cytochem* **18**: 116-119.

Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L, Casteilla L. 1992. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *Journal of cell science* **103**: 931-942.

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng Y-H, Doria A et al. 2009. Identification and Importance of Brown Adipose Tissue in Adult Humans. *New England Journal of Medicine* **360**: 1509-1517.

DeFronzo RA, Ferrannini E, Simonson DC. 1989. Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: Contributions of excessive hepatic glucose production and impaired tissue glucose uptake. *Metabolism: clinical and experimental* **38**: 387-395.

Dietz J, Schwartz J. 1991. Growth hormone alters lipolysis and hormone-sensitive lipase activity in 3T3-F442A adipocytes. *Metabolism: clinical and experimental* **40**: 800-806.

Dwarki VJ, Montminy M, Verma IM. 1990. Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. *EMBO J* **9**: 225-232.

Eguchi J, Wang X, Yu S, Kershaw EE, Chiu PC, Dushay J, Estall JL, Klein U, Maratos-Flier E, Rosen ED. 2011. Transcriptional Control of Adipose Lipid Handling by IRF4. *Cell metabolism* **13**: 249-259.

Elks ML, Manganiello VC. 1985. A role for soluble cAMP phosphodiesterases in differentiation of 3T3-L1 adipocytes. *Journal of Cellular Physiology* **124**: 191-198.

Erion DM, Ignatova ID, Yonemitsu S, Nagai Y, Chatterjee P, Weismann D, Hsiao JJ, Zhang D, Iwasaki T, Stark R et al. 2009. Prevention of hepatic steatosis and hepatic insulin resistance by knockdown of cAMP response element-binding protein. *Cell metabolism* **10**: 499-506.

Everett LJ, Lay JL, Lukovac S, Bernstein D, Steger DJ, Lazar MA, Kaestner KH. 2013. Integrative genomic analysis of CREB defines a critical role for transcription factor networks in mediating the fed/fasted switch in liver. *BMC Genomics* **14**.

Exton JH, Friedmann N, Wong EH-A, Brineaux JP, Corbin JD, Park CR. 1972. Interaction of Glucocorticoids with Glucagon and Epinephrine in the Control of Gluconeogenesis and Glycogenolysis in Liver and of Lipolysis in Adipose Tissue. *J Biol Chem* **247**: 3579-3588.

Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. 2009. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell metabolism* **9**: 203-209.

Fisher AA, Ye D, Sergueev DS, Fisher MH, Shaw BR, Juliano RL. 2002. Evaluating the Specificity of Antisense Oligonucleotide Conjugates A DNA ARRAY ANALYSIS. *J Biol Chem* **277**: 22980-22984.

Frühbeck G. 2008. Overview of adipose tissue and its role in obesity and metabolic disorders. *Methods Mol Biol* **456**: 1-22.

Gagnon A, Antunes TT, Ly T, Pongsuwan P, Gavin C, Lochnan HA, Sorisky A. 2010. Thyroid-stimulating hormone stimulates lipolysis in adipocytes in culture and raises serum free fatty acid levels in vivo. *Metabolism: clinical and experimental* **59**: 547-553.

Gastaldelli A, Baldi S, Pettiti M, Toschi E, Camastra S, Natali A, Landau BR, Ferrannini E. 2000. Influence of obesity and type 2 diabetes on gluconeogenesis and glucose output in humans: a quantitative study. *Diabetes* **49**: 1367-1373.

Ghorbani M, Claus TH, Himms-Hagen J. 1997. Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of diet-induced obesity in rats treated with a β3-adrenoceptor agonist. *Biochemical Pharmacology* **54**: 121-131.

Ghorbani M, Himms-Hagen J. 1997. Appearance of brown adipocytes in white adipose tissue during CL 316,243-induced reversal of obesity and diabetes in Zucker fa/fa rats. *Int J Obes Relat Metab Disord* **21**: 465-475.

Gonzalez GA, Montminy MR. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**: 675-680.

Goodman HM, Bray GA. 1966. Role of thyroid hormones in lipolysis. *American Journal of Physiology -- Legacy Content* **210**: 1053-1058.

Green H, Meuth M. 1974. An established pre-adipose cell line and its differentiation in culture. *Cell* **3**: 127-133.

Grujic D, Susulic VS, Harper M-E, Himms-Hagen J, Cunningham BA, Corkey BE, Lowell BB. 1997. β3-Adrenergic Receptors on White and Brown Adipocytes Mediate β3-Selective Agonist-induced Effects on Energy Expenditure, Insulin Secretion, and Food Intake A STUDY USING TRANSGENIC AND GENE KNOCKOUT MICE. *J Biol Chem* **272**: 17686-17693.

Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E, Sattler W, Magin TM, Wagner EF, Zechner R. 2002. Hormone-sensitive Lipase Deficiency

in Mice Causes Diglyceride Accumulation in Adipose Tissue, Muscle, and Testis. *J Biol Chem* **277**: 4806-4815.

Hai TW, Liu F, Allegretto EA, Karin M, Green MR. 1988. A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. *Genes & development* **2**: 1216-1226.

Hajer GR, Haeften TWv, Visseren FLJ. 2008. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *European Heart Journal* **29**: 2959-2971.

Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM. 1997. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *PNAS* **94**: 8878-8883.

Hany TF, Gharehpapagh E, Kamel EM, Buck A, Himms-Hagen J, Schulthess GKv. 2002. Brown adipose tissue: a factor to consider in symmetrical tracer uptake in the neck and upper chest region. *Eur J Nucl Med* **29**: 1393-1398.

Harms M, Seale P. 2013. Brown and beige fat: development, function and therapeutic potential. *Nature medicine* **19**: 1252-1263.

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN, Klein WH. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**: 501-506.

Herzig S, Hedrick S, Morantte I, Koo S-H, Galimi F, Montminy M. 2003. CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-[gamma]. *Nature* **426**: 190-193.

Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P et al. 2001. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* **413**: 179-183.

Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, Cinti S. 2000. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *American Journal of Physiology - Cell Physiology* **279**: C670-C681.

Hoeffler JP, Meyer TE, Yun Y, Jameson JL, Habener JF. 1988. Cyclic AMP--Responsive DNA-Binding Protein: Structure Based on a Cloned Placental cDNA. *Science* **242**: 1430-1433.

Hogan S, Himms-Hagen J. 1980. Abnormal brown adipose tissue in obese (ob/ob) mice: response to acclimation to cold. *Am J Physiol* **239**: E301-E309.

Hu E, Liang P, Spiegelman BM. 1996. AdipoQ Is a Novel Adipose-specific Gene Dysregulated in Obesity. *J Biol Chem* **271**: 10697-10703.

Hummler E, Cole TJ, Blendy JA, Ganss R, Aguzzi A, Schmid W, Beermann F, Schütz G. 1994. Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 5647-5651.

Hurst HC, Jones NC. 1987. Identification of factors that interact with the E1A-inducible adenovirus E3 promoter. *Genes & development* **1**: 1132-1146.

Iourgenko V, Zhang W, Mickanin C, Daly I, Jiang C, Hexham JM, Orth AP, Miraglia L, Meltzer J, Garza D et al. 2003. Identification of a family of cAMP response

element-binding protein coactivators by genome-scale functional analysis in mammalian cells. *PNAS* **100**: 12147-12152.

Jensen MD, Haymond MW, Rizza RA, Cryer PE, Miles JM. 1989. Influence of body fat distribution on free fatty acid metabolism in obesity. *Journal of Clinical Investigation* **83**: 1168-1173.

Karamanlidis G, Karamitri A, Docherty K, Hazlerigg DG, Lomax MA. 2007. C/EBPβ Reprograms White 3T3-L1 Preadipocytes to a Brown Adipocyte Pattern of Gene Expression. *J Biol Chem* **282**: 24660-24669.

Karamitri A, Shore AM, Docherty K, Speakman JR, Lomax MA. 2009. Combinatorial Transcription Factor Regulation of the Cyclic AMP-response Element on the Pgc-1α Promoter in White 3T3-L1 and Brown HIB-1B Preadipocytes. *J Biol Chem* **284**: 20738-20752.

Karlsson M, Contreras JA, Hellman U, Tornqvist H, Holm C. 1997. cDNA Cloning, Tissue Distribution, and Identification of the Catalytic Triad of Monoglyceride Lipase EVOLUTIONARY RELATIONSHIP TO ESTERASES, LYSOPHOSPHOLIPASES, AND HALOPEROXIDASES. *J Biol Chem* **272**: 27218-27223.

Kim J-Y, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, Durand JL, Li H, Li G et al. 2007. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *Journal of Clinical Investigation* **117**: 2621-2637.

Klaus S. 2001. Brown Adipose Tissue: Thermogenic Function and Its Physiological Regulation. in *Adipose Tissue*, pp. 56-82. Landes Bioscience.

Koo S-H, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P et al. 2005. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* **437**: 1109-1111.

Kozak UC, Kopecky J, Teisinger J, Enerbäck S, Boyer B, Kozak LP. 1994. An upstream enhancer regulating brown-fat-specific expression of the mitochondrial uncoupling protein gene. *Molecular and cellular biology* **14**: 59-67.

Landsberg L, Saville ME, Young JB. 1984. Sympathoadrenal system and regulation of thermogenesis. *Am J Physiol* **247**: E181-189.

Langin D, Dicker A, Tavernier G, Hoffstedt J, Mairal A, Rydén M, Arner E, Sicard A, Jenkins CM, Viguerie N et al. 2005. Adipocyte Lipases and Defect of Lipolysis in Human Obesity. *Diabetes* **54**: 3190-3197.

Le Lay J, Tuteja G, White P, Dhir R, Ahima R, Kaestner KH. 2009. CRTC2 (TORC2) Contributes to the Transcriptional Response to Fasting in the Liver but Is Not Required for the Maintenance of Glucose Homeostasis. *Cell metabolism* **10**: 55-62.

Lee D, Lay JL, Kaestner KH. 2014. The transcription factor CREB has no non-redundant functions in hepatic glucose metabolism in mice. *Diabetologia* **57**: 1242-1248.

Lee KY, Russell SJ, Ussar S, Boucher J, Vernochet C, Mori MA, Smyth G, Rourk M, Cederquist C, Rosen ED et al. 2013. Lessons on Conditional Gene Targeting in Mouse Adipose Tissue. *Diabetes* **62**: 864-874.

Liu X, Pérusse F, Bukowiecki LJ. 1998. Mechanisms of the antidiabetic effects of the β3-adrenergic agonist CL-316243 in obese Zucker-ZDF rats. *American Journal of*

Physiology - Regulatory, Integrative and Comparative Physiology **274**: R1212-R1219.

Lockie SH, Stefanidis A, Oldfield BJ, Perez-Tilve D. 2013. Brown adipose tissue thermogenesis in the resistance to and reversal of obesity. *Adipocyte* **2**: 196-200.

Lowell BB, S-Susulic V, Hamann A, Lawitts JA, Himms-Hagen J, Boyer BB, Kozak LP, Flier JS. 1993. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* **366**: 740-742.

Magnusson I, Rothman DL, Katz LD, Shulman RG, Shulman GI. 1992. Increased rate of gluconeogenesis in type II diabetes mellitus. A 13C nuclear magnetic resonance study. *Journal of Clinical Investigation* **90**: 1323-1327.

Montminy MR, Bilezikjian LM. 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328**: 175-178.

Montoliu L, Blendy JA, Cole TJ, Schütz G. 1994. Analysis of the cAMP response on liver-specific gene expression in transgenic mice. *Fundam Clin Pharmacol* **8**: 138-146.

Mund RA, Frishman WH. 2013. Brown Adipose Tissue Thermogenesis: [beta]3-Adrenoreceptors as a Potential Target for the Treatment of Obesity in Humans. *Cardiology in Review November/December 2013* **21**: 265-269.

Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. 2001. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1504**: 82-106.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. 1996. The Transcriptional Coactivators p300 and CBP Are Histone Acetyltransferases. *Cell* **87**: 953-959.

Osuga J-i, Ishibashi S, Oka T, Yagyu H, Tozawa R, Fujimoto A, Shionoiri F, Yahagi N, Kraemer FB, Tsutsumi O et al. 2000. Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *PNAS* **97**: 787-792.

Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. 2010. Chronic Peroxisome Proliferator-activated Receptor γ (PPARγ) Activation of Epididymally Derived White Adipocyte Cultures Reveals a Population of Thermogenically Competent, UCP1-containing Adipocytes Molecularly Distinct from Classic Brown Adipocytes. *J Biol Chem* **285**: 7153-7164.

Philippe J, Missotten M. 1990. Functional characterization of a cAMP-responsive element of the rat insulin I gene. *J Biol Chem* **265**: 1465-1469.

Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. 1998. A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis. *Cell* **92**: 829-839.

Qi L, Saberi M, Zmuda E, Wang Y, Altarejos J, Zhang X, Dentin R, Hedrick S, Bandyopadhyay G, Hai T et al. 2009. Adipocyte CREB Promotes Insulin Resistance in Obesity. *Cell metabolism* **9**: 277-286.

Qiao L, Kinney B, Schaack J, Shao J. 2011. Adiponectin inhibits lipolysis in mouse adipocytes. *Diabetes* **60**: 1519-1527.

Ren X, Lutfy K, Mangubat M, Ferrini MG, Lee ML, Liu Y, Friedman TC. 2013. Alterations in phosphorylated CREB expression in different brain regions following short- and long-term morphine exposure: relationship to food intake. *Journal of Obesity* **2013**.

Rim JS, Kozak LP. 2002. Regulatory Motifs for CREB-binding Protein and Nfe2l2 Transcription Factors in the Upstream Enhancer of the Mitochondrial Uncoupling Protein 1 Gene. *J Biol Chem* **277**: 34589-34600.

Rosen ED, MacDougald OA. 2006. Adipocyte differentiation from the inside out. *Nature Reviews Molecular Cell Biology* **7**: 885-896.

Rothwell NJ, Saville ME, Stock MJ. 1982. Effects of Feeding a "Cafeteria" Diet on Energy Balance and Diet-Induced Thermogenesis in Four Strains of Rat. *J Nutr* **112**: 1515-1524.

Rothwell NJ, Stock MJ. 1979. A role for brown adipose tissue in diet-induced thermogenesis. *Nature* **281**: 31-35.

Russell TR, Ho R. 1976. Conversion of 3T3 fibroblasts into adipose cells: triggering of differentiation by prostaglandin F2alpha and 1-methyl-3-isobutyl xanthine. *Proceedings of the National Academy of Sciences of the United States of America* **73**: 4516-4520.

Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, Iwanaga T, Miyagawa M, Kameya T, Nakada K et al. 2009. High Incidence of Metabolically Active Brown Adipose Tissue in Healthy Adult Humans Effects of Cold Exposure and Adiposity. *Diabetes* **58**: 1526-1531.

Screaton RA, Conkright MD, Katoh Y, Best JL, Canettieri G, Jeffries S, Guzman E, Niessen S, Yates Iii JR, Takemori H et al. 2004. The CREB Coactivator TORC2 Functions as a Calcium- and cAMP-Sensitive Coincidence Detector. *Cell* **119**: 61-74.

Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scimè A, Devarakonda S, Conroe HM, Erdjument-Bromage H et al. 2008. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454**: 961-967.

Song Y, Altarejos J, Goodarzi MO, Inoue H, Guo X, Berdeaux R, Kim J-H, Goode J, Igata M, Paz JC et al. 2010. CRTC3 links catecholamine signalling to energy balance. *Nature* **468**: 933-939.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. 2001. The hormone resistin links obesity to diabetes. *Nature* **409**: 307-312.

Student AK, Hsu RY, Lane MD. 1980. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J Biol Chem* **255**: 4745-4750.

Sutherland EW, Robison GA. 1969. The Role of Cyclic AMP in the Control of Carbohydrate Metabolism. *Diabetes* **18**: 797-819.

Thiel G, Al Sarraj J, Stefano L. 2005. cAMP response element binding protein (CREB) activates transcription via two distinct genetic elements of the human glucose-6-phosphatase gene. *BMC Molecular Biology* **6**.

Thonberg H, Fredriksson JM, Nedergaard J, Cannon B. 2002. A novel pathway for adrenergic stimulation of cAMP-response-element-binding protein (CREB)

phosphorylation: mediation via alpha1-adrenoceptors and protein kinase C activation. *Biochem J* **364**: 73-79.

Trayhurn P. 1979. Thermoregulation in the diabetic-obese (db/db) mouse. The role of non-shivering thermogenesis in energy balance. *Pflugers Arch* **380**: 227-232.

Trayhurn P, Fuller L. 1980. The development of obesity in genetically diabetic-obese (db/db) mice pair-fed with lean siblings. The importance of thermoregulatory thermogenesis. *Diabetologia* **19**: 148-153.

Trayhurn P, James WP. 1978. Thermoregulation and non-shivering thermogenesis in the genetically obese (ob/ob) mouse. *Pflugers Arch* **373**: 189-193.

Tusher VG, Tibshirani R, Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *PNAS* **98**: 5116-5121.

van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JMAFL, Kemerink GJ, Bouvy ND, Schrauwen P, Teule GJJ. 2009. Cold-Activated Brown Adipose Tissue in Healthy Men. *New England Journal of Medicine* **360**: 1500-1508.

Vitali A, Murano I, Zingaretti MC, Frontini A, Ricquier D, Cinti S. 2012. The adipose organ of obesity-prone C57BL/6J mice is composed of mixed white and brown adipocytes. *J Lipid Res* **53**: 619-629.

Wajchenberg BL. 2000. Subcutaneous and Visceral Adipose Tissue: Their Relation to the Metabolic Syndrome. *Endocrine Reviews* **21**: 697-738.

Wedellová Z, Dietrich J, Siklová-Vítková M, Kološtová K, Kováčiková M, Dušková M, Brož J, Vedral T, Stich V, Polák J. 2011. Adiponectin inhibits spontaneous and

catecholamine-induced lipolysis in human adipocytes of non-obese subjects through AMPK-dependent mechanisms. *Physiol Res* **60**: 139-148.

Woolf TM, Melton DA, Jennings CG. 1992. Specificity of antisense oligonucleotides in vivo. *PNAS* **89**: 7305-7309.

Wu J, Boström P, Sparks Lauren M, Ye L, Choi Jang H, Giang A-H, Khandekar M, Virtanen Kirsi A, Nuutila P, Schaart G et al. 2012. Beige Adipocytes Are a Distinct Type of Thermogenic Fat Cell in Mouse and Human. *Cell* **150**: 366-376.

Wu Z, Huang X, Feng Y, Handschin C, Feng Y, Gullicksen PS, Bare O, Labow M, Spiegelman B, Stevenson SC. 2006. Transducer of regulated CREB-binding proteins (TORCs) induce PGC-1 transcription and mitochondrial biogenesis in muscle cells. *PNAS* **103**: 14379-14384.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC et al. 1999. Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. *Cell* **98**: 115-124.

Xue B, Coulter A, Rim JS, Koza RA, Kozak LP. 2005. Transcriptional synergy and the regulation of Ucp1 during brown adipocyte induction in white fat depots. *Molecular and cellular biology* **25**: 8311-8322.

Yadav VK, Oury F, Tanaka KF, Tanaka K, Thomas T, Wang Y, Cremers S, Hen R, Krust A, Chambon P et al. 2011. Leptin-dependent serotonin control of appetite: temporal specificity, transcriptional regulation, and therapeutic implications. *J Exp Med* **208**: 41-52.

Yeung HWD, Grewal RK, Gonen M, Schöder H, Larson SM. 2003. Patterns of 18F-FDG Uptake in Adipose Tissue and Muscle: A Potential Source of False-Positives for PET. *J Nucl Med* **44**: 1789-1796.

Young P, Arch JRS, Ashwell M. 1984. Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Letters* **167**: 10-14.

Zhang X, Odom DT, Koo S-H, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E et al. 2005. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 4459-4464.

Zhou XY, Shibusawa N, Naik K, Porras D, Temple K, Ou H, Kaihara K, Roe MW, Brady MJ, Wondisford FE. 2004. Insulin regulation of hepatic gluconeogenesis through phosphorylation of CREB-binding protein. *Nature medicine* **10**: 633-637.

Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A et al. 2004. Fat Mobilization in Adipose Tissue Is Promoted by Adipose Triglyceride Lipase. *Science* **306**: 1383-1386.