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Role of Exchange Protein Directly Activated by cAMP Isoform 1 in Energy Homeostasis

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Role of Exchange Protein Directly Activated by cAMP Isoform 1 in Energy Homeostasis

by

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Dedication

To my family for their support and encouragement.

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Cyclic AMP-mediated signaling pathways are important for maintaining metabolic homeostasis. The effects of cAMP are mediated mainly by protein kinase A (PKA) and the more recently discovered exchange protein directly activated by cAMP (Epac). Epac has two isoforms, the ubiquitously expressed Epac1 and the more tissue restricted Epac2. The biological functions of Epac1 have been revealed, including cardiac stress, chronic pain, cancer and infections. However, the roles of Epac1 in energy balance are relatively unclear. To investigate the integrated metabolic functions of Epac1 in leptin-mediated energy balance *in vivo*, we have generated floxed alleles for Epac1 and a global loss of function mutant for Epac1. Epac1 global deficiency mice are more resistant to high fat diet (HFD)-induced obesity, with reduced adiposity and enhanced glucose sensitivity. Although Epac1 knockout (KO) mice have significantly lower plasma leptin, an important hormone for controlling energy homeostasis, the leptin sensitivity in hypothalamus is greatly enhanced compared to wild-type (WT) littermates. These results demonstrate Epac1 plays an important role in regulating adiposity and energy balance.

To investigate if the apparent phenotypes of Epac1 KO mice are due to the loss of Epac1 functions specifically in the central nervous system or in peripheral adipose

tissues, which is the dominant source for leptin production and secretion, we generated adipose tissue specific Epac1 knockout (AEKO) mice. Surprisingly, AEKO mice show more severe metabolic syndromes after being challenged by HFD, with increased average daily food intake, decreased energy expenditure and impaired glucose handling capability. Despite that AEKO mice on HFD display increased body weight; these mice have decreased circulating leptin levels as compared to the WT controls. *In vivo* and *in vitro* analyses further reveal that suppression of Epac1 decreases leptin mRNA expression and secretion via inhibiting CREB and Akt phosphorylation, respectively.

Taken together, our results suggest that Epac1 plays an orexigenic role in central nervous system (CNS) while adipose Epac1 has an anorectic role. Deletion of Epac1 in CNS results in a more potent anorectic effect which overcomes the orexigenic effect of Epac1 deficiency in adipose tissue.

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List of Abbreviations

AC	Adenylyl Cyclase
ACC	Acetyl-CoA Carboxylase
ADD1	Adipocyte Determination and Differentiation- dependent Factor 1
Agrp	Agouti- Related Protein
AMPK	AMP-activated Protein Kinase
AP-2β	Activator Protein-2β
ARC	Arcuate Nucleus
BAT	Brown Adipose Tissue
BMI	Body Mass Index
CCK	Cholecystokinin
C/EBP	CCAAT-Enhancer-Binding Protein
CNS	Central Nervous System
CREB	cAMP Response Element-Binding Protein
CRH	Corticotropin- Releasing Hormone
DIO	Diet Induced Obesity
DMH	Dorsomedial Hypothalamus
Epac	Exchange Protein Directly Activated by cAMP
ER	Endoplasmic Reticulum
FOSL2	Fos-related Antigen 2
GEF	Guanine Exchange Factor
GI	Gastrointestinal
GLP-1	Glucagon-like peptide -1

HFD	High Fat Diet
HSL	Hormone Sensitive Lipase
Icv	Intracerebroventricular
IHCL	Intrahepatocellular Lipid
JAK	Janus Kinase
IL-6	Interleukin-6
LH	Lateral Hypothalamus
LR	Leptin Receptor
LRP5	Lipoprotein Receptor- Related 5
MC3R	Melanocortin-3-Receptor
MC4R	Melanocortin-4-Receptor
MCH	Melanin Concentrating Hormone
MSC	Mesenchymal Stem Cell
α-MSH	α-Melanocyte Stimulating Hormone
NPY	Neuropeptide Y
PDE	Phosphodiesterase
PI3K	Phosphatidylinositol-3 Kinase
PKA	Protein Kinase A
POMC	Pro-opiomelanocortin
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PTP1B	Protein Tyrosine Phosphatase 1B
PVN	Paraventricular Nucleus
RBP4	Retinol binding protein- 4

SHP-2	Src Homology 2 Containing Phosphatase
SOCS3	Suppressor of Cytokine Signaling 3
SREBP-1	Sterol regulatory element binding protein 1
STAT	Signal Transducer and Activator of Transcription
T1R	Type 1 Taste Receptor
TAG	Triacylglycerol
TCF	T cell-specific transcription factor
TCF7L1	Transcription factor 7-like 1
TGF-β	Transforming Growth Factor-β
TNFα	Tumor necrosis factor- α
UCP-1	Uncoupling Protein-1
VMH	Ventromedial Hypothalamus
WAT	White Adipose Tissue
ZFP-423	Zinc-Finger Protein 423

Chapter 1 BACKGROUND AND SIGNIFICANCE

1.1 OBESITY OVERVIEW

1.1.1 Introduction

Obesity is now a severe health problem in the United States: nearly 35% of all adults and 17% of the youth are obese, and more than two-thirds of the adults have body weights problems.[1] The World Health Organization introduced Body Mass Index (BMI) as a measurement of tissue mass, particularly body fat. The BMI is defined as body weight in kilograms divided by the square of height in meters. An individual with a BMI equal to or more than 25 is considered overweight while BMI no less than 30 is considered obese.[2] In 2013, the American Medical Association officially classified obesity as a disease.[3] Obesity is also associated with various other diseases, such as cardiovascular diseases, stroke, type 2 diabetes and certain types of cancer, among the top ten disease-associated mortalities in the US[4] [5]. Around 5 % to 10% of the total US health care spending goes to meet direct medical costs of obesity and its related diseases.[6] Therefore, it is imperative to understand the pathogenesis and to develop effective strategies to treat obesity.

The molecular etiology of obesity is still unclear; however, one key factor is chronic dysregulation of energy balance – caused by chronic excess energy intake over energy expenditure with the surplus energy stored as fat mainly in adipose tissue.

1.1.2 Obesity and Central Nervous System Regulation

The central nervous system (CNS), especially the hypothalamus, plays a crucial role in regulating energy homeostasis. Hypothalamus has several regions involved in regulation of food intake: arcuate nucleus (ARC), paraventricular nucleus (PVN),

dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), and ventromedial hypothalamus (VMH).[7] (**Figure 1.1**)

ARC is one of the key hypothalamic sites responsible for regulating food intake through two major neuronal populations. The orexigenic population expresses neuropeptide Y (NPY)/agouti-related protein (Agrp) and the anorectic population expresses pro-opiomelanocortin (POMC).[8] These two types of neuronal projections target other hypothalamic sites, forming a complicated network to regulate energy homeostasis (**Figure 1.2**). Activated POMC neurons release α-melanocyte stimulating hormone (α-MSH), which is the cleavage product of POMC and functions by binding to melanocortin-3 receptors (MC3R) and melanocortin-4 receptors (MC4R) to inhibit appetite and elevate energy expenditure.[9] POMC knockout mice have significant higher body weight compared to wide type (WT) littermates fed on either regular chow diet or high fat diet (HFD).[10] MC4R is expressed throughout the hypothalamus, with the most abundant expression in the PVN region.[11] MC4R knockout mice show severe metabolic syndromes with hyperphagia, excessive body weight, hyperglycemia and hyperleptinemia phenotypes.[12] In humans, about 6% of the childhood obesity have MC4R mutations, which is the top known pathogenesis of monogenic obesity.[13] Agrp is the natural antagonist of MC3R and MC4R[14]. Central ablation of NPY/Agrp neurons results in robust anorexia in adult mice.[15]

PVN is another important hypothalamic site for integration of metabolic signals to regulate energy homeostasis. Injection of Agrp into PVN in mice leads to long-lasting stimulated hyperphagia. In contrast, PVN microinjection of MSH analog significantly inhibited appetite.[16] Both NPY and α-MSH terminals from ARC project to DMH sites at relatively high levels. α-MSH fibers project from DMH to PVN, targeting thyrotroph in releasing hormone neurons.[17] DMH is an important hypothalamic site in relaying neuropeptides and integrating peripheral metabolic signals. Compared to lean mice with

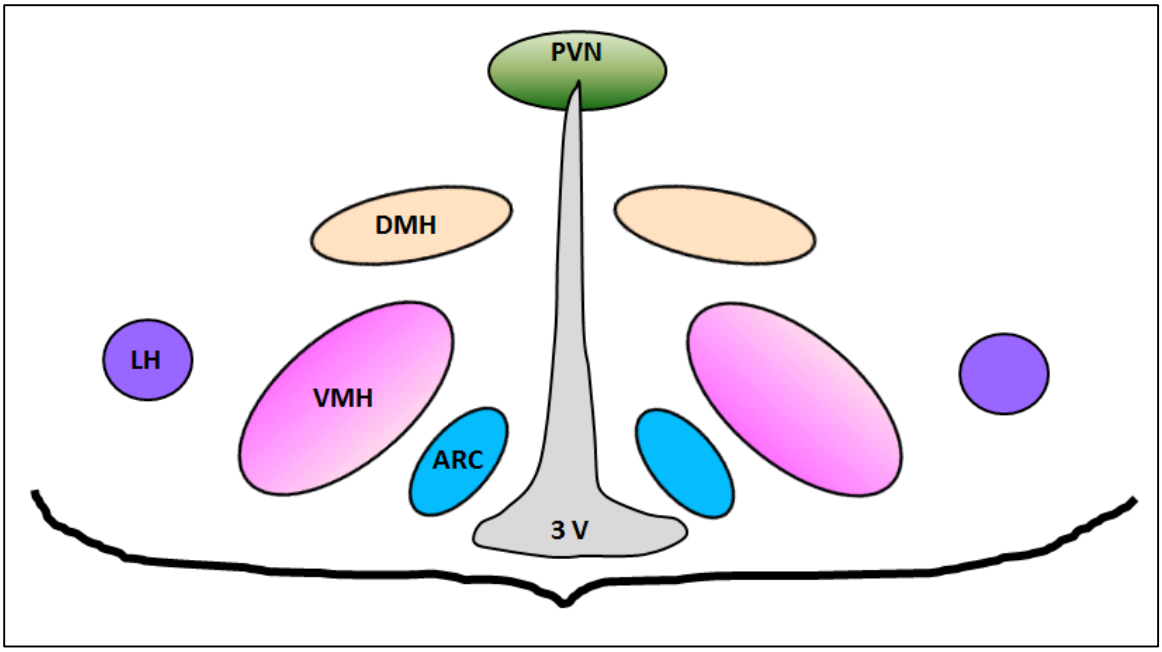

Figure 1.1 Schematic Diagram illustrating the regions of hypothalamus. 3V, third ventricle; ARC, arcuate nucleus; PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; VMH, ventromedial hypothalamus.

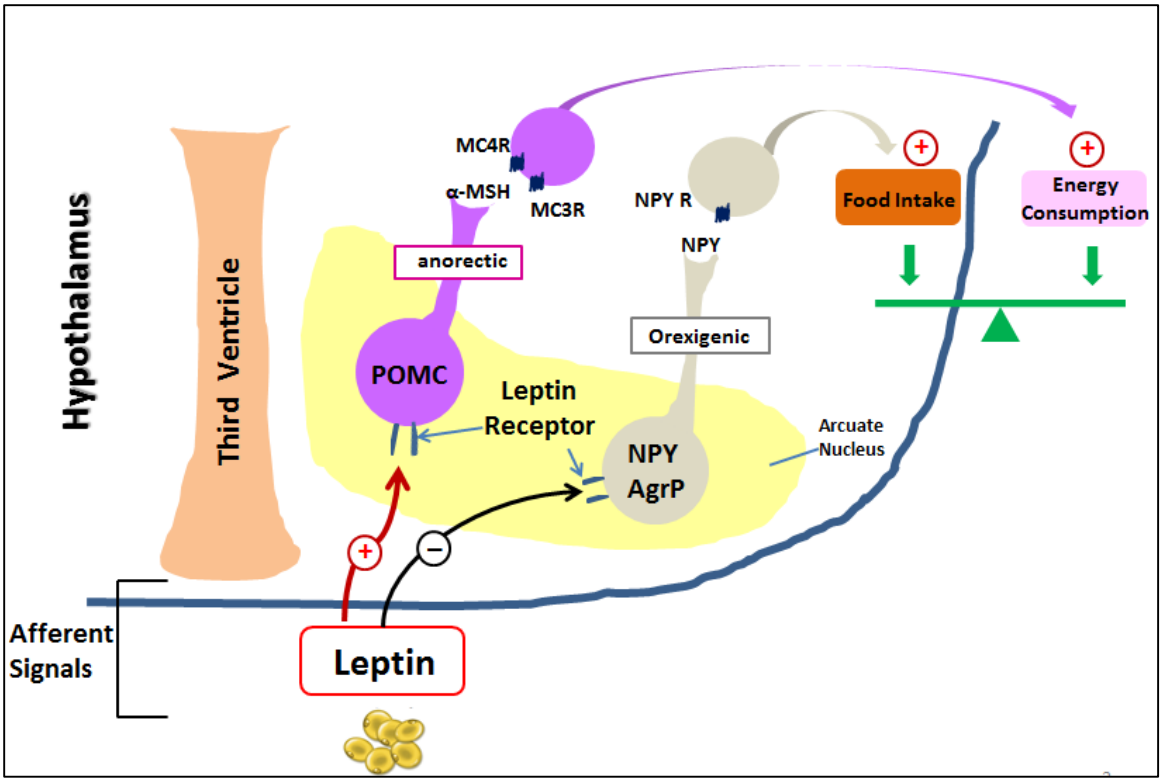

FIGURE 1.2 ROLES OF ARCUATE NUCLEUS NEURONS IN ENERGY HOMEOSTASIS. POMC neurons can be activated by adipocyte-derived hormone-leptin, while NPY/Agrp neurons can be inhibited by leptin. POMC neurons secrete the anorectic peptide α-MSH, which subsequently targets the MC3R and MC4R in PVN regions. On the other hand, NPY and Agrp are orexigenic peptides which are secreted from NPY/Agrp neurons.

restricted NPY mRNA expression in ARC, its expression drops by almost 50% in diet-induced obesity (DIO) mice with distribution to DMH and VMH regions as well.[18] In LH, both orexigenic NPY/Agrp and anorectic α-MSH neurons project from ARC to interact with melanin concentrating hormone (MCH) and orexins, which are all orexigenic peptides. Knocking out MCH receptors protects the mice from DIO by enhancing energy expenditure.[19] On the other hand, central administration of MCH to lateral ventricle induces hyperphagia.[20]

1.1.3 Roles of Peripheral Tissues in Obesity

Although CNS is the most important integrator on controlling food intake and energy expenditure, various peripheral signals from the gastrointestinal (GI) tract, muscles, oral cavity and adipose tissue are also involved in the regulation of energy homeostasis.

The tongue is an organ that tastes different flavors, such as sweetness and bitterness. Obese people with BMI up to 50 are more prone to sweet and fatty food due to lower sensitivity to sweetness.[21] Type 1 taste receptor (T1R) on the tongues have three isoforms: the heterodimers of T1R2 and T1R3 are responsible for sensing sweetness while the combinations of T1R1 and T1R3 detect umami. Obese people have genetic variations in the genes encoding T1R2[22].

Various hormones secreted from GI, such as ghrelin, cholecystokinin (CCK) and glucagon-like peptide -1 (GLP-1), have been discovered to regulate food intake. Ghrelin is secreted from the stomach and the only known orexigenic hormone from peripheral tissues. After crossing the blood brain barrier, ghrelin targets orexigenic NPY and Agrp neurons in ARC to stimulate appetite. Intracerebroventricular (Icv) injection of ghrelin in rats dose- dependently increases food intake, which can be impaired by blocking either NPY or Agrp activities.[23] Intraperitoneal or intravenous injection of ghrelin stimulates food intake in both rats[24] and human beings[25] respectively. On the other hand, it fails to

enhance food intake in vagotomy patients even after the infusion of ghrelin, which, underlying the requirement of an intact vagus nerve for the peripheral effects of ghrelin.[26]

Skeletal muscle is another organ highly impacted by obesity. There are two types of skeletal muscle fibers: type I and type II. The time needed to reach the peak tension is different for these two fibers: type I is slow twitching, while type II is fast twitching. Type II can be further divided into at least two sub-types: IIa and IIb. IIb is low in mitochondria while type I is abundant, and IIa has intermediate mitochondrial level. Obese people have significantly higher type IIb and lower type I fiber percentages as compared to lean people.[27] Similarly, type IIa fibers are decreased in Zucker rats with obesity and diabetes.[28] Moreover, skeletal muscle fatty acid oxidation is impaired in obese people as compared to lean control groups and this ability can be rescued by exercises[29] and PPARγ agonist drug rosiglitazone.[30] Fatty acid oxidation ability is crucial to skeletal muscles because type I fiber obtains ATP dominantly from oxidative metabolism due to high mitochondria levels while IIb fiber has the least dependence on it owing to low mitochondria level. Skeletal muscle fatty acid oxidation is an important predicator for obesity and type II diabetes. Obese individuals have fewer type I fibers and more type II fibers[27].

Obesity can cause excess fat accumulation in the liver, which is tightly related to the circulating free fatty acids from adipose tissue. The intrahepatocellular lipid (IHCL) has a positive relationship with total body adipose tissue mass. Obese and overweight people have statistically higher IHCL levels than lean people, and 1% incensement in total adipose tissue mass accounts for about 20% more IHCL.[31] The accumulation of triacylglycerol (TAG), which is derived from plasma non-esterified fatty acid in the liver, is the most important pathogenesis of non-alcoholic fatty liver disease. Thus, blocking fatty acid transport from adipose tissue is a crucial therapeutic target for fatty liver disease.[32]

1.2 ADIPOSE TISSUE

1.2.1 Overview

Adipose tissue is mainly composed of adipocytes, sparsely surrounded by preadipocytes, endothelial cells, fibroblasts and immune cells[33]. Adipose tissue is not only the major organ for energy storage, but also an important endocrine organ. The adipose derived hormones, such as leptin, adiponectin and resistin, all play a role in energy homeostasis. Dysfunctions of adipose tissue have been confirmed to be related to various diseases, including obesity, type 2 diabetes, and lipodystrophy.[34]

White adipose tissue (WAT) and brown adipose tissue (BAT) are the two primarily forms of adipose tissues, which differ in morphology, location and functions. WAT contains unilocular large droplets which occupy a majority of the adipocyte areas. On the other hand, BAT contains multiloculated adipocytes high in mitochondria, which are the sources of the brown color.[34] These two different adipose tissues are distributed in different areas of the body. WAT is mainly concentrated in subcutaneous areas and around visceral organs, and it is the primary adipose tissue in adults. On the other hand, BAT is normally distributed in periadrenal, paravertebral and supraclavicular areas.[35] In addition to be a key tissue for energy storage, WAT is also important for regulating diverse metabolic activities, such as satiety, lipid metabolism and glucose up-take in response to insulin stimulation. By contrast, BAT is mainly responsible for thermogenesis and energy expenditure.[34]

1.2.2 Adipogenesis

The process in which the mesenchymal stem cells (MSCs) differentiate into adipocytes is called adipogenesis, during which the cells increase both in the number and the size. Adipogenesis occurs in two steps: the commitment phase, which results in

preadipocytes from MSCs, and the terminal differentiation phase which leads to adipocytes.[34]

Cross-talk among key transcriptional factors and several signaling pathways, such as WNT signaling and transforming growth factor-β (TGF-β) signaling, has been discovered to be involved in the commitment step of adipogenesis.

One of the most important transcriptional factors is zinc-finger protein 423 (ZFP423). Enrichment of ZFP423 mRNA is an essential step for the commitment phase. ZFP423 level is high in 3T3-L1 preadipoytes, but not in the non-adipogenic NIH 3T3 fibroblasts. 3T3-L1 cells, originally derived from fibroblasts, can be induced to differentiate into adipocytes *in vitro* using defined stimuli and have been widely used as an *in vitro* model for studying adipogenesis[36]. In contrast, the ZFP423 expression level remains the same in the terminal differentiation phase, suggesting that this transcriptional factor may only play a role in the formation of the preadipocytes rather than mature adipocytes[37]. Most of the ZFP423 knockout mice are devoid of adipose tissue and die within one day of the birth, demonstrating the importance of this gene in adipogenesis *in vivo*.[38] Another transcriptional factor – transcription factor 7-like 1 (TCF7L1) has also been identified to play an important role in the terminal differentiation phase of adipogenesis, as knocking down transcription repressor TCF7L1 by siRNA impairs adipocyte differentiation, with enhanced accumulation of preadipocyte and decreased adipocyte markers in 3T3-L1 cells.[39]

The WNT signaling has two distinct pathways: canonical WNT/β-Catenin signaling inhibits adipogenesis while non- canonical WNT signaling has more complicated effects on adipogenesis. After WNT ligands bind to transmembrane receptors such as low -density lipoprotein receptor-related 5 (LRP5), LRP6 and Frizzled, the canonical WNT signaling pathway is activated. In the nucleus, T cell-specific transcription factors (TCFs) initiate transcription of target genes by recruiting β-catenin from cytosol. Retrovirus infection of WNT ligands or using pharmacological agents to

activate WNT signaling in 3T3-L1 preadipocytes blocked adipogenesis.[40] Mouse embryonic fibroblasts from LRP6 deficient mice show enhanced ability to differentiate into adipocytes.[41] Mice with overexpression of WNT in adipose tissue have diminished fat depots and are resistant to diet induced obesity.[42] The mechanisms of this inhibitory effect of WNT signaling are mainly due to the inhibition of several terminal differentiation regulators such as PPARγ and C/EBP family. WNT signaling has an alternative pathway which is β-catenin-independent. There are two WNT ligands in this non-canonical pathway: WNT5A and WNT5B. Like canonical WNT pathway, WNT5A can inhibit PPARγ activities, thus inhibiting adipogenesis. On the contrary, WNT5B inhibits the entry of β-catenin to the nucleus and thus has a stimulation effect on adipogenesis.[43]

In the terminal differentiation stage, the preadipoctyes are differentiated to mature adipocytes, under the control of various transcription factors and other factors. PPARγ, C/EBPα, C/EBPβ and C/EBPδ are called master regulators at this stage. The expression of C/EBPβ is stimulated quickly (<2h) in response to adipogenic stimuli such as glucocorticoids and cAMP elevating agents. Subsequently, C/EBPβ stimulates the expression of PPARγ which in turn triggers the expression of C/EBPα.[34] PPARγ knockout mice do not survive and deleting PPARγ in pre-adipocyte cells completely blocked adipocyte accumulation, demonstrating the importance of PPARγ in adipogenesis.[44]

1.2.3 Adipose Tissue as an Endocrine Organ

In addition to energy storage, adipose tissue is also an endocrine organ. It secretes several hormones, including leptin, adiponectin, resistin and retinol binding protein- 4 (RBP4), to regulate food intake and energy expenditure.

Adiponectin is primarily secreted from WAT, whose expression level is relatively low in the pre-adipocyte stage but enhances dramatically in the late terminal

differentiation stage. The plasma adiponectin level drops more than half in both obese mice and humans, underlying its potential physiological effects in the development of obesity.[45] Adiponectin is an anti-adiposity adipokine. In transgenic mice with mild overexpression of the adiponectin gene, body weights decreases significantly due to reduced adipocyte size and mass and increased energy expenditure.[46] On the other hand, the adiponectin knockout mice have the same phenotype as overexpression mice in body weight and adipocyte size[47], suggesting the possibility that some compensatory mechanism may be in play to counter the effects of the lost adiponectin.

Resistin, another adipokine secreted by adipose tissue, targets not only the adipocytes, but also skeletal muscle and liver to abolish insulin functions and induce glucose intolerance. Its expression is species dependent: resistin dominantly expresses in adipose tissue in rodents and bone marrow in humans, respectively. Its expression level is low in adipocytes in lean humans but can increase in obese patients.[48] Mice lacking resistin-encoding genes have lower fasting plasma glucose levels with similar body weight changes on either chow or HFD, due to intact insulin sensitivity in the liver. This anti-hyperglycemia activity can be impaired after the injection of recombinant resistin, demonstrating that resistin plays a role in glucose homeostasis.[49]

RBP4, secreted from adipose tissue, is another hormone highly related to insulin resistance and obesity. The plasma RBP4 levels are increased in both humans and mice with insulin resistant phenotype, which can be reversed by PPARγ agonist drug rosiglitazone.[50] Incubating the isolated adipocytes from healthy humans with RBP4 abolishes insulin sensitivity by down-regulating phosphorylation of the insulin receptor substrate 1 and ERK1/2, in a similar manner in diabetes patients.[51] However, detailed mechanisms about how RBP4 induces obesity and type 2 diabetes remain to be elucidated.

Lastly, adipose tissue can also secrete various cytokines such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6). Macrophages have two main types: M1 and

M2. M1 macrophages are classical activation macrophages which respond to inflammation. M2 macrophages are alternative activation macrophages which not only take part in wound healing and tissue repair, but also sensitize insulin functions in adipose tissue. Although obesity induces both M1 and M2 macrophage numbers in the adipose, M1 population is enhanced by almost 10 times over that of M2. This increased M1- to- M2 ratio is highly involved in insulin resistance in obese mice.[52] The obesity induced macrophages can trigger the secretion of TNFα and IL-6 from the adipose tissue, further impairing insulin sensitivity and adipose tissue functions.

1.3 LEPTIN

1.3.1 The History and Discovery of Leptin

The discoveries of two mutant mice – *ob/ob* and *db/db* in the 1950s – 60s raised the question of the etiology of obesity. Both mutant mice are obese and diabetic. In the 1970s, Coleman tried to find out the cause of the obese phenotype by conducting parabiosis experiments. Parabiosis of *ob/ob* mice to either *db/db* mice or normal control mice rescued the obese phenotype of *ob/ob* mice; on the other hand, parabiosis of *db/db* mice to either *ob/ob* mice or normal control mice worsened the *db/db* mice obese phenotype. These findings led Coleman to think that a circulating factor was responsible for obesity.[53] In 1994, Dr. Friedman's lab identified the *ob* gene through positional cloning, which led to the seminal discovery of leptin.[54]

1.3.2 Leptin, Its Production and Clearance

The 16kDa – leptin, predominantly secreted from white adipose tissue, is one of the most important hormones in regulating energy homeostasis.[54] The circulating leptin level is positively proportional to body fat mass.[55] The expression and production of leptin is tightly regulated by various factors such as physiological conditions, transcription factors and even pharmacological agents. Either feeding or obesity will

stimulate leptin mRNA and circulating leptin levels, while fasting decreases leptin production.[56, 57]Several transcription factors have been discovered to regulate leptin gene expression: CCAAT/enhancer-binding protein alpha (C/EBPα)[58], adipocyte determination and differentiation – dependent factor 1 (ADD1)/sterol regulatory element binding protein 1 (SREBP-1) and Fos-related antigen 2(FOSL2) positively modulate leptin gene transcription; on the other hand, activator protein-2β (AP-2β) down – regulates leptin gene expression. Various hormones and pharmacological agents, including insulin[57, 59, 60], glucose[61], glucocorticoids[59, 62], hexosamines[63], cytokines[64] up-regulate leptin production, whereas β3-adrenergic agonists[65] and leptin itself[66] have a negative effect.

The most important tissue for leptin clearance is the kidney which has abundant leptin receptor expression. Destroying kidney function by removing the kidney or ligating ureters results in dramatically decreased leptin clearance by almost 30% in rats.[67] In contrast to the relationship between plasma leptin and BWI, renal leptin extraction has a negative relationship with arterial plasma leptin. However, the increased plasma leptin levels in obese individuals are independent of the clearance rate.[68]

1.3.3 Leptin Receptors and Signaling

Leptin receptors belong to the Class I cytokine receptor family. Six isoforms of leptin receptors (LR a-f) encoded by one single *Lepr* gene have been discovered. LR-a, c, d and f are short forms, LR-b is the long form, and LR-e is the secreted form (**Figure 1.3**).[69] All the isoforms share a homologous extracellular leptin binding domain and differ in intracellular domains. The short forms of LRs mainly have two distinct functions: mediate the transport of leptin into brain via blood brain barrier,[70] and the internalization and degradation of leptin in lysosome.[71] The soluble LR-e only possesses extracellular

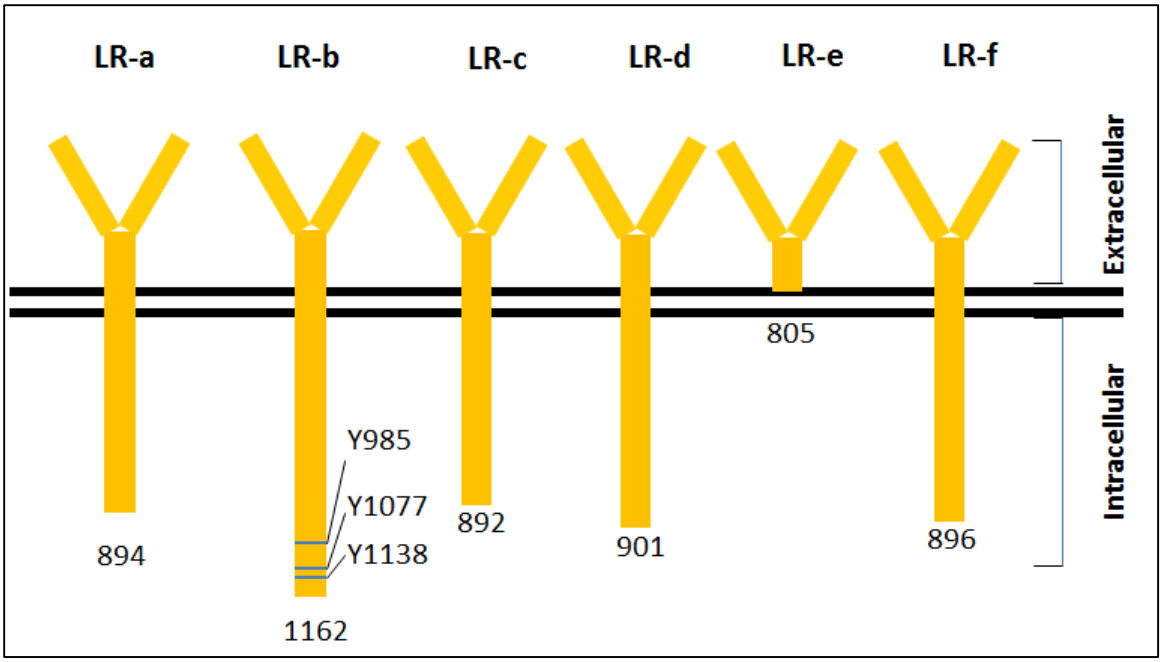

FIGURE 1.3 THE SIX ISOFORMS OF LEPTIN RECEPTORS. These isoforms share high homologous extracellular domains, with distinct intracellular domains. The LR-a, c, d and f are short forms of leptin receptors. The LR-b is the long form and the LR-e is the secreted form.

domain and is the main isoform for circulating leptin binding, thus blocking the transport of leptin to the CNS and its activity.[72]

The long form of leptin receptor LR-b is the one highly expressed in the hypothalamus and responsible for the metabolic functions of leptin via the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway.[69] After leptin binding to LR-b, JAK2 is recruited and activated, which not only phosphorylates JAK2 itself, but also three tyrosine phosphorylation sites on LR-b: Y985, Y1077 and Y1138. Each of these phosphorylation residues has its own distinct downstream signaling pathway. Y985 mediates the recruitment and activation of Src homology 2 containing phosphatase (SHP2), which controls the activation of extracellular-signal regulated kinase (ERK). This phosphorylation site is also the binding site of leptin signaling inhibitor-suppressor of cytokine signaling (SOCS3). Site-specific-loss-of-function mutation of Y985 in mice with enhances leptin sensitivity thus demonstrating the inhibitory effect of Y985 in leptin signaling.[73] Phosphorylation of Y1077 recruits and activates STAT5, and is responsible for leptin reproductive functions. The female mice with mutated Y1077 have impaired and elongated estrous cycling.[74] Y1138 mediates the major effects of leptin on energy balance. After being recruited by phosphorylated Y1138, the transcription factor STAT3 is tyrosine phosphorylated by JAK2, the p-STAT3 level has been used as a hallmark for leptin signaling activation. Disruption of STAT3 functions in the hypothalamus by mutating LR-b-Y1138 results in hyperphagia and obesity.[75] The Y1138-STAT3 pathway also activates the transcription and accumulation of SOCS3, attenuating leptin signaling via a feedback mechanism. In addition to SOCS3, various other factors such as the protein tyrosine phosphatase 1B (PTP1B), endoplasmic reticulum (ER) stress and some inflammatory signals can all attenuate the leptin signaling pathway.[69] (**Figure 1.4**) PTP1B dephosphorylates Jak2 and PTP1B deficiency mice are hypersensitive to leptin and resistant to DIO.[76] Obese mice have enhanced ER stress in hypothalamus and induction of ER stress by tunicamycin in

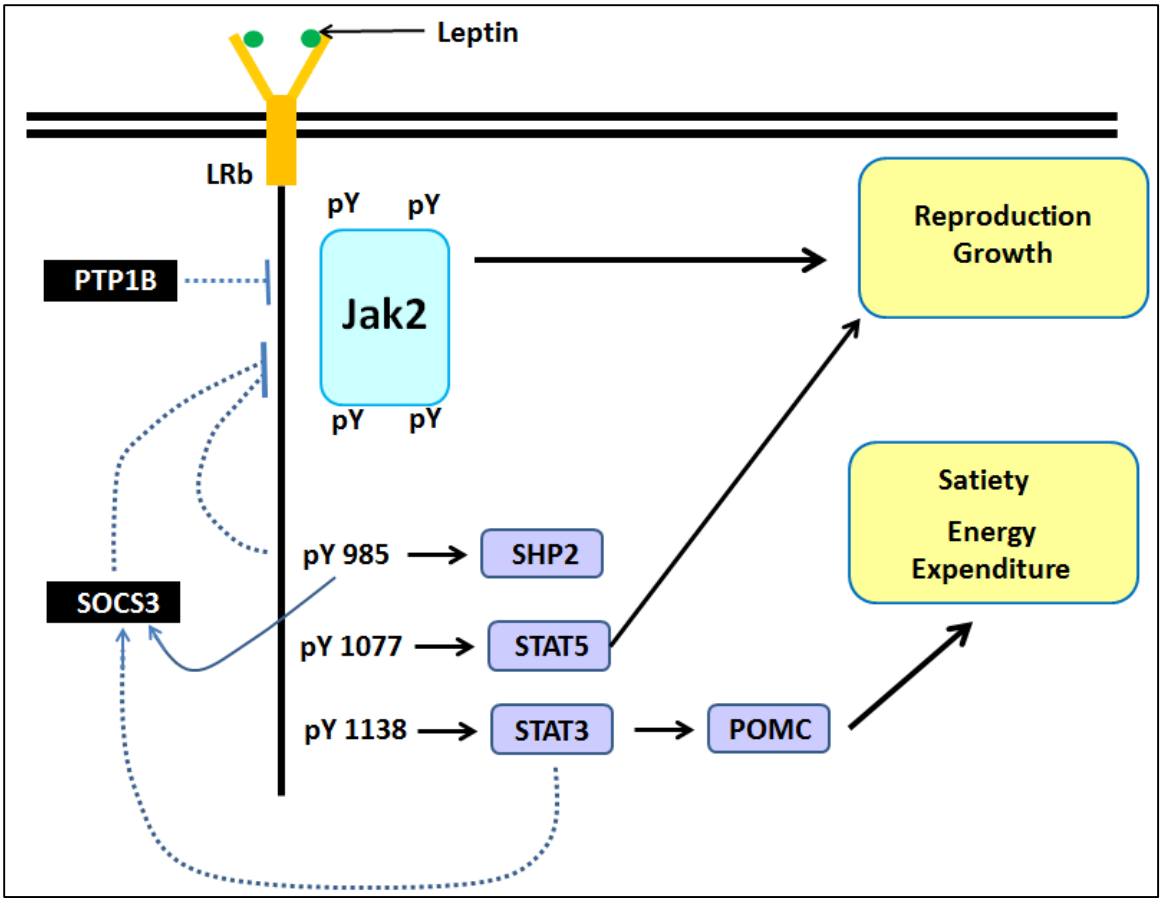

FIGURE 1.4 SCHEMATIC DIAGRAM OF LEPTIN SIGNALING PATHWAY AND LEPTIN RESISTANCE. In hypothalamus, leptin exerts its metabolic functions through Jak2-Y1138-SOCS3 signaling pathway. SOCS3 and PTP1B are negative regulators of leptin signaling. Dotted lines represent the feedback for inhibition in the LRb signaling.

the hypothalamus of lean mice also abolishes leptin sensitivity. [77] Mice fed on HFD rescued leptin sensitivity after administrating ginsenoside Rb1 by reducing TNFα and IL-6.[78]

1.3.4 The Central Metabolic Functions of Leptin

Leptin functions in CNS by regulating two major neuronal populations in hypothalamus, especially in ARC: NPY/Agrp and POMC neurons. POMC is anorexigenic peptide while NPY/Agrp are orexigenic peptides. LR-b is highly expressed in neurons expressing these peptides.[8] After treatment of 100nM leptin, POMC neurons are depolarized and trigger the production and secretion of α-MSH.[79] Specifically knocking out LR-b in POMC neurons resulted in mild obesity and hyperleptinemia.[80] On the other hand, leptin hyperpolarizes and inhibits NPY/Agrp neurons.[81] Agrp is the natural antagonist of MC3R and MC4R[14]. Ablation of NPY/Agrp neurons results in robust hypophagia in adult mice.[15] Impairment of LR-b-Y1138-STAT3 pathway decreased hypothalamic POMC mRNA levels while enhancing the Agrp mRNA level[75]. Disruption of MC4R functions by mutation results in obesity in both humans and mice.[82] In diet induced obese mice, the p-STAT3 levels dropped by almost half in POMC and Agrp neurons compared to control mice[83, 84]. All of these demonstrated the important role that leptin and its responsive neurons play in energy balance in the CNS. (**Figure 1.2**)

Besides leptin, orexigenic AMP-activated protein kinase (AMPK) is another important regulator of energy balance in the hypothalamus. Peripheral administration of leptin time-dependently suppresses the AMPK activity in the hypothalamus, including PVN and ARC by down-regulating the phosphorylation of acetyl-CoA carboxylase (ACC).[85]

1.3.5 Leptin Resistance and Obesity

The circulating leptin level is proportional to the total body fat mass. Obese individuals usually have higher leptin levels which dampen leptin's sensitivity of appetite and body weight suppression. This phenomenon is called leptin resistance[86]. Any factors impairing the leptin signaling pathway can induce leptin resistance, in which the inhibitory regulator-SOCS3 is highly related to leptin resistance. In a high fat diet induced leptin resistance state, the central expression of SOCS3 is also up-regulated in various hypothalamic leptin responsive neurons.[87] On the other hand, knocking out SOCS3 in the brain reverses diet induced obesity by sensitizing leptin signaling.[88] The inhibitory effect of SOCS3 on leptin pathway is not restricted in CNS only, but also occurs in peripheral tissues, especially in the skeletal muscle. Mice fed on HFD develop leptin resistance with SOCS3 up-regulation in skeletal muscle, whose phenotypes can be mimicked by overexpression of SOCS3 in mouse skeletal muscle.[89]

PTP1B is another important leptin signaling inhibitor and a candidate responsible for the development of leptin resistance. Knocking out PTP1B specifically in the brain reverses DIO, while removing PTP1B in the liver and muscles respectively has no such effects, underlying the particular role of PTP1B in CNS. Interestingly, knocking out PTP1B specifically in adipose tissue leads to increased body weight, demonstrating the differential roles of PTP1B in the brain and adipose tissue. [90]

1.4 cAMP AND ENERGY HOMEOSTASIS

The second messenger cAMP is produced upon the binding of various ligands, including β2-agonists and prostanoids, to the transmembrane Gs protein-coupled receptors. Subsequently, adenylyl cyclases (ACs) are activated to produce cAMP from adenosine triphosphate. On the other hand, phosphodiesterases (PDEs) catalyze the degradation of cAMP to 5'-AMP[91]. Protein kinase A (PKA) is the classical effector of cAMP, together with newly found Epac, mediate majorities of biological effects of cAMP, such as metabolism, proliferation and differentiation. (**Figure 1.5**)

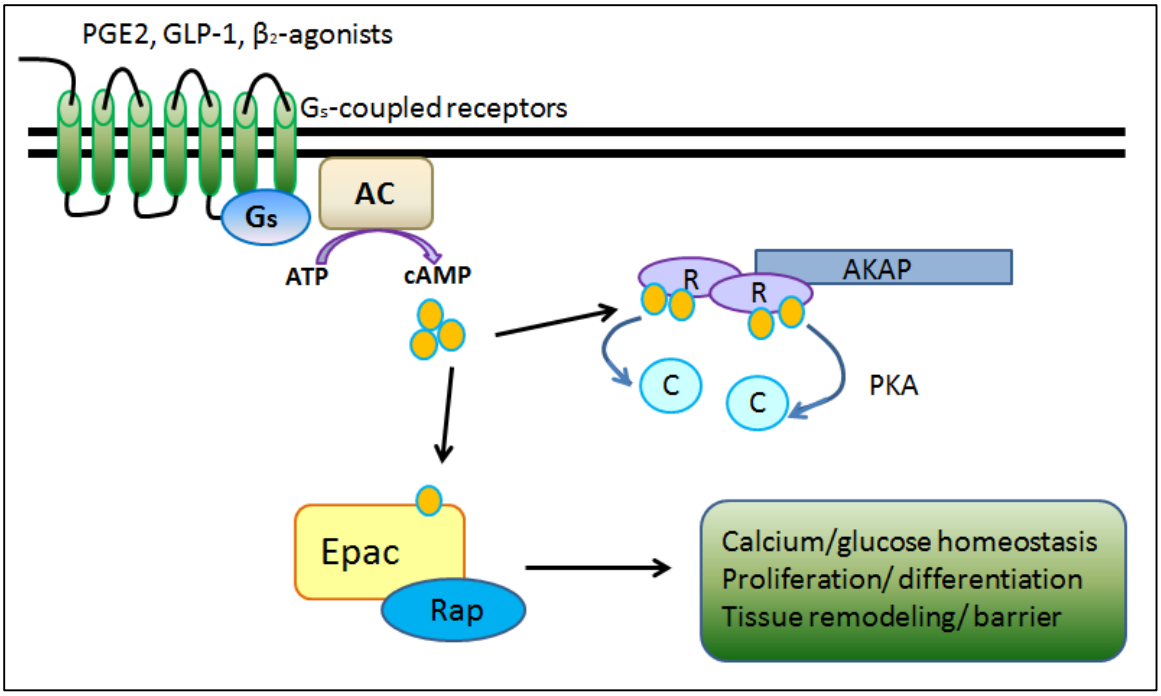

FIGURE 1.5 SCHEMATIC DIAGRAM OF CAMP SIGNALING. The production of cAMP is initiated upon binding of extracellular stimuli to the transmembrane Gs-coupled receptors. Subsequently, AC is activated to generate cAMP from ATP. On the other hand, PDEs catalyze the degradation of cAMP to 5'-AMP. The effects of cAMP are mainly mediated by PKA and Epac.

1.4.1 cAMP acts as an orexigenic second messenger

cAMP acts as either an orexigenic or anorectic second messenger depending on the regions and neurons in the hypothalamus.[92] Intracerebroventricular (i.c.v.) injection of cell-penetrating cAMP analog N6,2'-O-dibutyryl cAMP into the third ventricles of rats robustly stimulates the expression of NPY only in ARC and medial parvocellular portion of the paraventricular nucleus (mPVN), not affecting another orexigenic peptide galanin or the other regions in the hypothalamus.[93] The regulations of cAMP on NPY expression are confirmed by *in vitro* studies that cAMP can positively trigger NPY mRNA levels.[94] The site-specific orexigenic effects of cAMP in inducing food intake are also verified by some pharmacological evidences. Increasing intracellular cAMP levels by central injection cAMP analog 8-bromo-cAMP (8-BrcAMP) or cAMP-elevating agents (e.g. phosphodiesterase inhibitor IBMX and AC activator forskolin) significantly stimulate food intake in satiated rats. These food-enhancing effects of cAMP are only restricted in the PFH , LH and thalamus (THAL) regions of the hypothalamus.[95]

1.4.2 cAMP acts as an anorectic second messenger

On the other hand, the anorectic functions of cAMP are mainly restricted to the PVN region of the hypothalamus. The regulations of cAMP on some anorectic peptides are well studied. The intracellular cAMP level is increased after stimulation and activation of α-MSH and MC4R by central leptin injection.[96] The synthesis of cAMP is impaired by mutating MC4R and silencing its functions, leading to obesity and appetite disorders.[97]

Besides leptin, several other anorectic hormones in PVN regulate food intake by mediating cAMP production. PVN highly expresses GLP-1, which exerts its inhibitory effects on food intake and drinking by binding to G-protein coupled receptors to stimulate the production of cAMP.[98] *In vitro* studies further confirm that GLP-1 promotes cAMP production by binding to Gs-coupled receptors and activating AC.[99] Another

anorectic neuropeptide, corticotropin-releasing hormone (CRH) neurons, can be activated by i.c.v. injection of GLP-1. Both *in intro* and *in vivo* studies show that the CRH gene transcription and its secretion are regulated by PKA and its downstream effector cAMP response element-binding protein (CREB).[100] Thus, the binding of GLP-1 to the CRH neurons stimulates intracellular synthesis of cAMP, which further stimulates the secretion of CRH through PKA-CREB pathway to reduce food intake.

1.4.3 cAMP and Adipocyte Differentiation and Lipid Metabolism

The manipulations of cAMP on adipocyte differentiation are mainly through transcriptional regulators such as C/EBP, PPARγ, PPARδ. Stimulating 3T3-L1 pre-adipocytes without the cAMP elevating agent methylisobutylxanthine slows down the differentiation rate by delaying and down-regulating expressions of three C/EBP isoforms.[101] These results demonstrate that cAMP is required for full adipocyte terminal differentiation in 3T3-L1. During the terminal differentiation, C/EBPβ is activated only after the binding of phosphorylated-CREB to its proximal promoter. In MEF cells isolated from CREB knockout mice, not only is the differentiation abolished, but the expression of C/EBPβ is diminished as well.[102] Moreover, cAMP can trigger the expression of PPARγ as well. IBMX, used in combination with insulin and glucocorticoid in cocktail to stimulate adipocyte differentiation, not only increasing intracellular cAMP levels but also PPARγ transcription activity as well, through PKA – dependent and PKA-independent pathways.[103]

In mature adipocytes, the excess energy is stored as TAG. The hydrolysis of TAG is tightly regulated by cAMP. Various stimuli, including catecholamines, noradrenaline and adrenaline couple to surface receptors to activate Gs proteins and increase intracellular cAMP level, which regulates the phosphorylation of hormone sensitive lipase (HSL) and perilipin through PKA. As a result, TAG is hydrolyzed to free fatty

acids and glycerol. Phosphodiesterase inhibitors or cAMP analogs, which can elevate intracellular cAMP have been reported to potentiate 3T3-L1 adipocyte lipolysis.[104]

1.4.4 cAMP and Leptin Crosstalk

Besides the classical JAK2-STAT3 pathway, leptin is also known to signal through an alternative phosphatidylinositol-3 kinase (PI3K)/phosphodiesterase 3B (PDE3B) cascade[105]. Studies in liver and pancreas reveal a role of leptin in the regulation of cAMP.[106 107] PDE3B is one of the members of the PDE family, which degrades cAMP to AMP. PDE3B is expressed in various tissues including the brain, adipose tissue, liver, smooth muscles and macrophages.[108] In isolated rat pancreatic islets, GLP-1 potentiates insulin secretion by enhancing intracellular cAMP levels; however, this effect can be diminished by leptin. The treatment of leptin in HIT cells and primary hepatocytes stimulates PDE3B activity, which can be abated by PI3K inhibitor wortmannin or LY294002, demonstrating that leptin may activate PDE3B through PI3K.[109] This pathway also exists in the CNS. I.c.v. injection of leptin reduces intracellular cAMP levels by increasing PI3K and PDE3B activities. Furthermore, central administration of a specific PDE3 inhibitor, cilostamide, not only reverses leptin's metabolic functions and rescues intracellular cAMP levels, but also inhibits leptin induced hypothalamus p-STAT3 levels. These findings demonstrated that the classical JAK2-STAT3 pathway interacts with the PI3K-PDE3B-cAMP pathway, enabling leptin to exert its effects on food intake and body weight.[110]

1.5 PKA AND ENERGY HOMEOSTASIS

1.5.1 PKA Isoforms and Activation

The PKA holoenzyme is the classical effector of cAMP. In the absence of cAMP, PKA exists as a tetrameric holoenzyme that is consisted of a dimer of two regulatory (R)

subunits, each bound with one catalytic (C) subunits. In responding to the bind of cAMP to R subunits, the C subunits dissociate from the complex, and PKA is activated.

The PKA R subunits have two major isoforms, RI and RII, both of which have two minor variants encoded by separate genes, termed as RIα, RIβ, RIIα and RIIβ respectively. The expressions of these isoforms in mice are tissue specific: RIα is abundant in the CNS and heart, while RIβ expresses mostly in the brain and spinal cord. Similarly, RII is also expressed abundantly in the brain, with RIIα more restricted in the heart and RIIβ predominantly in the liver and adipose tissue. [111]

The C subunits have at least three isoforms: Cα[112], Cβ[113], and humans have another Cγ[114]. There are three splice variants of Cα, termed as Cα1, Cα2 and Cα-s respectively. Compared to the ubiquitously expressed of Cα, the splice variants of Cβ are expressed in a more complicated manner. Cβ1 is a ubiquitous form while Cβ2 is predominantly expressed in lymphoid cells and brains and Cβ3 is only found in the brain.[111 115]

1.5.2 PKA and Obesity

The roles of PKA in energy balance have been well documented. Several studies using mouse models have revealed the roles of PKA in obesity and energy homeostasis. RIIβ deficiency mice show lower body weight gains on both chow diet and HFD, with significant less fat mass owing to reduced triglyceride storage. The knockout of RIIβ does not affect the C subunit expressions; on the other hand, its deficiency has a compensatory upregulation effect on RIα, which leads to an increased basal PKA activity as RIα is more cAMP sensitive than RIIβ[116].

Similarly, RIIα knockout mice have significantly lower body weight as compared to WT littermates after being challenged by HFD. However, this RIIα deficiency has gender specific effects, with more robust metabolic protective effects in female mice. Female RIIα knockout mice already show lower body weight than WT controls on chow

diet, while male RIIα deficiency mice have to be challenged by HFD to manifest this phenotype.[117]

Knocking out all three splice variants of Cβ in mice shows no compensatory effects of Cα, but has gender specific effects. In contrast to RIIα knockout mice, the male rather than female Cβ deficiency mice show significantly lower body weights on the chow diet, although both genders are leaner when fed on HFD without food intake differences. On the other hand, the percentages of fat pads over whole body weights decrease in female knockout mice only; it remains the same in male mice, irrespective of the genotypes or food types. Lastly, Cβ deficiency protects mice against insulin intolerance by enhancing glucose clearance. Disruption of Cβ has the advantages of overcoming DIO and insulin resistance without affecting food intake and adiposity.[118]

In human, adipose tissue expresses RIIβ subunits abundantly, whose expression levels drops dramatically in obese individuals. In visceral adipose tissue, obese patients have significantly impaired PKA activity compared to non-obese individuals. This difference is not detected in subcutaneous fat.[119] Although RIIβ is the predominant subunit of PKA in both rodents and humans, the deficiency of this gene in adipose tissues has opposing effects. In humans, obese individuals have down-regulation of RIIβ expression without effecting RIα[119]; while in mice, RIIβ deficiency compensatorily up-regulates RIα and results in an unexpected lean phenotype[120]. However, Tissue-specific re-expression of RIIβ reveals some controversial metabolic effects of RIIβ compared to knockout studies. Re-expression of RIIβ, specifically in WAT and BAT, fails to change the phenotypes of RIIβ knockout mice, suggesting more complicated effects of RIIβ in adipose tissue than expected. On the other hand, re-expression of RIIβ in brain rescues the lean phenotype, indicating that the RIIβ in brain may be the one responsible for regulating body weight[120].

1.6 EXCHANGE PROTEIN DIRECTLY ACTIVATED BY CAMP

1.6.1 Epac Isoforms, Structures and Activation

It was a dogma for a long time that cAMP exerted its functions only through two down-stream effectors: protein kinase A (PKA) and cyclic nucleotide regulated channels. However, this dogma was broken in 1998 when the exchange protein directly activated by cAMP (Epac) was identified by two groups almost simultaneously.[121] [122] Epac proteins function as guanine exchange factors (GEFs) for its down-stream effectors, Ras like small GTPases, Rap1 and Rap2. In the absence of cAMP, Epac is in an auto-inhibitory inactive state, with the regulatory N-terminal directly interacts with the catalytic C terminal, blocking the binding of Rap. Upon binding of cAMP, it undergoes a conformational change, relieving the auto- inhibitory effect.[123] In its active state, Epac catalyzes the nucleotide exchange reaction between the inactive guanosine diphophate (GDP)-bound Rap and the active guanosine triphosphate (GTP)-bound Rap, leading to the activation of Rap.

Epac family has two members: Epac1 and Epac2, and there is also a related protein called Repac.[124] The multiple domain proteins Epac1 and Epac2 have an N-terminal regulatory region and a C-terminal catalytic region, while Repac only has a C-terminal catalytic region. In N-terminal, the Disheveled, Egl-10, and pleckstrin (DEP) domain is responsible for Epac's translocation from cytosol to plasma membrane with the regulation of cAMP[125]. (**Figure 1.6**) The DEP domain is flanked by cAMP-binding domains, CBD-A and CBD-B, in Epac2. However, Epac1 possesses only one CNB-B domain which is close to the C-terminal. CNB-A has a much lower affinity to cAMP binding, thus, is probably not involved in Epac2 activation, but plays an important role in subcellular localization.[126] It is the CDC-25-HD domain in C-terminal that possesses the guanine exchange factor activity of Epac proteins. CDC-25-HD is stabilized by the Ras exchange motif (REM) domain. A Ras association (RA) domain between CDC-25-HD and REM domains enables Epac2 bind to activated H-, K-, and N-Ras, thus triggers

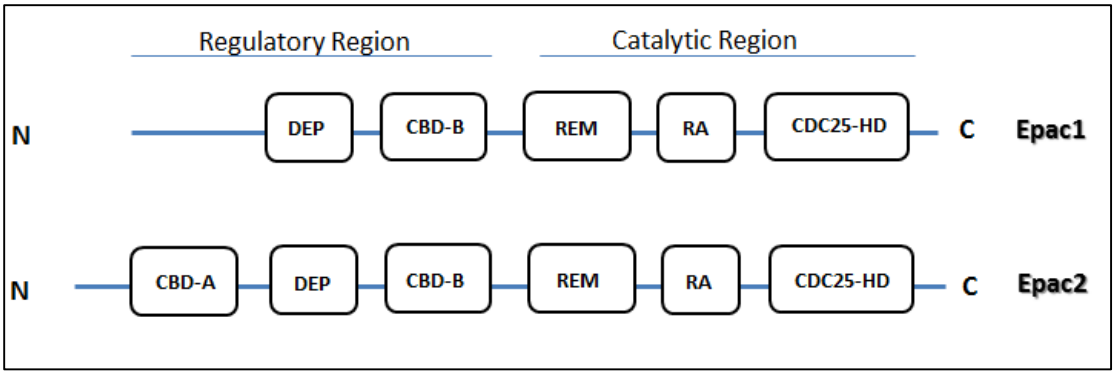

FIGURE 1.6 A SCHEMATIC MODEL OF EPAC DOMAINS. Epac1 and Epac2 are multiple domain proteins, which have an N-terminal regulatory region and a C-terminal catalytic region. CBD: cyclic AMP binding domain; DEP: Disheveled, Egl-10, and pleckstrin domain; REM: Ras exchange motif domain; RA: Ras association domain.

translocation of Epac2 and activation of Rap1 in cells.[127, 128] The function of RA domain in Epac1 remains to be elucidated[129]. While Epac proteins can activate both Rap1 and Rap2, Epac1 has higher affinity for Rap2; Epac2 and Repac bind more efficiently to Rap1.[124] Interestingly, double deletion of Epac1 and 2 is required to impair the activation of Rap1 in the forebrain of mice.[130]

Both Epac1 and Epac2 have several different splicing variants. Human Epac1 has three known splicing variants. Variant 1 (6239 bp) is the longest form, which encodes Epac1a with 923 amino acids. Variant 2 (5773 bp) and variant 3 (6003 bp) encode the same isoform of Epac1b with 881 amino acids. The differences of functions and distributions of Epac1 isoforms need to be elucidated[129]. Compared to Epac1, the isoforms of Epac2 are better studied. The full-length Epac2 is referred to as Epac2A. The adrenal-specific isoform Epac2B has no CNB-A domain.[126] The liver-specific isoform Epac2C lacks both CNB-A and DEP domains and is only 3.5kb compared to 4.0kb of Epac2A.[131] However, similar to Epac2A, both Epac2B and Epac2C proteins are functional and possess GEF activity towards Rap1.

1.6.2 Epac Protein Tissue Distributions

Epac1 is a widely expressed protein, with its highest expression in the kidney, while Epac2 is more tissue-specific, found especially in the brain, pancreas and adrenal glands[121, 122, 132]. The gene expression levels of Epac1 and Epac2 change during development in some tissues. For example, both Epac gene expression levels are significantly increased in adult lungs compared with fetal stages, although Epac1 is expressed more abundantly than Epac2 in adult lungs[133]. The expression levels of Epac1 genes in the brain diminish significantly during the development. Such a phenomenon also occurs in the kidney with Epac2 genes. It is very interesting to see that although the expression level of Epac1 is much lower than Epac2 in human adult brains, it is Epac1, not Epac2 that is expressed in the septum and thalamus.[124] In the kidney, Epac1 and

Epac2 proteins are expressed in various tubular segments, except for thin descending limbs of Henle's loop in both rats and humans with different distribution patterns in the sub-compartment of cells.[134] The different distributions of Epac1 and Epac2 suggest distinguished functions of these two proteins in various tissues.

1.6.3 Epac Protein Subcellular Localizations

The subcellular localization of Epac1 is cell cycle – and cAMP concentration – dependent.[135] Epac1is targeted to the nuclear envelope, mitochondria and cytoskeleton during interphase, when the concentration of intracellular cAMP is relatively high.[135-137] In the metaphase of mitosis, when intracellular cAMP level is relatively low, Epac1 disassembles from membrane fractions and translocates to mitotic spindle and centrosomes. The localization of Epac1 is regulated by the DEP domain, since the deletion of this domain (72-148) makes Epac1 diffusible across the cytoplasm. Epac1 contains a putative mitochondrial targeting sequence at its N-terminus, and the mitochondrial targeting of Epac1 is DEP-independent.[135, 138] Besides, deletion of Epac1 CBD results in deficiency of cytoskeleton targeting, showing that the CBD domain may also play a role in Epac1 localization[136]. On the other hand, Epac2A translocates from cytosol to plasma membrane by cAMP-induced, active Ras- mediated recruitment in a PKA and PI3K-independent manner.[126, 127] Epac2B is mainly targeted in the cytoplasm, indicating that the additional CNB-A domain in Epac2A plays a role in its plasma membrane localization.[126] In cortical neurons, Epac2 can be translocated from cytosol to the plasma membrane by coexpression with NL3, which can also trigger Epac2-mediated Rap-GEF activity.[139] Bers and coworkers used Epac1 knockout (KO) mice, Epac2 KO mice and double knockout mice plus a specific fluorescent Epac ligand 8-[Pharos-575]-2'-O-methyladenosine-3'5'-cyclic monophosphate (ϕ-O-Me-cAMP) to elucidate the localization and translocation of these two isoforms in the heart. Generally speaking, Epac1 is the isoform that mainly resides in peri-nucleus and involves in the gene

transcriptional activity, while Epac2 is mainly expressed in the heart's T tubules and is responsible for arrhythmias.[140]

1.6.4 Epac and Adipocyte Differentiation *in vitro*

As an antilipolytic hormone, insulin mediates glucose uptake, translocation of GLUT-4 from cytosol to plasma membrane and lipogenesis in adipocytes in a PDE3B-dependent manner as these effects of insulin can be impaired by PDE inhibitor OPC3911.[141] The inhibitory effects of OPC3911 can also be mimicked by Epac agonist 8-pCPT-2'-O-Me-cAMP in a PKA-independent manner.[142] Contrary to its effects on AKT phosphorylation in HEK cells[138] and skeletal muscle[143], treatment of Epac agonist mimics PDE inhibition and diminishes insulin-mediated AKT phosphorylation in rat adipocytes[144], and increases critical energy homeostasis regulator – AMPK phosphorylation and activity[145] and its downstream molecule – the fatty acid metabolism rate – limit hormone – ACC phosphorylation.[144]

During adipocyte differentiation, PKA and Epac function synergistically to augment adipose differentiation master regulator - PPARγ expression[146] and transcription,[103] and cAMP-mediated, insulin and dexamethasone-dependent adipogenesis in 3T3-L1 preadipocytes and mouse embryo fibroblast.[147, 148] While PKA acts to inhibit Rho/Rho-kinase, Epac is involved in the activation of Rap, which reverses the decrease of AKT phosphorylation and adipogenesis by PKA and Rho-kinase inhibitor.[147] The opposing effects of Epac on AKT phosphorylation in mature rat adipocytes and 3T3-L1 preadipocytes suggest different Epac-mediated signaling pathways are involved.

1.6.5 Epac and metabolic hormones *in vitro*

Fukuda et al[149] revealed the involvement of Epac in leptin's classical energy homeostasis effects in the hypothalamus for the first time. The phosphorylation of

STAT3 in hypothalamus was greatly weakened by forskolin treatment, and this inhibitory effect was even greater with the combination of a low dose of leptin, which had little effect on pSTAT3 alone. Epac agonist 8-pCPT-2'-O-Me-cAMP plus low concentration of leptin mimicked the effect of forskolin by upregulating leptin signaling inhibitor SOCS3 and PTP1B dose-dependently, and suppressing leptin target- POMC neurons depolarization in a PKA-independent manner.[149]

Besides leptin, Epac has been also discovered to be related to several other hormones linking to energy balance. Adiponectin is another hormone secreted from the adipose tissue which plays a role in energy homeostasis. In contrast to leptin, the circulating adiponectin level is down-regulated in obese patients. Some *in vitro* and *ex vivo* studies reveal a regulatory role of Epac on adiponectin secretion. In 3T3-L1 adipocytes and human adipocytes, Epac mediates forskolin and IBMX induced adiponectin secretion, which is PKA-independent and can be mimicked by Epac specific agonist. [150 150 145 127 -136]

The orexigenic hormone ghrelin is secreted from the stomach, which can be potentiated by glucagon and norepinephrine through PKA-independent Epac activation. The ghrelin secretion from primary rat stomach cells increases significantly after pre-treatment of Epac specific agonist while the PKA specific inhibitors H89 and RP-cAMP have no effects on ghrelin secretion.[151] Ghrelin receptors and LR-b co-express in rat nodose ganglia neurons. Leptin activity can be impaired by ghrelin through de-phosphorylation of STAT3. This inhibitory effect of ghrelin is mediated by Epac1 stimulated SOCS3. Knocking down Epac1 levels in rat neurons not only decreases SOCS3 expression, but also impairs the inhibitory effect of ghrelin on leptin.[152] These results further confirm the metabolic functions of Epac in regulating both orexigenic and anorectic hormones centrally and peripherally.

1.7 SIGNIFICANCE

Our studies are significant because our group was the first to report the metabolic functions of Epac1 *in vivo* by using global Epac1 KO mice. Besides, we are also the first to investigate the specific role of Epac1 in peripheral adipose tissue by using adipose tissue specific Epac1 knockout mice. Our studies provide a potential therapeutic target of Epac1 in the obesity.

Chapter 2 Materials and Methods

*Note: This Chapter contains a portion of the papers:

Yan, J.; Mei, F. C.; Cheng, H.; Lao, D. H.; **Hu, Y**.; Wei, J.; Patrikeev, I.; Hao, D.; Stutz, S. J.; Dineley, K. T.; Motamedi, M.; Hommel, J. D.; Cunningham, K. A.; Chen, J.; Cheng, X., Enhanced leptin sensitivity, reduced adiposity, and improved glucose homeostasis in mice lacking exchange protein directly activated by cyclic AMP isoform 1. *Mol Cell Biol* **2013,** *33* (5), 918-26.

Hu, Y.; Mei, F.; Kim, ER.; Wang, H.; Tong, Q.; Jin, J.; Chen, J.; Cheng, X., Role of exchange protein directly activated by cAMP isoform 1 in energy homeostasis: regulation of leptin expression and secretion in white adipose tissue. Submiteed to *Mol Cell Biol*

Animal Studies – Mice with C57BL6 background were used in this study. The mice were housed on a 12/12 h light/dark cycle in pathogen-free facilities with free access to food and water. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (UTMB) at Galveston and or the University of Texas Health Science Center at Houston (UTHealth).

Generation of Global Epac1 Knockout Mice – Epac1 KO mice were generated using the Cre-loxP system targeting Exons 3-5 previously described[153]. Briefly, to target Epac1 gene, two loxP sites were inserted into introns 2 and 5 to flank exons 3-5. A neomycin resistance cassette sandwiched by two FRT sites was placed in intron 5 for positive selection while. A diphtheria toxin A cassette was used for negative selection. The targeting DNA in the linearized form was introduced into R1 mouse embryonic stem (ES) cells by electroporation. A 470-bp DNA probe amplified from the 5' recombinant arm was used to hybridize with the G418-selected ES cell genomic DNA digested by restriction enzyme EcoRV. The primers used for PCR amplification were: 5'-GAAGCCAGGCAACGAGATT-3' (sense) and 5'-AGGCACGAGCTTTACGGTAG-3' (antisense). WT allele had a 17-kb band in gels while the recombinant allele had a 8-kb band (**Figure 2.1**).

Two C57BL/6 blastocysts were microinjected with verified recombinant ES cell clones and implanted into pseudopregnant foster mice. Male chimeras and female black Swiss mice were mated to generate germ line transmission (fneo/+). WT and Epac1 deficiency mice ($Epac1^{-/-}$) were obtained from heterozygous mice ($Epac1^{+/-}$) which were generated from protamine-Cre carrier mice cross breeding. Epac1 knockout ($Epac1^{-/-}$) mice used in this study were back-crossed for more than 12 generations to the C-57BL/6 background.

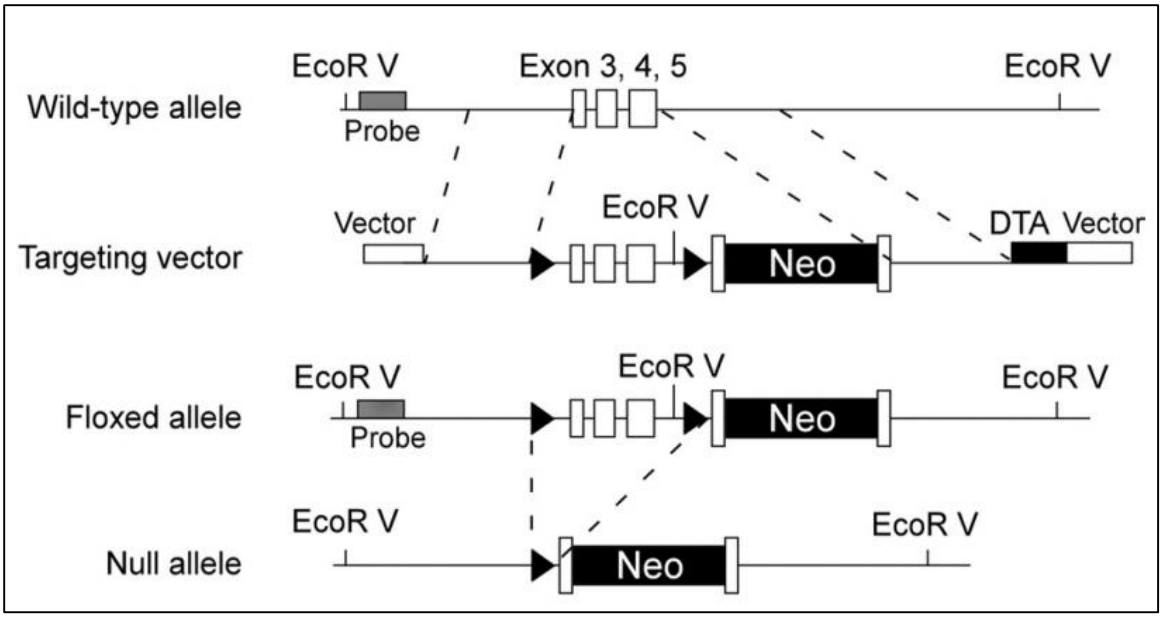

FIGURE 2.1 SCHEMATIC MODEL OF GENERATION OF EPAC1 KNOCKOUT MICE. Triangles represent the two *loxP* sites which flanked Epac1 exons 3 to 5. Open rectangles represent the two Frt sites which flanked a neomycin resistance cassette.

Adipose Tissue Specific Epac1 Knockout Mice – $Epac1^{LoxP/LoxP}$ mice on C57BL/6 background with two LoxP sits flanking exons 3 and 5 of Epac1 gene were generated as described previously.[153] Transgenic *Cre* line driven by the adipocyte – specific *aP2/Fabp4* promoter [B6.Cg-Tg (Fabp4-Cre)1Rev/J; JAX Stock NO. 005069] was obtained from Jackson Lab.[154] Homozygous $Epac1^{LoxP/LoxP}$ mice were crossed with aP2-Cre mice to generate the adipose specific $Epac1^{LoxP/LoxP}$: $aP2\text{-}Cre^{+/-}$ progeny, referred to as AEKO (**Figure 2.2**). The $Epac1^{LoxP/LoxP}$ littermates without *Cre* gene expression were used as controls and are referred to as WT controls.

Food Intake and Body Weight Analyses – Only male mice were used in this study. Briefly, age- and gender-matched mutant Epac1 and WT littermates were fed on regular chow diet (Teklad 7912, with 17% of energy from fat, 25% of energy from protein and 58% of energy from carbohydrate) until postnatal day 24, at which point, the mice were either fed with a HFD (ResearchDiet D12492) with 60% of calories from fat and 20% of energy from protein and carbohydrate or continued on with chow diet, respectively. The Body weight and food intake were measured every 4 days.

To compare body weight change between groups, Two-Way Repeated ANOVA was utilized to analyze the data. To compare food intake between groups, the average daily food intake of each mouse was calculated. The equation is:

$$\text{Average daily food intake (g)} = \frac{The\ amount\ of\ food\ consumed\ during\ 4\ days\ (g)}{4\ x\ The\ number\ of\ the\ mice}$$

Determination of Whole Body Fat Content – A Siemens Inveon MultiModality micro-computed tomography (micro-CT) system (Siemens Preclinical Solutions, Knoxville, TN) was used for mouse whole body scanning. Mice, under anesthesia with 1

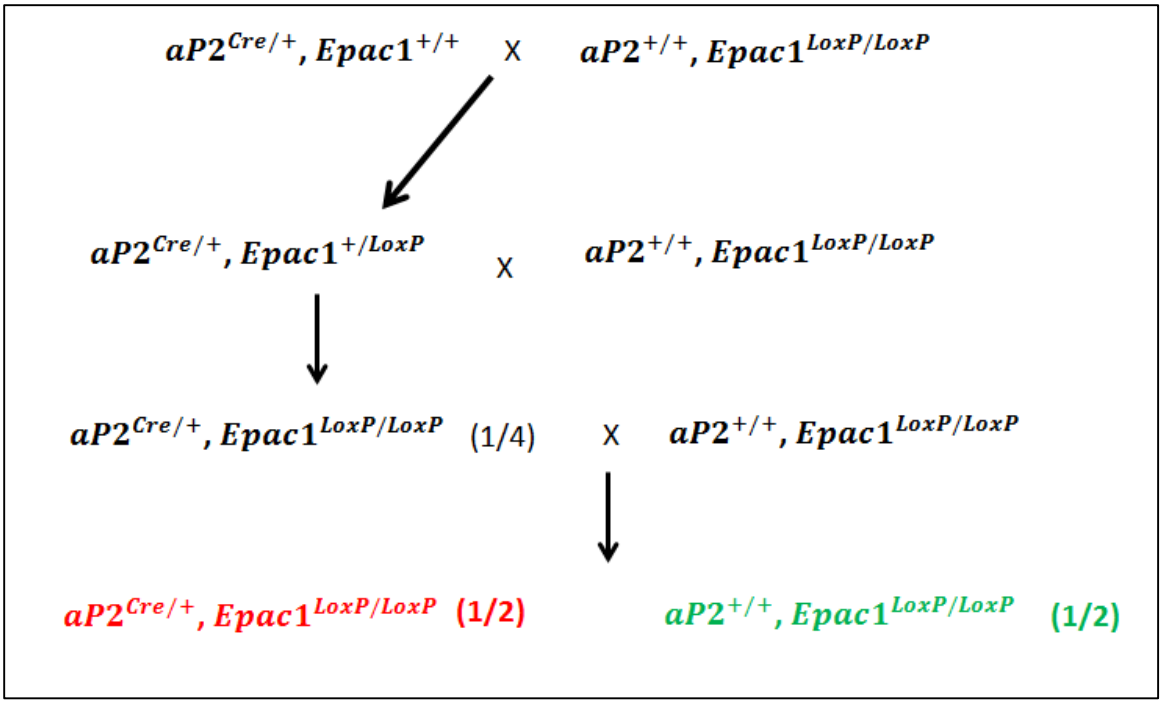

FIGURE 2.2 A SCHEMATIC MODEL OF GENERATION OF ADIPOSE TISSUE SPECIFIC EPAC1 KNOCKOUT MICE. $Epac1^{LoxP/LoxP}$ mice were crossed with Fabp4/aP2-Cre mice to generate adipose tissue specific knockout mice.

to 2% of isoflurane carried by O_2 through the nose cone, were individually scanned in a prone position. The settings of the high-resolution micro-CT imaging were: 70 kVp, 500μA and 360° rotation at a 1,000-ms exposure time. The whole-body adipose tissue volume was determined in a three-dimensional volume punch mode using Siemens Syngo multimodality workplace VE36a software. Mouse body length was measured as the distance between C1 and L6 vertebrae.

Plasma Leptin Measurements – After a 6-h fasting, the retro-orbital blood was collected. The plasma leptin concentration was determined by R&D Quantikine mouse leptin immunoassay kits (R&D system, MN). For the plasma from HFD mice, a 1:60 dilution with Calibrator Diluent RD5-3 from the kit was used for the final measurement. For the plasma from newly weaned mice fed on regular chow diet, a 1:2 dilution was used. Standard curve was generated by measurements of mouse leptin standard at the concentrations of 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL and 62.5 pg/mL used for the quantification sample leptin levels.

Plasma Insulin Measurements – After a 6-h fasting, the retro-orbital blood was collected. The plasma insulin concentration was measured by Ultrasensitive Mouse Insulin ELISA kit (Crystal Chem Inc.) Insulin standards at the concentrations of 3.2 ng/mL, 1.6 ng/mL, 0.8 ng/mL, 0.4 ng/mL, 0.2 ng/mL and 0.1 ng/mL were used for quantification of sample insulin levels.

Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT) – OGTT and ITT were performed as described previoulsy[153]. For OGTT, mice were fasted overnight and for ITT mice were fasted 4 hours. In OGTT, mice were orally administered D-glucose (ICN Biomedicals, Inc) at the dose of 1 g/kg body weight. In ITT, mice were administered intraperitoneally (*i.p.*) human insulin (Eli Lilly) at the dose of 0.5 mU/g

body weight. The blood glucose concentration was determined from the tail blood by OneTouch Ultra blood glucometer at various time courses.

Energy Expenditure Study – This study was performed in comprehensive lab animal monitoring system (CLAMS; Columbus Instruments), as described previously[155]. Briefly, 4 – 5 week-old mice (n=6 for each group) were housed individually in the chambers of CLAMS with free access to water and food at room temperature. The animals were accustomed in their cages for two days followed by four days of data collection. Mice were initially fed on chow diet and switched to HFD for the last two days. Food intake, O_2 consumption, CO_2 production, body weight and beam break data were collected.

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9 System to Knock out Epac1 in 3T3-L1 cells – Online design tool (http://crispr.mit.edu/) was used to identify optimal single guide RNA (sgRNA) sequences. Two different sgRNA sequences were picked with highest scores. Oligo 1: GTCATCTCCCTCGTGCAACGTGG; Oligo 2: GCGGCTAGTTGGCCGATGGGTGG. These two oligos were cloned to pLKO vector. The 3T3-L1 cells were transfected pHAGE-EF1a-Cas9-IRES-Blast plasmid with lipofectamine 2000 (Thermo Fisher Scientific Inc.). Blasticidin (Calbiochem, VWR) was used to select cells stably expressing Cas9. Cas9-expressing 3T3-L1 cells then were transfected with sgRNAs to knock out Epac1. Epac1 knockout cells were selected by Puromycin and confirmed by RT-qPCR and Western Blot.

Leptin Secretion Assay – 3T3-L1cells were used for adipocyte differentiation as described before[153]. Leptin secretion assay was performed using differentiated 3T3-L1 adipocytes as described previously[60]. Briefly, 3T3-L1 pre-adipocytes were maintained at

37 ℃ with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS. Adipocyte differentiation was induced after two days the cells became 100% confluent, with the cocktail of 5 μg/ml insulin, 0.5 mM 3- isobutyl-1-methylxanthine (IBMX), 1μM dexamethasone (DEX) and 5 μM Rosiglitazone. The cells were kept in the full cocktail for 3 days and then switched to the medium with 10% FBS plus insulin and Rosiglitazone for 2 more days. The 3T3 adipocytes were then kept in DMEM/10% FBS medium for 1 more week. During this time, manipulations were performed to knock out Epac1 by CRISPA-Cas9 or inhibiting Epac1 activity by adding specific inhibitor ESI-09, respectively. Cell culture supernatant was collected and concentrated by Amicon Ultra 0.5 ml centrifugal filters (Merck Millipore Ltd.) and used for measurement of leptin concentration. The leptin levels were normalized to the total protein in the culture supernatant.

Western Blot – Tissues were homogenized in RIPA buffer (50mM Tris-HCl, pH 7.5; 150mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 0.1% SDS; 0.5% Na-deoxycholate; 50mM sodium floride plus 1% phosphatases inhibitor cocktail I, 0.5% phosphatase inhibitor cocktail II, and 1% protease inhibitor cocktail [Sigma-Aldrich, MO]) to prepare tissue lysate. Total cellular protein concentration was determined using the bicinchoninic acid (BCA) assay. Samples with equal total proteins (10μg) were analyzed by SDS-PAGE, transferred to PVDF membranes and probed by specific antibodies. The antibodies used were: Epac1 (4155; Cell Signaling), phosphorylated STAT3 tyrosine 705 (9131; Cell Signaling), phosphorylated AKT Ser473 (9271; Cell Signaling), phosphorylated CREB Ser133 (9198; Cell Signaling), GAPDH (10r-g109A, Fitzgerald).

Reverse-Transcription Quantitative Real-time PCR (RT-qPCR) – Total RNA in white adipose tissue, macrophages, and cells were isolated with TRIzol (Invitrogen) and

then subjected to EconoSpin spin column for RNA extraction (Epoch Life Science). The RNA concentrations were determined by Nanodrop (Thermo Scientific). The cDNA was synthesized by reverse transcription step from RNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). The iTaq Unversal SYBR Green Supermix kit (Bio-Rad) was used for quantitative PCR. The primers used were: mouse GAPDH: 5'-TGGAAAGCTGTGGCGTGAT-3' (sense) and 5'- TGCTTCACCACCTTCTTGAT-3' (antisense); mouse Epac1: 5'-GGAAGCTCCTGCACCGCCATCTCTT-3' (sense) and 5'-AAGATCCCATCCACTAGCTCCCGGC-3' (antisense); mouse Leptin: 5'-GAGACCCCTGTGTCGGTTC-3' (sense) and 5'-CTGCGTGTGTGAAATGTCATTG-3' (antisense). Comparative cycle threshold (Ct) method is used for data analysis[156]. The calculations of delta Ct (dCt) and delta delta Ct (ddCt) are shown below:

dCt = Ct(goi) – Ct(ref)

Here, *goi* is the gene of interest; *ref* is the reference gene.

ddCt = dCt(exp) – dCt(cal)

Here, *exp* is the gene for the particular experimental sample and *cal* is the same gene for the calibrator sample.

The linear fold change between the experimental and calibrator samples is 2^{-ddCt}

Statistical Analysis – Results are presented as mean ± standard error of the mean (SEM), with $p<0.05$ regarded as statistically significant. A student t test was used to compare between two groups. One –way or two-way ANOVA and Bonferroni post hoc test were used to compare among groups.

Chapter 3 Deletion of Epac1 enhances leptin sensitivity, reduces adiposity and improves glucose homeostasis *in vivo*

*Note: This Chapter contains a portion of the paper:

Yan, J.; Mei, F. C.; Cheng, H.; Lao, D. H.; **Hu, Y**.; Wei, J.; Patrikeev, I.; Hao, D.; Stutz, S. J.; Dineley, K. T.; Motamedi, M.; Hommel, J. D.; Cunningham, K. A.; Chen, J.; Cheng, X., Enhanced leptin sensitivity, reduced adiposity, and improved glucose homeostasis in mice lacking exchange protein directly activated by cyclic AMP isoform 1. *Mol Cell Biol* **2013,** *33* (5), 918-26.

3.1 INTRODUCTION

Obesity is an increasingly alarming health problem in the United States as it is closely related to the leading causes of morbidity and mortality, such as cardiovascular diseases, type 2 diabetes, hypertension, depression and cancer[1]. To date, few effective treatment options are available for obesity. A better understanding of the molecular mechanisms of obesity development and effective, safe therapeutic interventions are needed. At the physiological level, obesity is the result of a prolonged small imbalance between energy intake and energy expenditure. The identification of the leptin gene (Ob) provided a milestone in our understanding of obesity at the molecular level[54]. Leptin, an appetite suppressing hormone derived from adipose tissues, plays a key role in regulating food intake and energy expenditure. Leptin functions by binding to receptors on the arcuate nucleus (ARC) of the hypothalamus and activating the JAK/STAT3 signaling pathway[69]. While leptin administration decreases food intake and reduces body weight in lean humans and animals, circulating leptin concentrations are correlated with percentage of body fat and greatly elevated in obese individuals[86], suggesting that obese persons are insensitive to leptin production in much the same way type 2 diabetes patients are resistant to the effects of insulin. Therefore, understanding the molecular/cellular mechanisms underlying leptin resistance and developing therapeutic interventions targeting leptin resistance is a key issue in fighting obesity.

Cyclic AMP mediated signaling pathways are important for maintaining metabolic homeostasis and have been implicated in regulating leptin production and secretion[157], as well as food consumption[92]. In eukaryotic cells, the effects of cAMP are mediated mainly by two ubiquitously expressed intracellular cAMP receptors, the classic protein kinase A/cAMP-dependent protein kinase (PKA/cAPK) and the more recently discovered exchange protein directly activated by cAMP/cAMP-regulated guanine nucleotide exchange factor (Epac/cAMP-GEF). A recent study reveals that activation of

Epac proteins by an Epac-selective cAMP analog, 8-CPT-2'-O-Me-cAMP, interferes with leptin signaling in hypothalamus, suggesting that Epac may contribute to the pathophysiology of leptin resistance and represent a novel pharmacological target for obesity[149]. To investigate the functional significance of exchange protein directly activated by cAMP isoform 1 (Epac1) in leptin-mediated energy balance *in vivo*, we have generated a global loss of function mutant for Epac1 to test its functional role in regulating adiposity and energy balance.

3.2 RESULTS

Global $Epac1^{-/-}$ mice are partially resistant to diet-induced obesity (DIO) – To determine if Epac1 plays a general role in energy balance *in vivo*, we monitored the body weight and average daily food intake of $Epac1^{-/-}$ and WT littermates. Compared to their WT controls, Epac1 null mutant mice appeared normal with similar body weights at birth up to three weeks of age, when the mice were weaned and started on the high fat diet or standard rodent chow. However, the $Epac1^{-/-}$ mice on chow diet gradually gained less body weight compared to WT controls, and the difference became significant around week 5, eventually reached approximately 85% of the body weight of wild-type littermates (**Figure 3.1 A and B**). Similar observations were made for the HFD-fed mice. After on HFD for 7 weeks, the knockout mice showed significantly lower body weight, and maintained at about 90% of the littermates. (**Figure 3.1 C and D**)

The reduced body weight of Epac1 KO mice were most likely not due to the result of overall growth retardation or developmental defects as the $Epac1^{-/-}$ mice had the same body length as the wild-type mice.**(Figure 3.2 A)** Consistent with the body weight change, the $Epac1^{-/-}$ mice had statistically lower average food intake than WT controls on chow diet; however, although there was a similar trend, the average daily food intake in mice fed on HFD was not statistically different (**Figure 3.2B**).

$Epac1^{-/-}$ mice reduced adiposity. – To ascertain if decreased body weight in $Epac1^{-/-}$ mice is due to reduced adipose tissue accumulation, we examined the adiposity of the animals. As shown in **Figure 3.3 A, B**, the epididymal fat pads from $Epac1^{-/-}$ mice were smaller and significantly less than those from wild-type. Furthermore, the whole body fat mass of Epac1 null mice on HFD were significantly less than those from wild-type controls as determined by **Figure 3.3 C, D**. Moreover, the difference in calculated total fat mass was larger than the difference in body weight

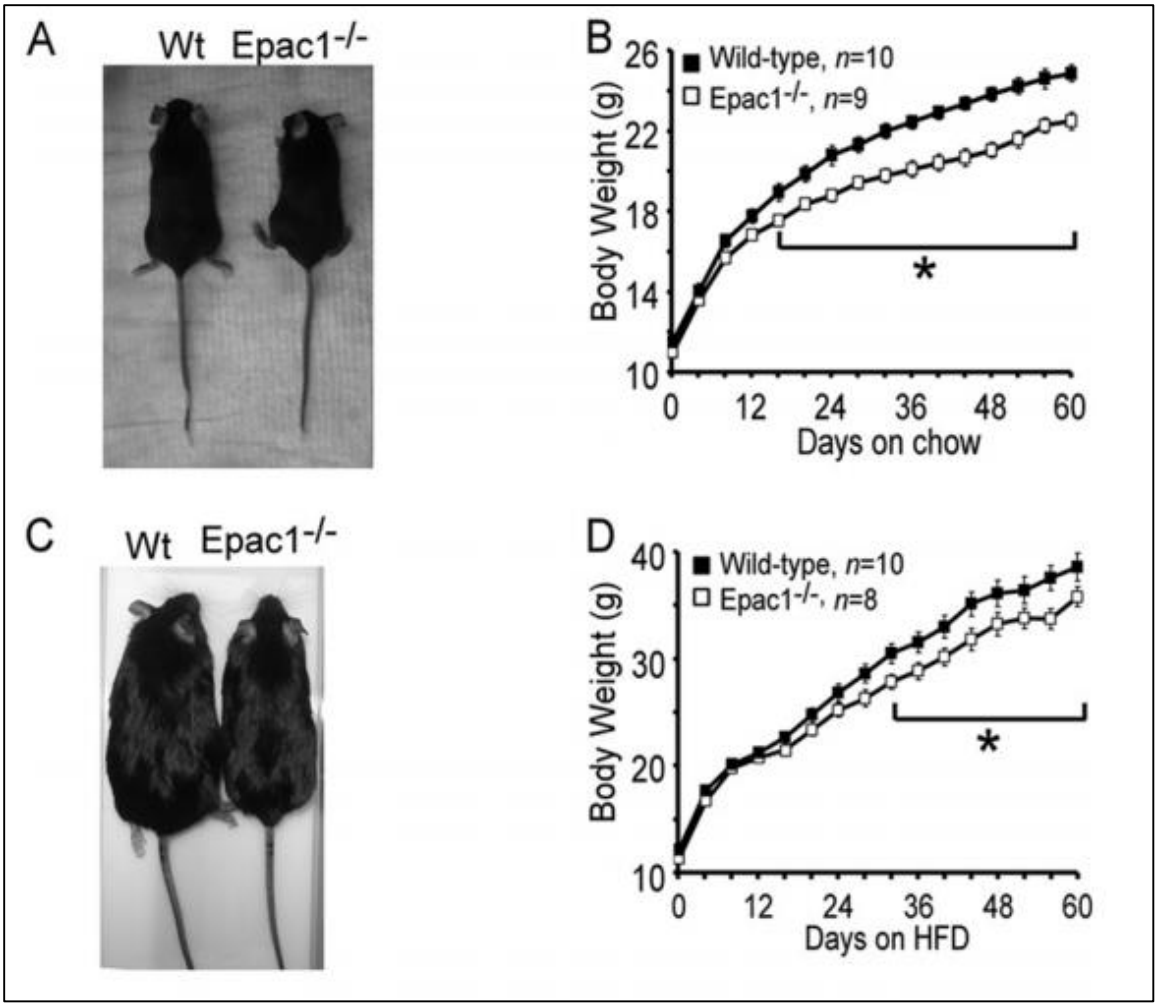

FIGURE 3.1 GLOBAL EPAC1 KNOCKOUT MICE HAVE LOWER BODY WEIGHT ON EITHER CHOW DIET OR HFD. (A) Aged matched 10-month-old Epac1 -/- and WT mice fed on chow diet. The $Epac1^{-/-}$ mouse was obviously smaller than WT control with similar body length. (B) Growth curves of WT and Epac1 -/- mice fed on a chow diet. Data are means ± standard deviations (SD). (C) Aged matched 10-month-old $Epac1^{-/-}$ and WT mice fed on HFD. The $Epac1^{-/-}$ mouse was obviously smaller than WT control with similar body length. (D) Growth curves of WT and $Epac1^{-/-}$ mice fed on HFD. Data are means ± SD. *Figure is adopted from Yan et al.*[153]

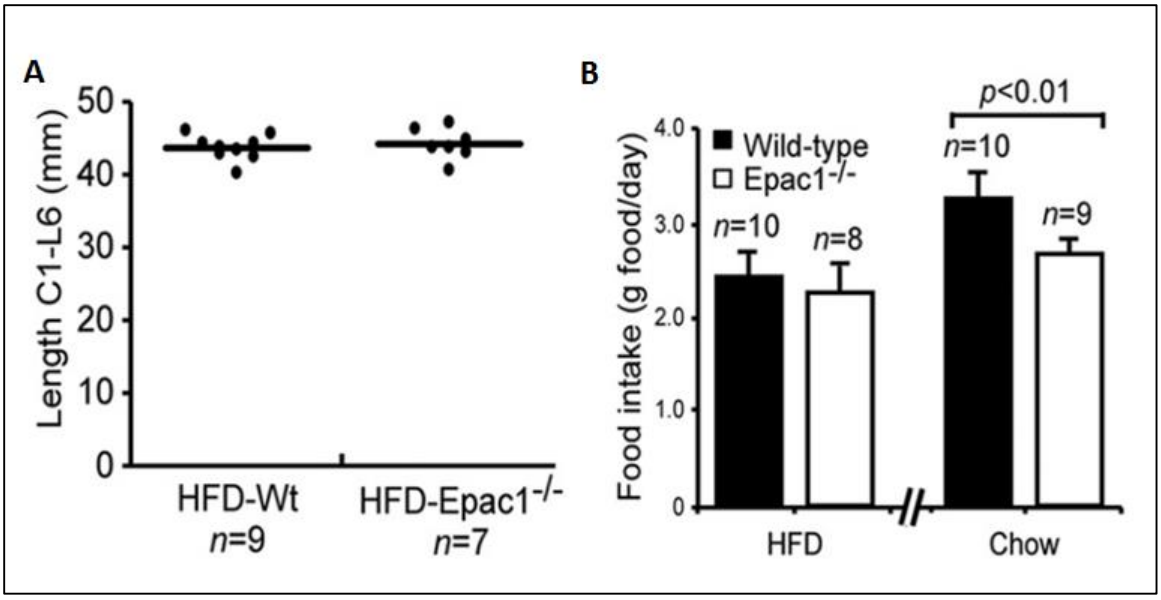

FIGURE 3.2 DELETION OF EPAC1 DECREASES FOOD INTAKE OF MICE FED ON CHOW DIET. (A) Epac1 KO and WT littermates had similar body length after 10 weeks on HFD. The body length was measured from the C1 to L6 vertebrae. (B) Average daily food intakes of WT and Epac1 KO mice on HFD and chow diets. Data are means ± SD. *Figure is adopted from Yan et al.*[153]

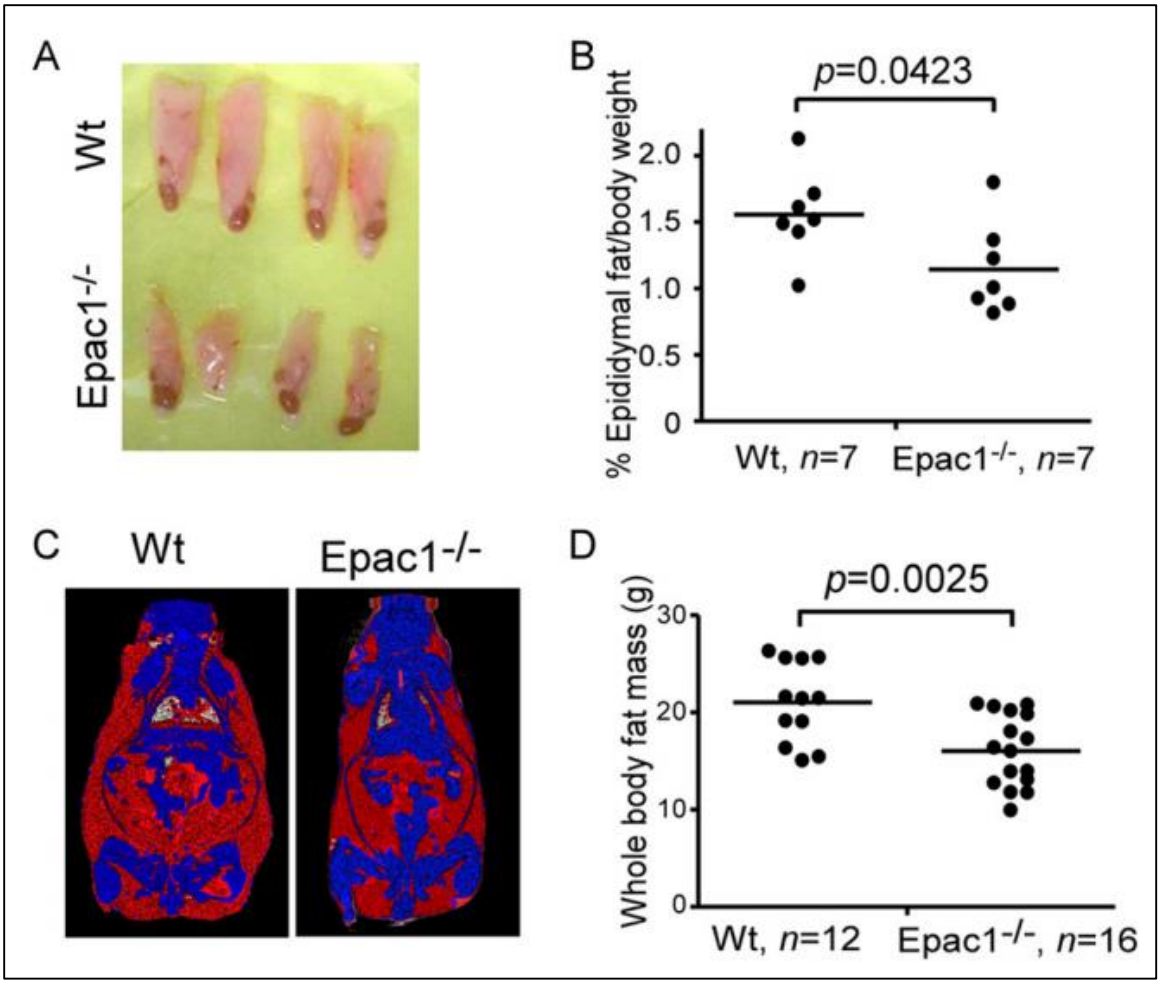

FIGURE 3.3 ADIPOSITY IS IMPAIRED IN EPAC1 DEFICIENT MICE. (A) The epididymal fat pads from WT and $Epac1^{-/-}$ mice. (B) Normalization of epididymal fat by dividing whole body weight. (C) 3D reconstruction images of micro-CT scanning. (D) Whole body fat mass of $Epac1^{-/-}$ and WT mice. *Figure is adopted from Yan et al.*[153]

between Epac1 null and WT mice. Taken together, these data suggest that the decreased body weight of $Epac1^{-/-}$ mice is due to a decline in fat mass accumulation.

To investigate if the apparent reduction in Epac1 null mice is the result of impaired adipocyte differentiation or a reduction of adipocyte size, we performed ex vivo adipocyte differentiation analyses using mouse embryonic fibroblast (MEF) cells isolated from both wild-type and $Epac1^{-/-}$ mice. As shown in **Figure 3.4 A**, $Epac1^{-/-}$ and WT MEFs differentiated into mature adipocytes with similar efficiencies, suggesting that Epac1 deficiency did not impede adipose differentiation. On the other hands, adipocytes from epididymal white fat tissue of Epac1 null mice were significantly smaller than those from the wild-type counterparts (**Figure 3.4B**).

Epac1 null mice display reduced plasma leptin levels. – The adipose-secreted hormone leptin is one of the most important hormones to regulate energy homeostasis. The plasma leptin levels are positively correlated with the body fat mass. In order to investigate whether the phenotypes of lower body weight, food intake and reduced adipose mass in Epac1 KO mice were related to plasma leptin levels, we determined the plasma leptin levels in WT and Epac1 KO mice on both chow diet and HFD. The average plasma leptin levels were 3.97 ± 0.78 ng/ml (means ± standard error of the mean [SEM]) and 1.01 ± 0.26 ng/ml in WT and Epac1 KO mice on the chow diet, respectively. The plasma leptin levels in Epac1 KO mice were significantly lower than those in age- and gender-matched WT littermates (**Figure 3.5 A**). Similar observations were made on the mice fed on HFD. The average plasma leptin levels were 83.16 ± 5.76 ng/ml of WT mice, which were statistically higher than those in Epac1 KO littermates with 66.15 ± 3.52 ng/ml (**Figure 3.5 B**).

Loss of Epac1 sensitizes leptin/STAT3 signaling. – To determine if Epac1 deficiency affects leptin signaling *in vivo*, we probed the basal and stimulated pSTAT3 Y705 levels in response to intracerebroventricular (ICV) injection of leptin by immunoblotting analysis. As shown in **Figure 3.6**, the basal and the stimulated levels of

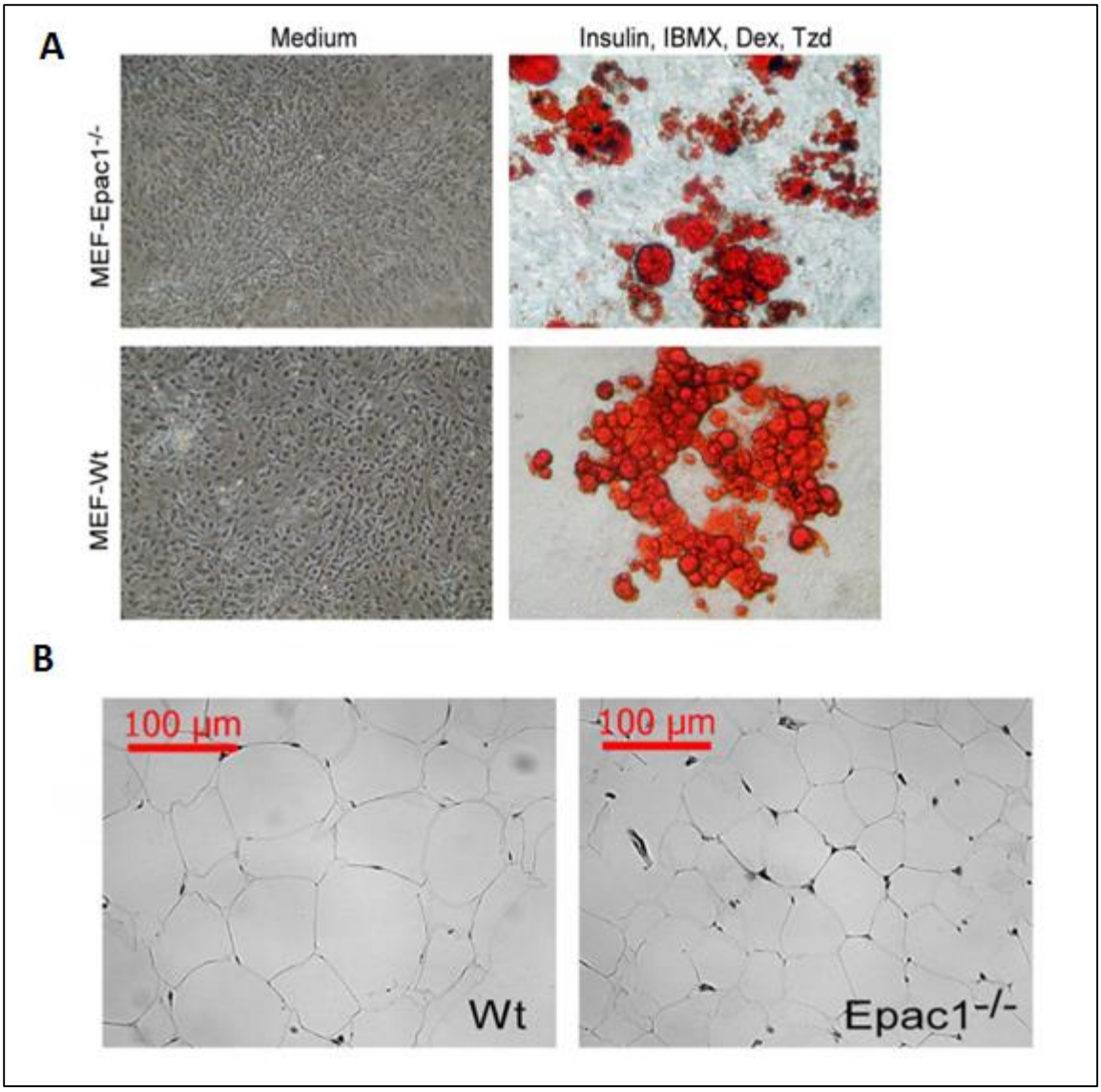

FIGURE 3.4 EPAC1 DEFICIENCY REDUCES ADIPOSITY. (A) In vitro differentiation of adipocytes from mouse embryonic fibroblasts. (B) Hematoxylin and eosin staining of paraffin-embedded epididymal white adipose tissue. *Figure is adopted from Yan et al.*[153]

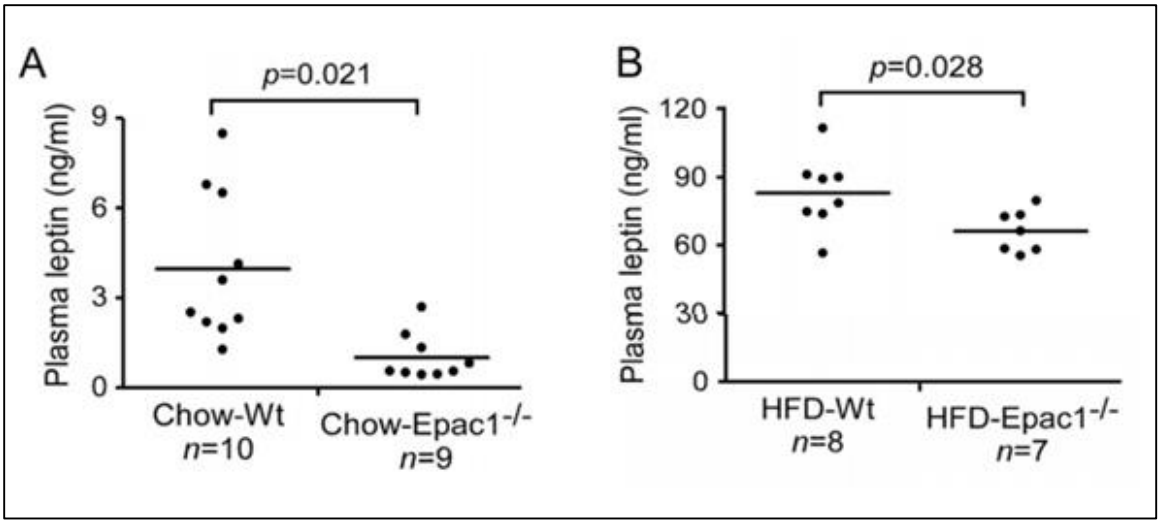

FIGURE 3.5 DISRUPTION OF EPAC1 DOWN-REGULATES PLASMA LEPTIN LEVELS. Age- and gender- matched WT and Epac1 KO mice were fasted overnight and the blood was collected for measuring plasma leptin levels. (A) The plasma leptin levels from 16-week-old mice fed on chow diet. (B) The plasma leptin levels from 28-week-old mice fed on HFD. *Figure is adopted from Yan et al.*[153]

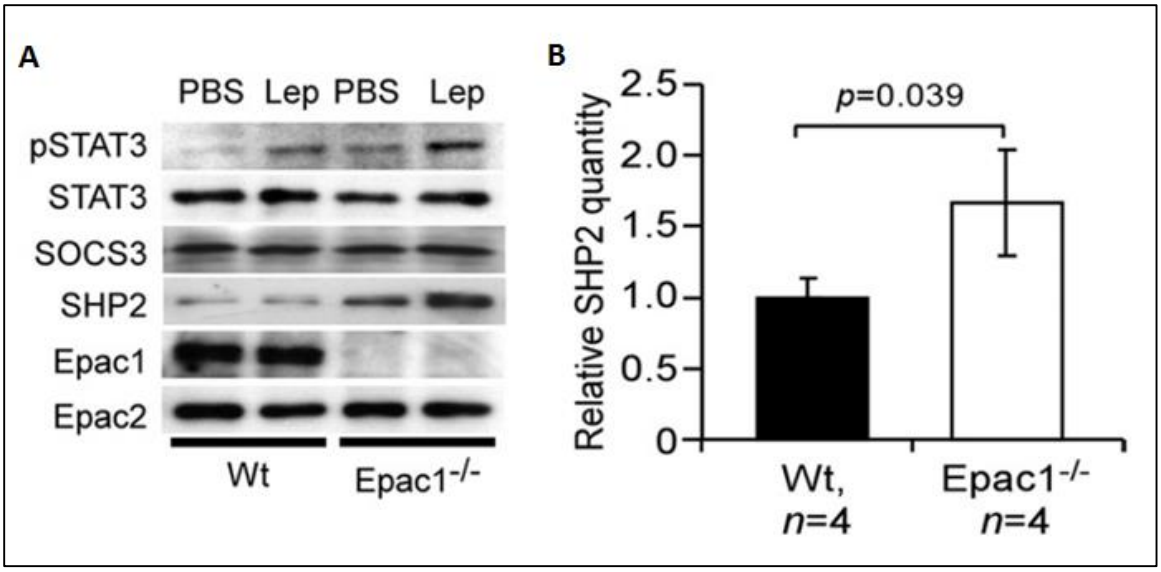

FIGURE 3.6 HEIGHTENED LEPTIN SIGNALING IN EPAC$1^{-/-}$ ***HYPOTHALAMUS***. (A) Western blotting of pSTAT3 Y705, total STAT3, SOCS3, SHP2, Epac1 and Epac2 in the hypothalamus 30 min after ICV injection of vehicle (PBS) or leptin (5 μg) into the lateral ventricle of a 3-week-old WT or $Epac1^{-/-}$ mouse. (B) Quantitation of SHP2 Western blotting results for 4 pairs of male mice at 3 to 4 weeks of age. Data are means ± SEM. *Figure is adopted from Yan et al.*[153]

pSTAT3 Y705 were both increased in the $Epac1^{-/-}$ hypothalamic tissue. These data are consistent with a recent report by Fukuda, et al. showing that pharmacological activation of Epac-Rap1 with an Epac selective agonist blunts leptin signaling in hypothalamus[149]. To probe the potential mechanism by which Epac1 deficiency promotes leptin signaling, we measured the level of suppressor of cytokine signaling 3 (SOCS3), whose expression has been shown to be under the control of Epac1 in vascular endothelial cells[158]. However, SOCS3 expression in hypothalamus was not significantly affected by Epac1 knockout (**Figure 3.6**). On the other hand, we found that the expression level of tyrosine phosphatase SHP2, another important regulator of leptin-Jak2-Stat3 signaling, was reproducibly increased in the hypothalamic tissue of $Epac1^{-/-}$ mice.

$Epac1^{-/-}$ mice show improved glucose homeostasis against HFD stress. – When the glucose handling capability of wild-type and $Epac1^{-/-}$ mice was compared using the oral glucose tolerance test (OGTT), similar results were obtained for $Epac1^{-/-}$ and WT mice on the standard chow diet. (**Figure 3.7 A**) However, $Epac1^{-/-}$ mice showed a significantly improved glucose handling capability after fed on HFD for 15 weeks. The basal blood glucose in $Epac1^{-/-}$ mice after overnight fasting was significantly lower than that in WT mice. What's more, the blood glucose levels in $Epac1^{-/-}$ mice were statistically lower than those in WT control groups at every time point after glucose administration. The blood glucose of $Epac1^{-/-}$ mice returned to the baseline in 2 hours while it remained elevated in the WT mice. (**Figure 3.7 B**) These data suggest that $Epac1^{-/-}$ mice are more resistant to HFD-induced glucose intolerance.

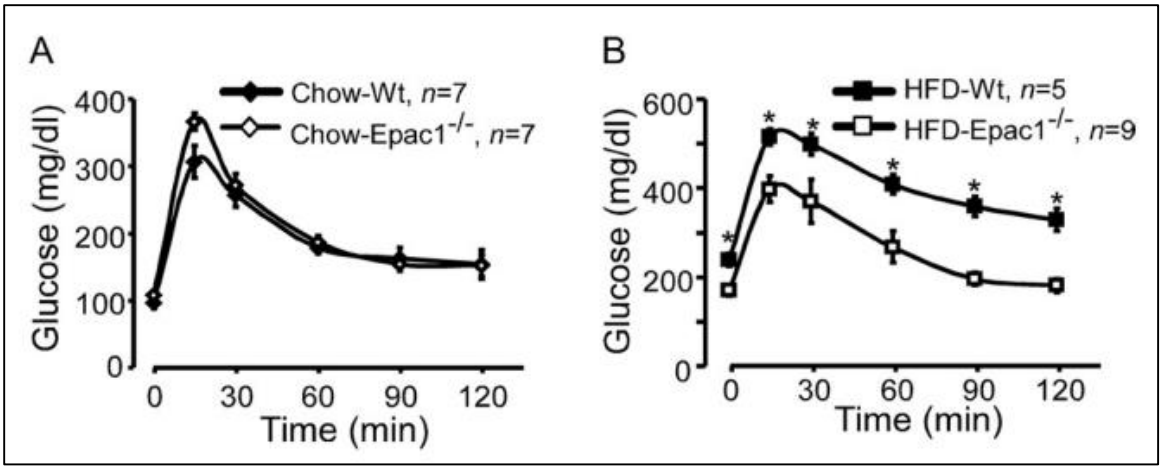

FIGURE 3.7 $Epac1^{-/-}$ ***MICE ARE RESISTANT TO HFD-INDUCED GLUCOSE INTOLERANCE.*** (A) WT mice and $Epac1^{-/-}$ mice have similar glucose clearing capability on regular chow diet. Data are means ± standard error of the mean (SEM). (B) $Epac1^{-/-}$ mice have better glucose clearting capability on HFD. Data are means ± SEM. *Figure is adopted from Yan et al.*[153]

3.3 DISCUSSION

In this study, we show that the body weight gains in global Epac1 knockout mice slow down and become significantly less than WT controls on either chow or HFD. The body weight reduction observed in Epac1 null mice is most likely due to the reduction of adiposity because the estimated whole body fat mass reduction by CT-imaging in the $Epac1^{-/-}$ is greater than the total body weight difference between Epac KO and WT mice. Epac1 deficiency also results in smaller epididymal adipocyte size *in vivo*.

Studies using 3T3-L1 preadipocytes *in vitro* suggest that Epac1 is involved in regulating adipocyte differentiation[33, 41, 46]. However, our results using isolated MEF cells *ex vivo* demonstrate that Epac1 does not play a critical role in adipogenesis. The decreased adiposity observed in Epac1 null is most likely due to reduced accumulative food intake as WT mice have more average daily food intake than Epac1 KO mice, although the difference was not significant when they were fed on HFD due to relative small sample volumes.

This heightened basal leptin signaling activity and sensitivity is further validated by a more pronounced food intake and body weight reduction of $Epac1^{-/-}$ mice in response to high dose intraperitoneal leptin injection. It has been well documented that enhanced leptin sensitivity confers resistance to diet induced obesity and improves glucose tolerance[83, 87, 159]. Indeed, our studies show that $Epac1^{-/-}$ mice are markedly protected from HFD induced fasting hyperglycemia and glucose intolerance. Therefore, Epac1 may be a critical intermediate by which a HFD causes impaired glucose control.

Epac1 KO mice have significantly lower plasma leptin levels compared to age- and gender- matched WT controls either on regular chow diet or HFD. This is consistent with the reduced adiposity in KO mice since circulating leptin levels are proportional to the total fat mass. Interestingly, despite the lower plasma leptin level, the leptin/JAK/STAT3 signaling activity, as measured in pSTAT3 level, is enhanced in

$Epac1^{-/-}$ mouse at steady state *in vivo*, a sign of a heightened basal level of leptin signaling activity. Moreover, leptin ICV injection leads to an even more robust increase in pSTAT3 in $Epac1^{-/-}$ hypothalamus, indicating an enhanced leptin sensitivity. While it has been reported that in vascular endothelial cells Epac1 activation upregulates SOCS3, which subsequently suppresses IL-6 mediated STAT3 phosphorylation[160], the level of SOCS3 in hypothalami in $Epac1^{-/-}$ mice is not significantly changed. However, SHP2, another important signaling molecule involved in regulating leptin signaling, is significantly upregulated in $Epac1^{-/-}$ mice. While SHP2 can act both as a positive and negative regulator of leptin signaling *in vitro*[161], several *in vivo* studies using tissue-specific SHP2-null mouse models demonstrate that SHP2 functions as a positive regulator sensitizing leptin signaling in the brain[162]. Therefore, our study reveals a novel mechanism by which inactivation of Epac1 promotes leptin signaling via upregulation of SHP2.

In line with the fact that Epac1 deficiency mice are more protected against DIO and have heightened leptin sensitivity, these mice are protected against glucose intolerance when challenged by HFD. Epac1 KO mice not only have lower basal blood glucose level, but also clear glucose from blood significantly faster than wild-type mice after glucose challenge.

In summary, we are the first to discover that Epac1 knockout mice are more resistant to high-fat diet induced obesity, hyperleptinemia, glucose intolerance and insulin resistance. Our work demonstrates the relevance of intracellular cAMP on leptin resistance. Specifically, Epac1 appears to blunt the leptin signaling, thus decreasing the responsiveness of leptin receptor and perpetuating a cycle leading to increased body weight. However, several important questions remain. For example, it is not clear if the apparent phenotypes of Epac1 null mice are due to the loss of Epac1 functions specifically in peripheral adipose tissues, in the central nerve system or both. Addressing

this question requires careful dissections of tissue-specific Epac1 knockout mice, which is the focus of the next chapter.

Chapter 4 Role of Epac1 in Energy Homeostasis: Regulation of Leptin Expression and Secretion

*Note: This Chapter contains a portion of the paper:

Hu, Y.; Mei, F.; Kim, ER.; Wang, H.; Tong, Q.; Jin, J.; Chen, J.; Cheng, X., Role of exchange protein directly activated by cAMP isoform 1 in energy homeostasis: regulation of leptin expression and secretion in white adipose tissue. Submiteed to *Mol Cell Biol*

4.1 INTRODUCTION

Adipose tissue is not only the main reservoir for fat storage, but also an important endocrine organ that secrets various adipose-derived hormones, adipokines. As one of the most important and widely studied adipokines, leptin plays a critical role in energy balance by acting on receptors in the arcuate nucleus of the hypothalamus to suppress appetite and to increase energy expenditure.[54] [163] While the circulating leptin levels is proportional to the total body fat mass in rodents and human, in obese patients a reduced sensitivity to leptin leads to a failure in suppressing food intake despite high fat stores and blood leptin levels. This apparent condition of leptin resistance is one of the major drivers in developing obesity. [69]

Recent studies have implicated the involvement of exchange protein directly activated by cAMP (Epac1) mediated signaling in leptin resistance. [149] [164] As a major intracellular sensor of second messenger cAMP, pharmacological activation of Epac proteins, but not protein kinase A the classic cAMP receptor, dampens leptin signaling in mice hypothalamus by reducing signal transducer and activator of transcription 3 (STAT3) phosphorylation levels.[149] Consistent with the pharmacological studies, global Epac1 knockout mice exhibit with an increased leptin sensitivity, decreased food intake and overall adiposity despite a reduced serum leptin levels.[153] These findings establish the importance of Epac1 in leptin signaling and energy homeostasis.

Epac1 is ubiquitously expressed[129], with abundant expressions both in the adipose tissue and the central nervous system, which represents the main source of leptin production and the major target of leptin signaling, respectively. To date, it is not clear if the apparent phenotypes of Epac1 null mice are due to the loss of Epac1 functions specifically in peripheral adipose tissues, in the central nervous system or both. In this study, we investigate the adipose tissue specific functions in energy balance and leptin signaling by using adipose tissue specific Epac1 knockout mice.

4.2 RESULTS

AEKO mice display increased body weight and food intake on high fat diet (HFD). -To ascertain the functional role of Epac1 in adipose tissue, we generated adipose specific Epac1 knockout mice (AEKO) using the *Cre/LoxP* site – specific recombination system. Disruption of Epac1 expression in WAT was confirmed by Western blot analyses whereas the expression Epac1 in hypothalamus was not affected (**Figure 4.1 A**). The AEKO mice and WT controls had similar body weights from birth to postnatal day 24, when the mice were weaned and switched to a HFD. The AEKO mice gradually gained more body weight than WT controls, and became significantly heavier than WT mice around week 6 (**Figure 4.1 B**). In agreement with the heavier body weight phenotype of AKEO mice on HFD, the average daily food intake of AEKO mice were significantly higher than that of the WT littermates (**Figure 4.1 C**). No significant difference in body weight between AEKO and WT group was observed when mice were fed on regular chow diet (**Figure 4.1 D**).

Deficiency of Epac1 in adipose tissue down-regulates energy expenditure. **-**To determine the potential causes of the apparent difference in body weight, we examined the effects of adipose tissue specific Epac1 deletion on energy homeostasis by monitoring the energy expenditure using the CLAMS system. As shown in **Figure 4.2A &B**, while both groups of mice had higher oxygen (O_2) consumption rates at night when the animals were most active regardless of the food sources; mice fed on HFD had a higher O_2 consumption rate than mice fed on regular chow. Importantly, the AEKO mice showed significantly lower O_2 consumptions when compared to WT counterparts under HFD or chow diet. Similar observations were made for carbon dioxide (CO_2) production. The AEKO mice had statistically lower CO_2 production rate on both chow and HFD (**Figure 4.2 C&D**). The calculated respiratory exchange rate, defined as ratio between O_2 consumptions and CO_2 production, was lower in HFD fed mice for both groups (**Figure**

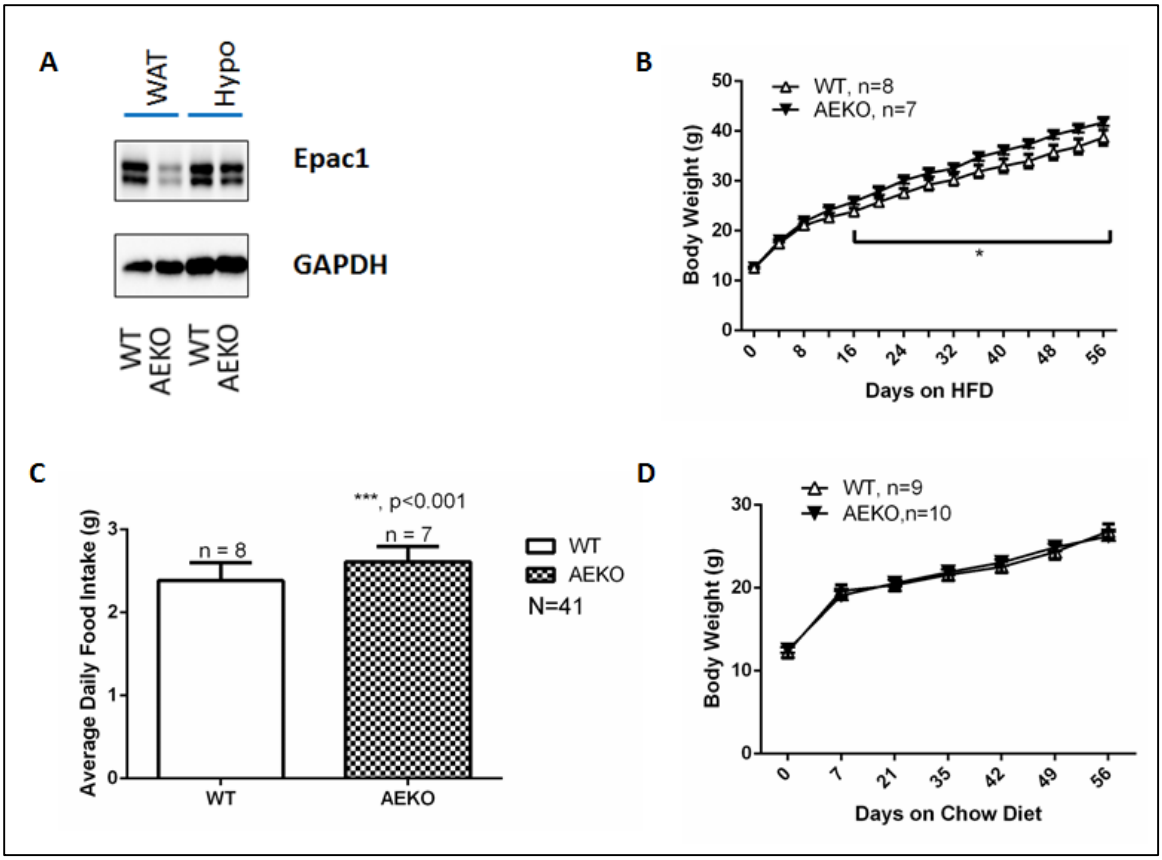

FIGURE 4.1 DISRUPTION OF EPAC1 IN ADIPOSE TISSUE INCREASES BODY WEIGHT GAINS AND FOOD INTAKE ON HFD. (A) Immunoblotting result shows that Epac1 has been knocked out in adipose tissue, with its level intact in hypothalamus. (B) Body weight changes of WT and AEKO mice on HFD. Data are means ± standard error of the mean (SEM). *, $p<0.05$ by two way repeated ANOVA. (C) Average daily food intake of WT and AEKO mice on HFD for 41 days. Data are means ± standard deviations (SD). ***, $p<0.001$ by student *t* test. Days (N) = 41. (D) Body weight changes of WT and AEKO mice on regular chow diet. Data are means ± standard error of the mean (SEM).

4.2 E). This is consistent with the fact that mice on HFD burned more fatty acids while mice on chow diet utilized carbohydrates as the predominant fuel source. There was no significant difference in respiratory exchange rate between AEKO and WT mice under HFD or chow diet. Taken together these data suggest that AEKO mice have an overall lower energy expenditure than the WT controls while maintaining a balanced carbohydrate/lipid oxidation ratio.

To determine if the apparent lower energy expenditure phenotype observed in AEKO mice was associated with a decrease in overall physical activities, we monitored and compared the spontaneous locomotor activity between AEKO and WT mice under HFD or chow diet. While there were no significant changes in the average total activity counts between AEKO and WT cohorts, AEKO mice showed a slight increase in voluntary locomotor activity consistently across all groups (**Figure 4.3 A & B**). Similarly, no statistically significant difference in food intake between AEKO and WT mice was observed although AEKO mice had slightly higher food intake than WT mice when fed on HFD (**Figure 4.3 C**).

***Loss of Epac1 in adipose tissue reduces plasma leptin levels*. -**To investigate if aforementioned phenotypes of reduced energy expenditure and increased body weight and food intake were related to change in leptin, one of the most important adipose-derived hormones for regulating energy homeostasis, we determined plasma leptin levels. As shown in **Figure 4.4 A**, the measured plasma leptin level (95.6 ± 3.4 ng/ml) of 15-week-old AEKO mice fed on HFD for 12 weeks was significantly lower than that (109.5 ± 2.5 ng/ml) of the age-matched WT controls. The reduced leptin levels in AEKO mice are particularly significant considering that these mice have increased body weights, which would normally be associated with an increase in leptin level. Therefore, these results suggest that Epac1 may be directly involved in regulating production and/or secretion of leptin in adipose tissue, and are consistent with the finding that plasma leptin levels were significantly lower in newly weaned naïve AEKO mice than those in age-

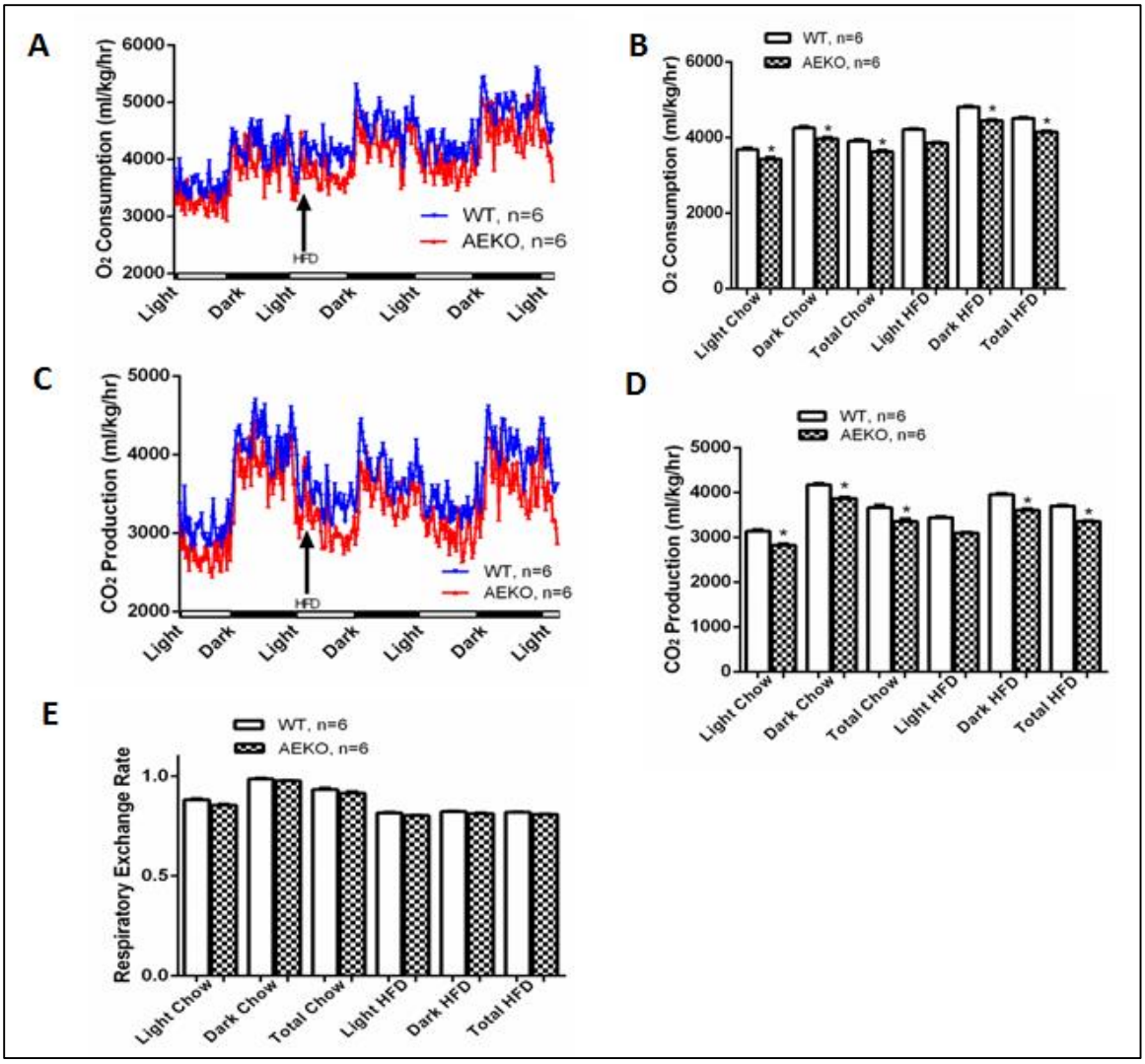

FIGURE 4.2 DELETION OF EPAC1 IN ADIPOSE TISSUE DOWN-REGULATES ENERGY EXPENDITURE. (A) Energy expenditure of WT and AEKO mice measured by O_2 consumption at age of 5 weeks by CLAMS. (B) Average O_2 consumption of WT and AEKO mice. Data are means ± SEM. *, $p< 0.05$ by student *t* test. (C) Energy expenditure of WT and AEKO mice measured by CO_2 production at the age of 5 weeks by CLAMS. (D) Average CO_2 production of WT and AEKO mice. Data are means ± SEM. *, $p<0.05$ by student *t* test. (E) The respiratory exchange rate of WT and AEKO mice. Data are means ± SEM.

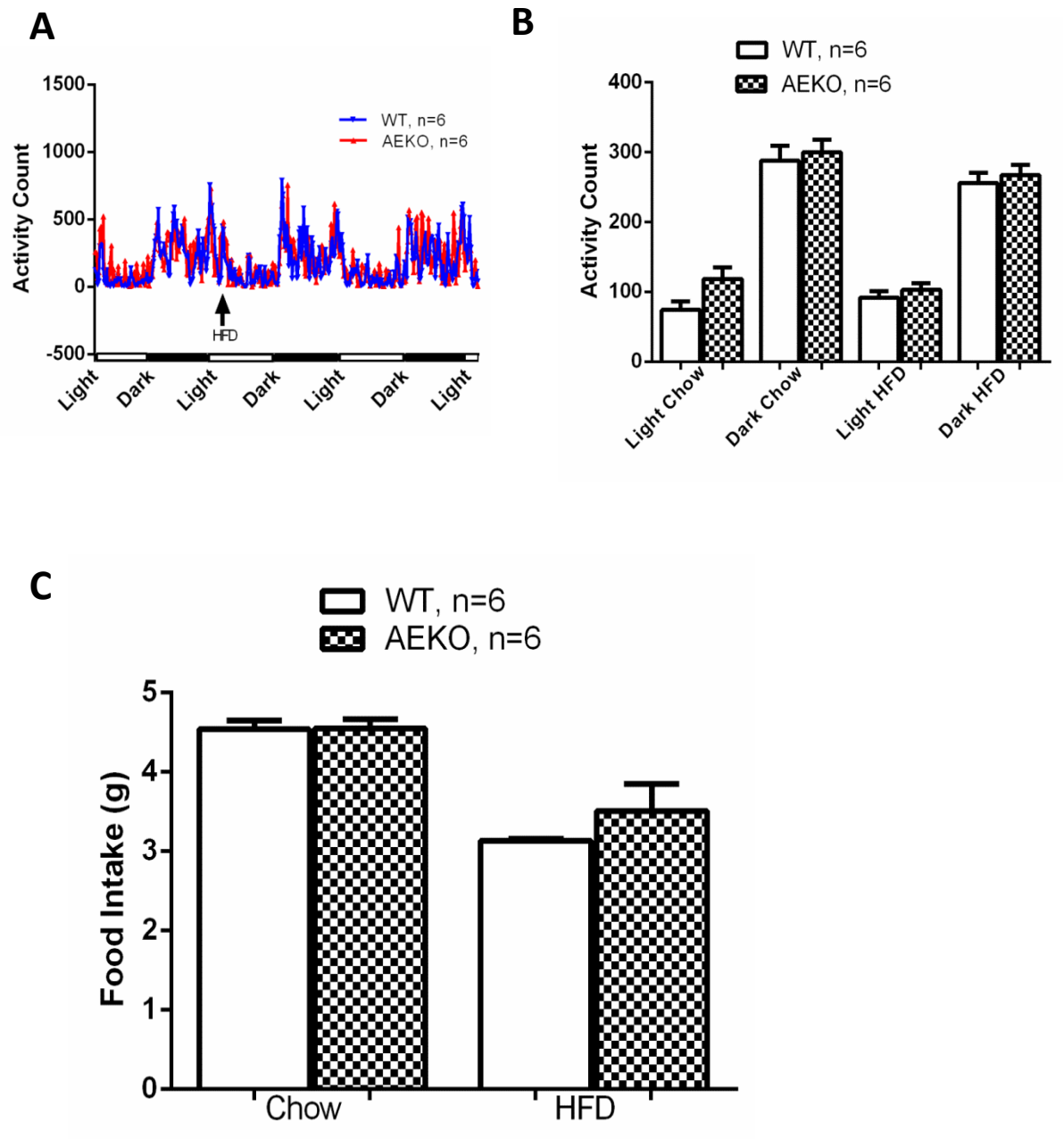

FIGURE 4.3 AEKO AND WT MICE HAVE SIMILAR LEVELS OF SPONTANEOUS LOCOMOTOR ACTIVITY AND FOOD INTAKE. (A) Spontaneous activity profiles of WT and AEKO mice. (B) Average spontaneous locomotor activity of WT and AEKO mice. Data are means ± SEM. (C) Daily food intake of WT and AEKO mice on regular chow diet and HFD.

matched WT mice on the postnatal day 24 when there was no body weight difference between the two groups (**Figure 4.4 B**).

***Epac1 deletion and inhibition in 3T3-L1 adipocytes reduce leptin secretion*.-**To confirm that the apparent reduction in plasma leptin levels observed in AEKO mice was due to direct Epac1 deletion, not secondary effects such as *in vivo* compensation and/or adaptation. We performed *in vitro* analyses using 3T3-L1, a model pre-adipocyte cell line widely used for studying adipocyte differentiation and functions such as leptin secretion[60]. As shown in **Figure 4.5 A**, suppressing Epac1 activity in differentiated 3T3-L1 adipocytes using an Epac-specific inhibitor, ESI-09[165 166], led to dose-dependent inhibition of leptin secretion. Furthermore, we used the CRISPR-Cas9 gene editing system to disrupt Epac1 gene in fully differentiated 3T3-L1 adipocytes with two distinct sgRNA sequences to minimize potential interference of Epac1 deletion in 3T3-L1 differentiation, as well as potential off-target effects. Consistent with the pharmacological data, knocking out Epac1 in 3T3 adipocytes significantly suppressed leptin secretion in differentiated 3T3-L1 adipocytes (**Figure 4.5 B**). Taken together, these data demonstrate a direct role of Epac1 in leptin secretion, supporting the *in vivo* observation of reduced plasma leptin levels in AEKO mice.

***AEKO mice have more severe HFD induced glucose intolerance*. -** In light of our finding that deletion of Epac1 in adipose tissue reduces plasma leptin and disrupts energy balance, we investigated the effects of adipose specific deletion of Epac1 on glucose homeostasis in HFD fed mice by oral glucose tolerance test. After overnight fasting, AEKO mice had slightly higher basal blood glucose levels than WT controls. Following a single dose orally administration of glucose, the blood glucose levels increased dramatically and peaked at 15 or 30 min in AEKO and WT mice, respectively (**Figure 4.6 A**). Starting from 30 min, the blood glucose levels were significantly elevated in AEKO mice compared to WT controls. Two hours after the glucose challenge, the blood glucose levels of WT mice almost returned to the baseline while

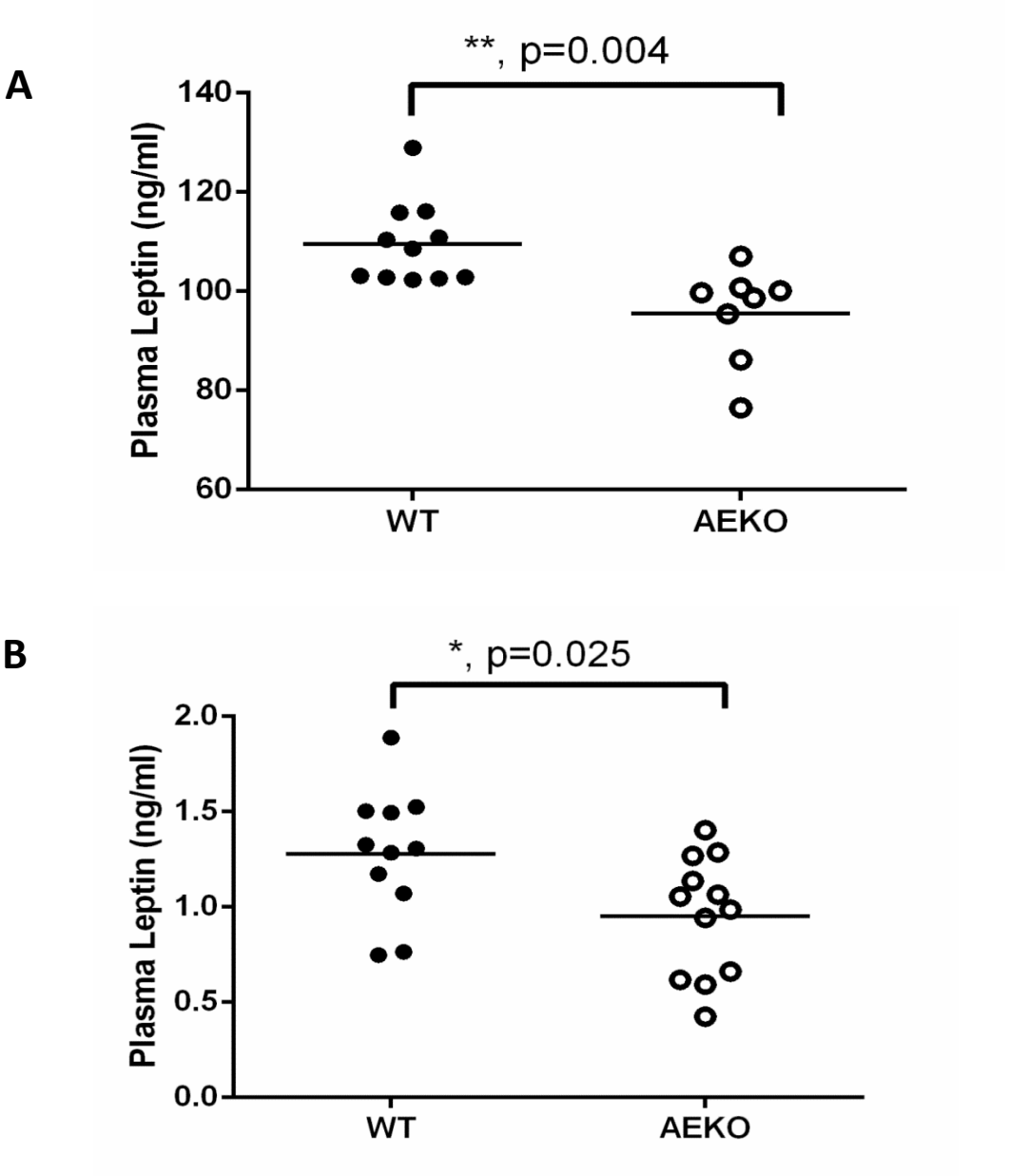

FIGURE 4.4 DELETION OF EPAC1 IN ADIPOSE TISSUE REDUCES PLASMA LEPTIN LEVELS. (A) 6-hour fasting plasma leptin levels are shown for 16-week-old AEKO mice and WT littermates on HFD. Data are means ± SEM. **, $p<0.01$ by student *t* test. (B) Plasma leptin levels of WT and AEKO mice at the age of 25 days on chow diet. Data are means ± SEM. *, $p<0.05$ by student *t* test.

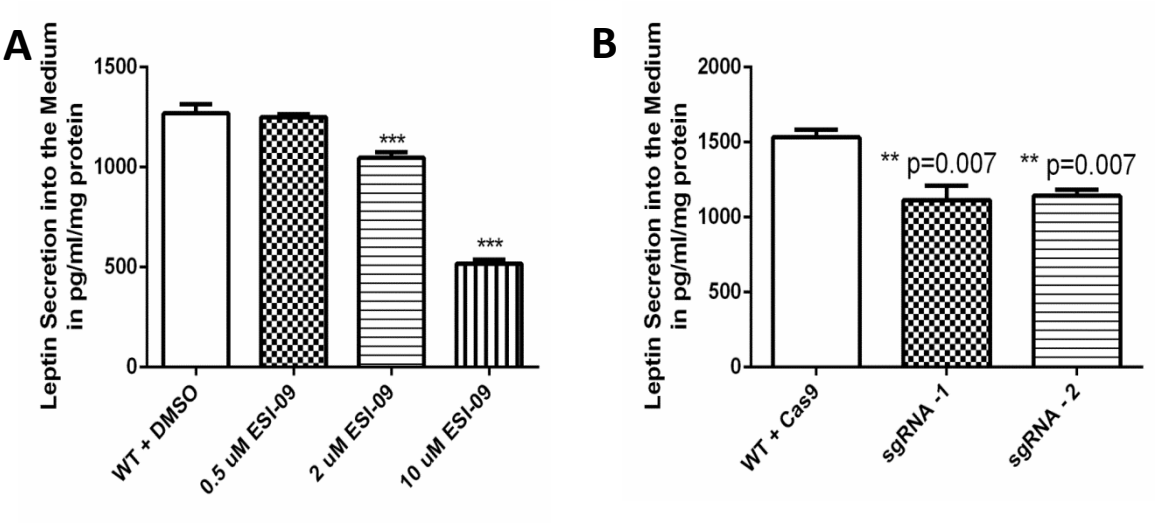

FIGURE 4.5 EPAC1 DELETION AND INHIBITION IN 3T3 ADIPOCYTES REDUCE LEPTIN SECRETION. (A) The secreted leptin levels by 3T3-adipocytes are dose-dependently decreased by treatment with an Epac specific inhibitor ESI-09. Data are means ± SEM. ***, $p<0.001$ by One-Way ANOVA. (B) The secreted leptin levels by 3T3-adipocytes are decreased by disruption of Epac1 by CRISPR-Cas9 gene editing system. Data are means ± SEM. **, $p<0.01$ by One-Way ANOVA.

AEKO mice still had significant higher blood glucose level. In parallel to glucose levels, the plasma insulin levels were also monitored. No significant differences were observed between WT and AEKO mice (**Figure 4.6 B**). Furthermore, insulin tolerance test was performed to determine the insulin sensitivity, as shown in **Figure 4.6 C**. While the blood glucose levels were slightly elevated in AEKO mice starting from 15 min after insulin administration; these changes were not statistically significant. Taken together, these data suggest that AEKO mice have a compromised glucose clearance capability while maintaining insulin sensitivity.

Disruption of Epac1 down-regulates leptin mRNA expression. **-**To determine if Epac1 plays a role in controlling leptin expression at the transcriptional level, we measured the leptin mRNA expression levels in WAT of AEKO and WT mice using real-time PCR. Epac1 deficiency in WAT resulted in a marked reduction in leptin mRNA levels (**Figure 4.7 A**). To further confirm this *in vivo* observation of diminished expression of leptin mRNA in response to Epac1 deletion, leptin expression in 3T3-L1 cells in response to manipulations of Epac1 using pharmacological and genetic approaches. As shown in **Figure 4.7 B**, treatment of 3T3-L1 cells with forskolin, a cAMP elevating agents and H89, a PKA selective inhibitor, resulted in significant increases in leptin mRNA levels. On the other hand, deletion of Epac1 gene in 3T3-L1 cells using the CRISPR-Cas9 gene editing system led to a dramatic decrease in leptin mRNA levels (**Figure 4.7 C**).

Deletion or inhibition of Epac1 in adipocytes suppresses AKT and CREB phosphorylation. **-**To determine the potential mechanisms by which Epac1 may employ in regulating leptin expression and secretion, we investigated the activation status of signaling pathways down-stream of cAMP/Epac/Rap1 in adipose tissues from AEKO and WT mice. Among multiple related signaling molecules probed, we consistently observed a reduced phosphorylated AKT/PKB level at Ser473, as well as a reduced phosphorylated CREB level at Ser133 in AEKO WAT as compared to that of WT control (**Figure 4.8**).

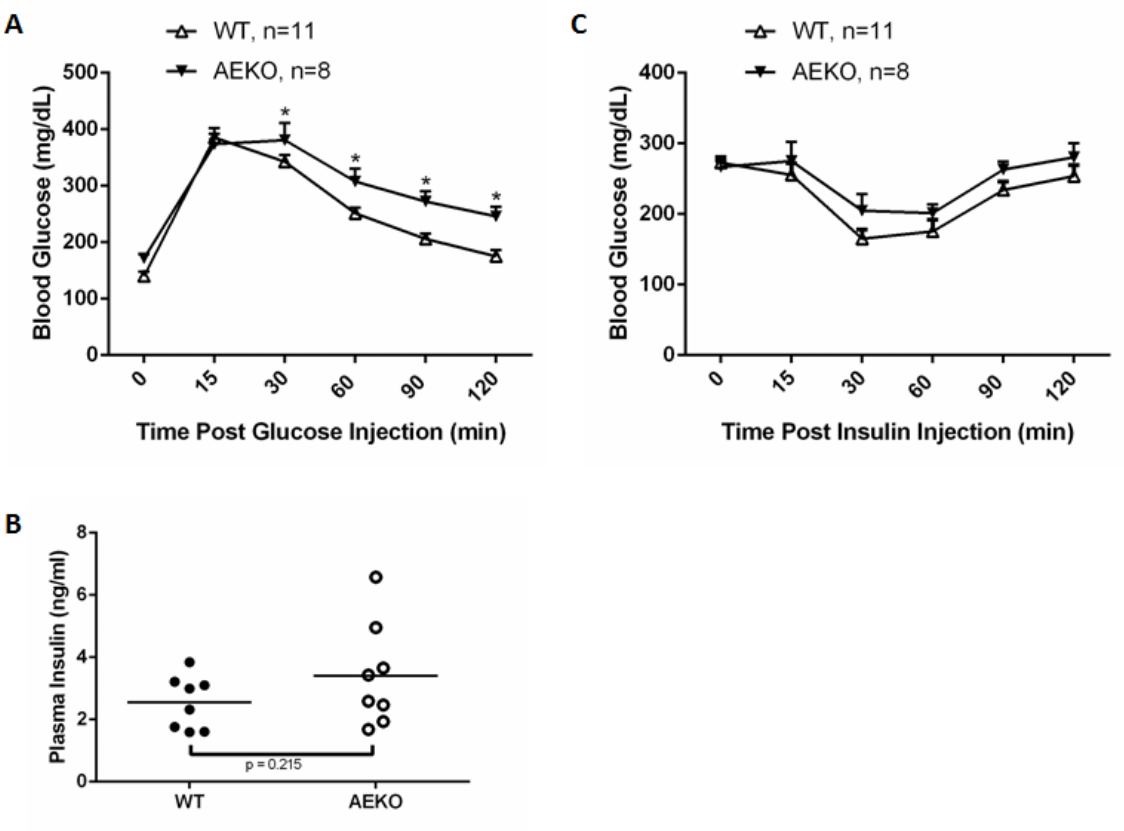

FIGURE 4.6 AEKO MICE HAVE MORE SEVERE HFD INDUCED GLUCOSE INTOLERANCE. (A) Oral glucose tolerance test results for 14-week-old HFD mice. Data are means ± SEM. *, $p<0.05$ by Two Way repeated ANOVA. (B) Plasma insulin levels for 16-week-old mice fed on HFD. (C) Insulin tolerance test results for 15-week-old HFD mice.

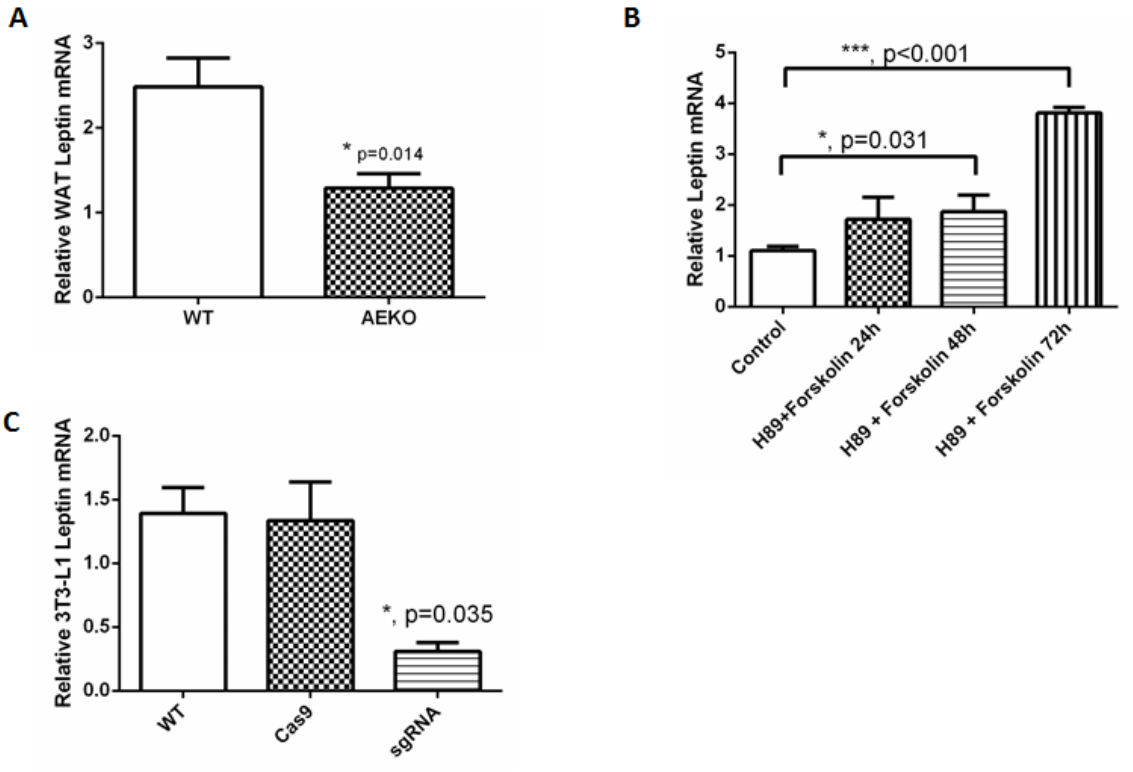

FIGURE 4.7 DISRUPTION OF EPAC1 IN ADIPOCYTES DOWN-REGULATES LEPTIN MRNA LEVELS. (A) Deletion of Epac1 in adipose tissue abolishes leptin transcription level. Data are means ± SEM. *, $p<0.05$ by student *t* test. (B) Activation of Epac1 activity by 10μM forskolin and 5μM H89 together up-regulates leptin transcription levels in 3T3-L1 cells. Data are means ± SEM. *, $p<0.05$ and ***, $p< 0.001$ by One-Way ANOVA. (C) Disruption of Epac1 in 3T3-L1 cells by CRISPR-Cas9 gene editing system abolishes leptin transcription level. *, $p<0.05$ by One-Way ANOVA.

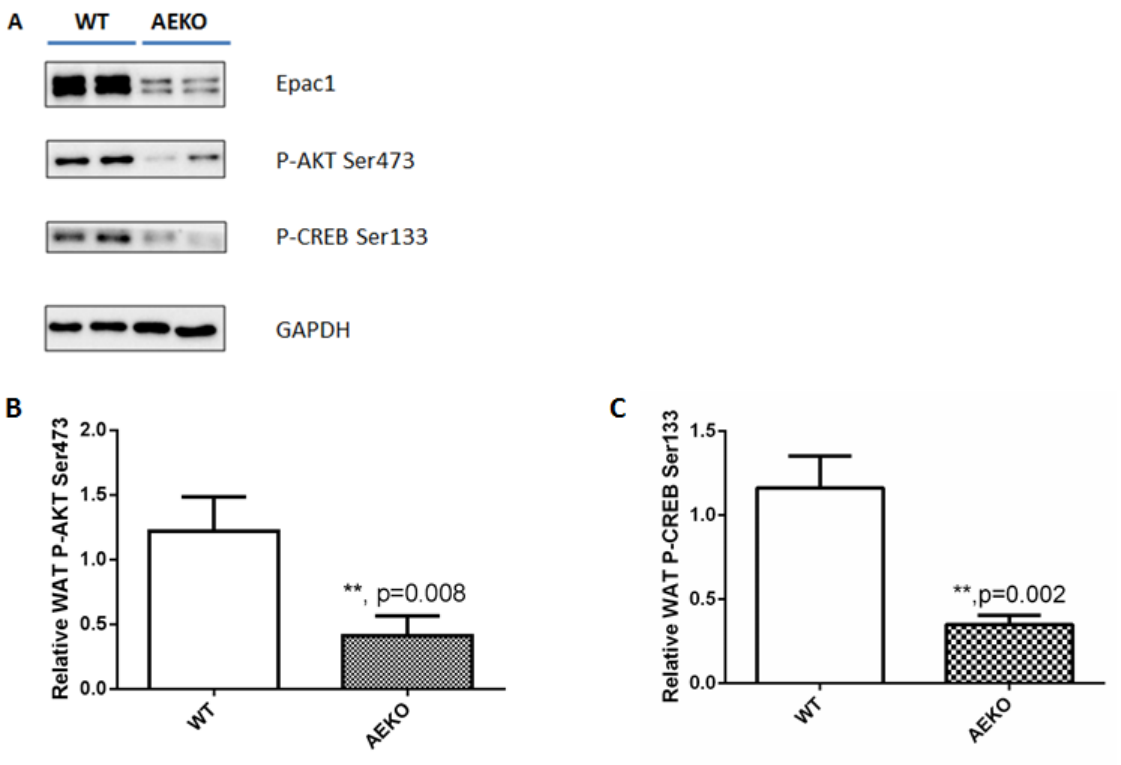

FIGURE 4.8 DISRUPTION OF EPAC1 IN ADIPOSE TISSUE IMPAIRED LEPTIN SECRETION AND TRANSCRIPTION SIGNALING. (A) Western blotting of WAT of p-AKT Ser473 which takes part in leptin secretion and p-CREB Ser133 which is responsible for leptin transcription. (B) Quantitation of p-AKT Ser473 for 8 pairs of male mice at 16-week age on HFD. Data are means ± SEM. **, $p<0.01$ by student *t* test. (C) Quantitation of p-CREB Ser133 for 5 pairs of male mice at 16-week age on HFD. Data are means ± SD. **, $p<0.01$ by student *t* test.

4.3 DISCUSSION

Leptin plays a critical role in maintaining energy balance and glucose homeostasis under normal physiological conditions. Dysregulation of leptin signaling in conditions such as leptin resistance disrupts energy homeostasis and leads to metabolic disorders, including obesity. Therefore, elucidating the signaling network important for the regulation of leptin functions is crucial for understanding the molecular etiology of obesity, as well as for developing new therapeutics for this growing health burden. Recent studies based on genetic and pharmacological manipulations have implicated Epac1 as an important signaling molecule for modulating leptin signaling in hypothalamus[149 153 164]. However, the roles of Epac1 in peripheral tissues, particularly in WAT, the major tissue responsible for leptin production, remain unknown. To bridge this gap in our knowledge, we generated conditional adipose tissue specific Epac1 knockout mice using the transgenic Cre line driven by the adipocyte protein 2 (αP2) gene promoter[154]. Although αP2-Cre has been widely used for generating conditional adipose specific knockout models[167], it has been reported that expression of αP2-driven Cre recombinase can be detected in macrophages[168] and brains[169] in certain instances. Therefore, in addition to confirm the knockdown Epac1 in WAT, special attentions were devoted to monitor the expression levels of Epac1 in hypothalamus and macrophages, and we did not notice changes of Epac1 expression levels in both macrophages and hypothalamus. Hence, the apparent phenotypes observed in AEKO mice should be predominantly due to the deficiency of Epac1 in peripheral tissues, in particular WAT.

Similar to Epac1 global knockout mice, AEKO mice showed decreased plasma leptin levels. Surprisingly, when challenged by HFD, AEKO mice eat more and showed significantly higher body weight gains, which is opposite to phenotypes observed in mice with global Epac1 deletion. Conventional Epac1 KO mice have heightened leptin sensitivity and reduced food intake, and are partially resistant to HFD-induced obesity[153].

These unexpected opposing phenotypes offer important clues on the specific roles of Epac1 in various tissues. While phenotypes observed in the global Epac1 knockout mice reflect the integrated outcomes of Epac1 deletion from the whole body, including both CNS and peripheral effects, our AEKO mice showed only the specific consequences of Epac1 deletion in adipose tissues. It appears that Epac1 deletion in adipose tissues reduces circulating leptin levels, which explain the apparent phenotypes of AEKO mice, as these animals have normal Epac1 expression in hypothalamus and a reduced leptin level would lead to increased food intake and decreased energy expenditures. On the other hand, since CNS, especially hypothalamus, is the most important area for integrating metabolic signals and maintaining energy homeostasis, deletion of Epac1 in hypothalamus results a more potent anorectic effect capable of overcoming the orexigenic effect of adipose tissue Epac1 deficiency in the global knockout mice[153]. Our results further support a dominant role of CNS over peripheral tissues in regulating energy balance as in the case of leptin resistance, although in the opposite direction.

In addition to energy balance, leptin has also been implicated in glucose homeostasis[170]. Consistent with this notion, AEKO mice fed on HFD are compromised in clearing glucose in OGTT test while maintaining normal insulin sensitivity (**Figure 4.6 C**). It is of particular interest to note that despite increased body weight, AEKO mice on HFD have decreased overall leptin levels, which can explain all observed phenotypic changes in energy balance and glucose homeostasis. Therefore, our study highlights an important and previously unknown role of Epac1 in regulation of leptin production in WAT.

Physiological levels of leptin usually correlate with adiposity and regulated by food consumption and insulin, among other signals. As an important stress signal, second messenger cAMP is known to regulate leptin production[171 172]. Increases in intracellular cAMP levels have been shown to down-regulated leptin mRNA levels and to suppress leptin secretion in 3T3 adipocytes and mesenchymal stem cells[173 174]. This inhibitory

effect of cAMP on leptin production and secretion is PKA-dependent, as inhibition of PKA is capable of rescuing cAMP-mediated down-regulation of leptin. On the other hand, the role of Epac1, another important cAMP effector, in regulating adipocyte leptin production has not been explored. Our current studies based on *in vivo* tissue specific Epac1 knockout and *in vitr*o cellular analyses reveal that Epac1 plays a stimulatory role in adipose leptin production. Suppression of Epac1 by genetic manipulations and pharmacological inhibition in adipose tissue and adipocytes diminish leptin mRNA expression levels and reduce leptin secretion. These observations suggest that cAMP-mediated leptin regulation is more complex and dynamics than that was previously recognized as Epac1 and PKA, the two distinct cAMP sensors, can act antagonistically in cAMP-mediated leptin regulation. Therefore, the net outputs of leptin production under the control of cAMP signaling are determined by the dynamic distribution/activation of PKA and Epac1 in adipose tissue, which provides a more integrative and broad-range regulation.

Although the precise mechanism of cAMP mediated regulation of leptin production is remain to be determined, our study suggests a potential role of AKT, which is known to promote leptin production in 3T3-L1 and primary adipocytes in response to insulin[175 176]. The levels of AKT phosphorylation in primary adipocytes and 3T3-L1 with Epac1 knocked out are significantly lower than the WT controls, consistent with the reduced leptin production observation. Moreover, our previous studies reveal that Epac1 and PKA are capable of regulating AKT activation antagonistically down-stream of cAMP: while Epac1 activates AKT, PKA inhibits it[138 177]. Therefore, it is very likely that Epac1 and PKA differentially control leptin production, through a common down-stream effector, AKT.

In summary, we are the first to discover the specific metabolic roles of Epac1 in adipose tissue. Disruption of Epac1 in adipose tissue makes the mice more prone to HFD-induced obesity with reduced energy expenditure and glucose intolerance. In line with the

global Epac1 KO mice, the AEKO mice have decreased circulating leptin levels although these mice have more severe metabolic syndromes. *In vivo* and *in vitro* studies further suggest that suppression of Epac1 down regulates leptin mRNA expression and secretion via inhibiting CREB and Akt phosphorylation, respectively. Taken together, our results demonstrate that Epac1 plays an important role in regulating energy balance and glucose homeostasis by promoting leptin expression and secretion in WAT.

In summary, we demonstrated that disruption of Epac1 in adipose tissue suppresses leptin expression and secretion. Reduced leptin levels prompts the AEKO mice more prone to HFD-induced obesity by increasing food intake and decreasing energy expenditure.

Chapter 5 Summary and Future Direction

Chronic energy imbalance results in obesity. The adipose-derived hormone – leptin is one of the most important regulator on food intake and energy expenditure[54]. Circulating leptin level is proportional to the total body fat mass[178]. Leptin functions by binding to its receptors in hypothalamus to initiate a cascade of down-stream signaling pathways, including JAK2/STAT3, consequently inhibiting appetite and increasing energy expenditure[66]. Deficiency in either leptin or leptin receptor results in severe obesity[179].

Global Epac1 KO mice are protected against diet-induced obesity, partially due to the regulation on leptin signaling. Despite lower plasma leptin levels, the global Epac1 KO mice have heightened leptin sensitivity in hypothalamus by up-regulating pSTAT3. This heightened central leptin sensitivity reduces food intake and improve glucose homeostasis in Epac1 mutant mice[153].

To investigate the role of Epac1 plays in adipose tissue, where is the main source of leptin production and secretion, we generated adipose tissue specific Epac1 knockout (AEKO) mice. Surprisingly, AEKO mice are more prone to diet-induced obesity after being challenged by HFD, with increased appetite and reduced energy expenditure. Consistent with conventional Epac1 KO mice, AEKO mice also have reduced plasma leptin levels owing to the ability of Epac1 to directly regulate leptin expression and secretion in WAT. The reduced circulation leptin level can partially account for the diet-induced metabolic syndromes of AEKO mice.

The opposing phenotypes of body weight and food intake in conventional Epac1 KO mice and AEKO mice suggest a different role of Epac1 plays in CNS and adipose tissue. Epac1 in adipose tissue has an orexigenic effect while the CNS Epac1 has a more potent anorectic effect which can overcome the peripheral effects of Epac1 deficiency in conventional Epac1 KO mice.

While our studies reveal novel physiological functions of Epac1 in regulating leptin production and signaling, several important questions remain and warrant further investigation.

1) How does Epac1 exert its orexigenic effects and induce leptin resistance on CNS, especially in hypothalamus? Leptin functions by regulating the activation of two types of responsive neuron populations: the orexigenic NPY/Agrp neurons and anorectic POMC neurons[79]. How exactly Epac1 regulates these two neuron populations is unclear. To address these issues, CNS tissue specific Epac1 knockout mice, such as POMC specific Epac1 knockout mice are necessary.
2) How does Epac1 regulate leptin secretion and production in the adipose tissue? Compared to WT littermates, the HFD fed AEKO mice have reduced phosphorylation of AKT, which has been reported to mediate insulin-induced leptin secretion in 3T3-adipocytes[176]. However, the direct relation between pAKT and leptin secretion *in vivo* has not been reported.
3) What are the metabolic functions of Epac1 in other peripheral leptin targeted tissues, such as skeletal muscle? Conventional Epac1 KO mice showed suppressed masseter muscle hypertrophy induced by clenbuterol[180], suggesting that Epac1 plays a role in skeletal muscle. To investigate whether Epac1 has a metabolic function in this tissue, skeletal muscle specific Epac1 animal models will be useful.
4) Is Epac2 also involved in leptin functions and energy homeostasis? Despite no compensatory expression of hypothalamic Epac2 in conventional Epac1 KO mice[153], Epac2 is also abundantly expressed in hypothalamus where the leptin receptors are highly expressed. So far, no reports about Epac2's role in leptin signaling in CNS have been revealed.

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