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Qualitative and Quantitative Regulation of the Leptin Gene in vivo

Yi-Hsueh Lu

The Rockefeller University
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Qualitative and Quantitative Regulation of the *Leptin* Gene *in vivo*

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Yi-Hsueh Lu
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Yi-Hsueh Lu, Ph.D.
The Rockefeller University 2016

The high prevalence of obesity is a major public health concern worldwide. Conventional strategies have failed to provide solid solutions, greater than 90% of individuals who lost weight by diet and exercise eventually regained the weight (Arner and Spalding, 2010a). As such, biological factors including genetic contributions have become the research focus on pathogenesis of obesity and the target of effective disease control.

Central to energy homeostasis is the mechanism by which animals regulate and/or response to fluctuation in energy intake and output. Adipose tissues participate in metabolic regulation by serving as an energy store and by secreting adipokines such as leptin and adiponectin. Leptin functions as the afferent signal to the central nervous system in a negative feedback loop that maintains homeostatic control of adipose tissue mass. Leptin gene expression is highly correlated with cellular lipid content in adipocytes but the transcriptional mechanisms controlling leptin expression *in vivo* are poorly understood. Leptin-BAC Luciferase transgenic mice combining with other computational and molecular techniques were used to identify transcription regulatory elements including a CCAAT-binding protein Nuclear Factor Y (NF-Y). The function of NF-Y in adipocyte was studied *in vitro* with 3T3-L1 cells and *in vivo* with adipocyte-specific knockout of *NF-Y*. The results showed that NF-Y binding site is essential for transcriptional regulation of *leptin* and NF-Y is also an adipogenic factor important for adipocyte development *in vivo* (Chapter 3 and Chapter 4).

Finally, since obesity is defined as excess accumulation of adipose tissue, understanding developmental control of adipose tissue size and body adiposity will aid the study on disease progress of obesity. We employed a blastocyst complementation method where wild-type ES-cell is injected into AZIP lipodystrophic blastocyst. The result suggested that while leptin production adipocyte-autonomous, adipose tissue expandability and glucose metabolism is controlled by other factors inv olving other cell types in adipose tissues and/or other organs (Chapter 5). In sum, the thesis identifies adipocyte specific quantitative and qualitative control of leptin transcription, at the same time presents the complexity of metabolic regulation *in vivo*.

ACKNOWLEDGEMENTS

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Lu, Y.H., Dallner, O.S., Birsoy, K., Fayzikhodjaeva, G., and Friedman, J.M. (2015). Nuclear Factor-Y is an adipogenic factor that regulates leptin gene expression. Molecular metabolism *4*, 392-405.

Zeng, W., Lu, Y.H., Lee, J., and Friedman, J.M. (2015). A Reanalysis of Parabiosis of Obesity Mutants in the Age of Leptin. Proceedings of the National Academy of Sciences of the United States of America *(under review)*.

CHAPTER 1: INTRODUCTION

1.1 Obesity as a Health Concern

In 2011-2012, the prevalence of obesity in the United States was 16.9% in youth and 34.9% in adults (Ogden et al., 2014). Obesity is a major cause of morbidity and mortality in the United States. Studies show that the risk of death rises with increasing weight. Even moderate weight excess (10 to 20 pounds for a person of average height) increases the risk of death (Calle et al., 1999; Patel et al., 2014). It is estimated that the annual number of deaths attributable to obesity is about 280,000 in the United States with more than 80% of deaths in people with BMIs (body mass index, see below) of at least 30 kg/m^2 (Allison et al., 1999a). Aside from mortality rate, obesity substantially increases morbidity and impairs quality of life, and that clinical studies consistently show that weight loss improves health and happiness among obese people (Allison and Pi-Sunyer, 1995). In the midst of the energy crisis in the 70s, Hannon and Lohman expressed the cost of obesity in terms of the total fossil energy equivalent of the food calories saved by reducing the present degree of obesity to optimum body weight (2.3 billion pounds for the adult population in the US) (Hannon and Lohman, 1978). Later studies have shown that approximately 4% of direct healthcare costs are attributed to obesity itself (Allison et al., 1999b). Across all payers, per capita medical spending for the obese was $1,429 higher per year, or roughly 42 percent

higher, than for someone of normal weight, summating to a total of $147

billion dollars in 2008 (Finkelstein et al., 2009).

1.2 Definition of Obesity

Obesity is defined as excessive adiposity (body fat). The historical

method for estimating adiposity was by calculating a person's buoyancy,

which was done by measuring his or her body weight under water (Ward et

al., 1978). However, this method is cumbersome, so a surrogate measure

known as the body mass index (BMI) is now routinely used. BMI (weight in

kilograms per square of height in meters) is a convenient measure and useful

for assessing the weight status of a population over time (Romero-Corral et

al., 2008). The common classification system determines whether you are

underweight (<18.5 kg/m2), normal weight (18.5-24.9 kg/m2), overweight

(25-29.9 kg/m2), or obese (>30 kg/m2). There are three degrees of obesity:

class 1 (30.0–34.9kg/m2), class 2 (35.0–39.9kg/m2) and class 3 (≥40kg/m2)

(WHO, 2015).

However, BMI is often unreliable to assess an individual's weight class

because it does not account for body proportion, nor does it account for the

wide variation in the nature of obesity between different individuals and

populations (Kopelman, 2000). Particularly, BMI does not distinguish

between fat and muscle mass or adipose tissue distribution (so called 'pear-

shaped' versus 'apple-shaped'), both of which result in profound differences

in body metabolism. Other direct methods that measure adiposity include air-displacement plethysmography (commercial name BOD POD) and dual-energy x-ray absorptiometry (DEXA) (Fields et al., 2002). The waist-to-hip circumference ratio distinguishes between central obesity and other types of obesity, and it has been suggested that waist circumference should be measured along with BMI to adequately classify obesity-related health risk (Cervellati et al., 2014; Wakabayashi, 2013).

1.3 Obesity as Risk Factor

Despite some shortcomings, there is a close relationship between BMI and the incidence of several conditions caused by excess body fat. This relationship is approximately linear for a range of BMIs less than 30 (kg/m2), but all risks are greatly increased for those subjects with a BMI above 30 for both genders (Willett et al., 1999). Obesity increases the risk of several debilitating diseases, including type 2 diabetes mellitus, cardiovascular disease, and some cancers. The condition most strongly influenced by body weight is type 2 diabetes, where men with BMIs of 30 (kg/m2) or higher had a sevenfold higher risk of developing type 2 diabetes, and women with BMIs of 30 or higher had a 12-fold higher risk compared with their normal-weight counterparts (Guh et al., 2009). Numerous studies have demonstrated a direct association between excess body weight and coronary artery disease and ischemic stroke, though the contribution is multifactorial (Whitlock et al.,

2009). Obesity is associated with cancers of the esophagus, pancreas, colon and rectum, breast, endometrium, and kidney (Keum et al., 2015). Also, studies have shown correlation between obesity and depression, though it is likely that reciprocal interactions exist between the two conditions (Faith et al., 2011). To date, the integrated effect of obesity across physical, psychological, and social functioning as scored by a concept known as health-related quality of life (HRQoL) in adults and youths is an active field of research being pursued (Jia and Lubetkin, 2005; Swallen et al., 2005). Obesity can influence various aspects of reproduction, especially in women. Both infertility rates and pregnancy risks increase with BMI, including both natural and assisted pregnancies (Brewer and Balen, 2010). Encouragingly, however, numerous studies have shown that weight loss, even 5 to 10 percent of weight, offers meaningful health benefits to the obese person (Ben-Menachem, 2007).

1.4 The Obesogenic Environment

While the aforementioned studies were performed in the US, obesity is no longer a problem for developed countries alone. The largest expansion of obesity since the 1980s has occurred in developing countries in Latin America, Oceania, North Africa, and Asia (Finucane et al., 2011). This global upsurge of obesity is associated with the increased availability of food, especially energy-dense items with fat and sugar, as well as sedentary

lifestyle from industrialization and urbanization, best described as

the "obesogenic environment" (Kirk et al., 2010). Global changes in diet and

physical inactivity have been fueled by changes in agricultural practices, food

processing, marketing, transportation, and other aspects of urban planning.

It is worth noting that obesity often co-exists with malnutrition in low-

income urban areas, presenting a complex socioeconomic problem for

policymakers (Oliver and Hayes, 2005; Pearce et al., 2007; Robert and

Reither, 2004). However, since not everyone who has unlimited access to

food develops obesity, lifestyle and environment may be necessary but

insufficient to cause obesity.

One of the confounding phenomena is that people are well aware of

and are sincerely concerned about being obese, owing to exposure of media

and health campaigns (Kassirer and Angell, 1998). However, the battle

against obesity is largely failing. At any given time of the year, an astonishing

15 to 35 percent of Americans are trying to lose weight (Horm and Anderson,

1993). Despite all the investment of a staggering amount of time, money, and

effort, greater than 90% of individuals who lost weight by diet and exercise

eventually regained the weight (Arner and Spalding, 2010a). Moreover,

recent studies have suggested that individuals possess less control over their

own weight than they themselves believed, and that biological factors and

the modern environment are the main causes of obesity (King, 2011). Thus,

understanding the physiology of obesity is critical to effective obesity control.

Individual differences in a common environment are reflective of genetic contribution (FIGURE 1.1), as is established through family studies, investigations of parent-offspring relationships, and the study of twins and adopted children (Maes et al., 1997; Stunkard et al., 1990). These studies estimated the heritability of obesity is 40-70%, which is slightly less heritable than height (Farooqi and O'Rahilly, 2006). Just as nutritional improvement has increased the mean of adult height in many populations, environmentally-driven changes in body weight occur against a background of susceptibility to weight gain that is determined by genetic factors (Barsh et al., 2000; Bouchard et al., 1990; Comuzzie et al., 1996; Price and Gottesman, 1991). For instance, different inbred strains of mice respond differently to a high-fat diet (HFD) with varying caloric intake and weight gain (West et al., 1992). Moreover, in the presence of gene-environment interactions, the effects of high genetic susceptibility are amplified by a high-risk environment (Barsh et al., 2000). Several known mutations that lead to obesity in mice have been cloned, and some homologous mutations have been identified as rare causes of human obesity (see below). These genes and their products provide the molecular basis for obesity and thus promising therapeutic targets of a complex and highly heterogeneous disease. However, these are rare and severe conditions that do not account for common forms of body weight variation and obesity. Overall, the etiology of modern obesity arises from interactions of multiple genes, environmental factors, and behavior, which makes the search for obesity genes especially challenging. Recent
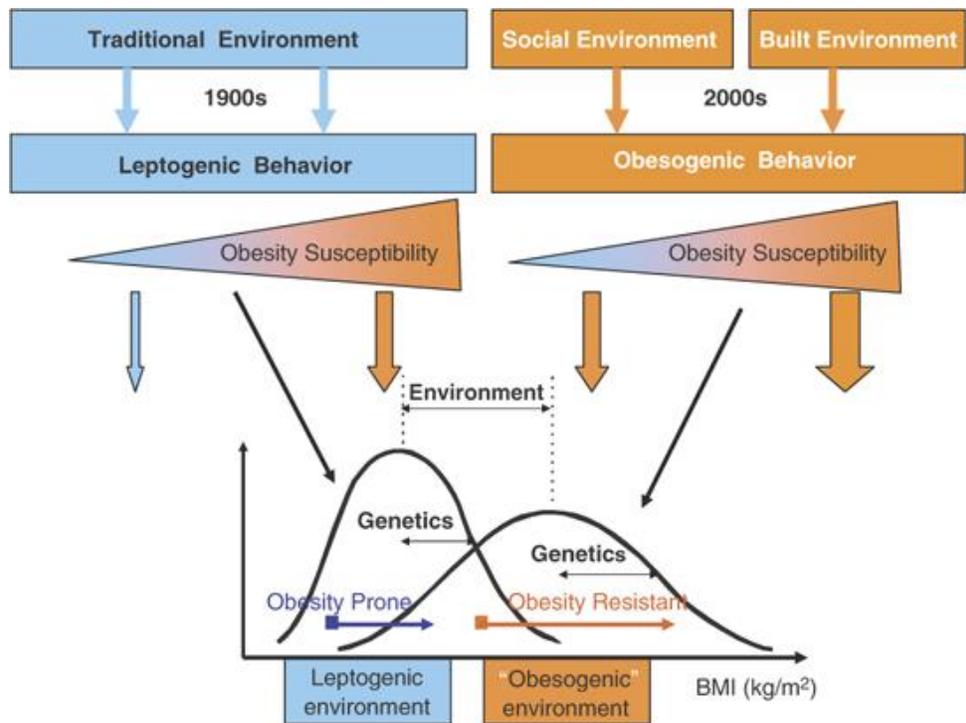
FIGURE 1.1 Potential effect of genes and environment on adiposity assessed by body mass index. The modern "obesogenic" environment on the right side is characterized by the increase availability of high-calorie density food and decreased need for physical activity. The variability in BMI will depend on the genetic susceptibility to weight gain of individuals. Note that due to gene-environment interaction, the distribution of BMI will have a higher mean and higher standard deviation under the high-risk obesogenic environment. Figure adopted from *Energy metabolism, fuel selection and body weight regulation* (Galgani and Ravussin, 2008).

human genome-wide studies estimate that common genetic variation accounts for approximately 20% of BMI variation (Locke et al., 2015).

The rapid growth in the obese population in a short period of time excludes the possibility of significant change in the population's genetic makeup. Therefore it is conceivable that obesity genes have long existed in the human genomes, which now become expressed as an obese phenotype

under modern environmental conditions. Several explanations for the existence of obesity genes have been proposed. If genetic predisposition to energy efficiency conferred a fitness advantage, the genes that enhanced energy storage (as body fat) and avoided energy expenditure would likely be favored by selection. Individuals with alleles of these "thrifty genes" (Neel, 1962) that favored efficient fat deposition would then survive subsequent famines, whereas those individuals with alleles that were inefficient would not survive. This view is supported by observations of animals that deposit a large amount of fat prior to hibernation and migration, presumably in prediction of reduced food availability (Klaassen and Biebach, 1994; Kunz et al., 1998; Martin, 2008; Moore and Kerlinger, 1987). In the modern environment, where food is abundant, the consequence is that people who carry the "thrifty" alleles more efficiently store energy by depositing enormous amounts of fat in preparation for a famine that never comes. The rise in obesity among different animal species living with or around humans in industrial societies may indicate that the "thrifty" alleles are a common survival strategy among many animal species (Klimentidis et al., 2011). It is worth noting that because food supplies were probably always low in preindustrial eras, the levels of obesity, even in those carrying the "thrifty" alleles, were probably quite modest, and individuals never became obese enough to experience the detrimental impacts of obesity on health. Others have proposed alternative scenarios where obesity genes arise as a side-product of other selection forces and genetic drift (the "drifty gene"

hypothesis) from a neutral evolutionary process (Himmshagen, 1979;

Rothwell and Stock, 1979; Speakman, 2008, 2013).

1.5 Historical Perspectives on Energy Homeostasis

At the beginning of the 20th century, the patriarchs of physiology,

Claude Bernard and Walter Cannon, developed the concept that the internal

environment of an organism remains constant, despite frequent fluctuations

in the surrounding variables. Organisms respond to perturbations from the

external environment with counteracting forces, a process that Cannon called

"homeostasis" (Woods and Ramsay, 2007). Subsequently, animal studies by

Gasiner and Mayer demonstrated that body weight remained remarkably

constant over long periods of time, despite environmental perturbations

including diet and physical exercise (Blundell and Bellisle, 2013). It was

thought that the animals adjusted the amount of food intake in order to

maintain constant body weight. Several theories were proposed to explain

the physiological connection between regulation of body weight and feeding

behavioral control. It was proposed that the beginning and terminating of

feeding is controlled by some satiety factor, but was felt dissatisfactory in

explaining long term body weight homeostasis.

Around the same time, studies of the brain identified two discrete

neurological pathways in which the hypothalamus controls feeding behavior

in rats. Electrolytic lesions in the ventromedial hypothalamus (VMH)

resulted in hyperphagia and profound body weight gains due to excessive fat

deposition (Hervey, 1959). Lesions in the ventrolateral hypothalamus (VLH)

resulted in hypophagia and weight loss (Bernardis and Bellinger, 1993).

Further studies showed that the "satiety center" and "feeding center" in the

hypothalamus not only control food intake, but also regulate body weight

and energy expenditure to maintain a body weight "set-point" (Keesey and

Hirvonen, 1997). Gordan Kennedy was the first to suggest that fat might

produce a signal that was sensed by the brain, where it was compared with a

target level of body adiposity; any discrepancy between the target and signal

would subsequently trigger changes in intake or expenditure that would

regulate the levels of body fat to bring its signal back in line with the target

(Kennedy, 1953). This "lipostatic" model was based on a simple negative-

feedback system, yet the signal produced by adipose tissue remained elusive.

1.6 Discovery of Leptin

The existence of an appetite suppressing hormone was demonstrated

by the results of a set of parabiosis experiments (FIGURE 1.2; a technique

that allows continuous, low-volume blood exchange between two live

animals) between genetically *obese* (*ob*) and *diabetic* (*db*) mice (Coleman,

1973; Coleman and Hummel, 1969).
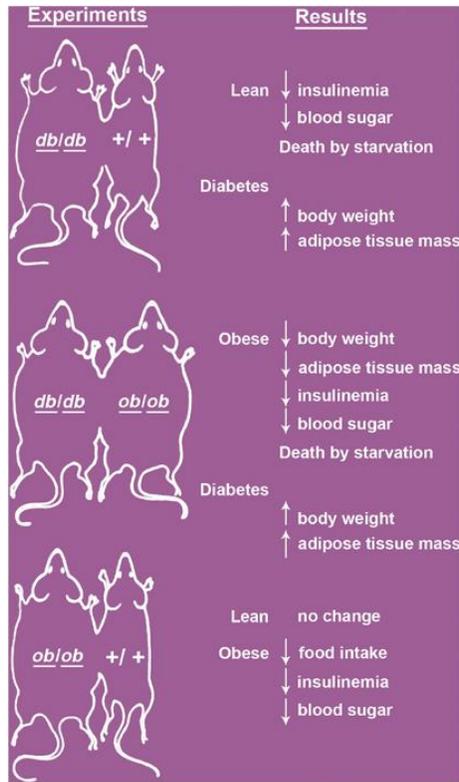
FIGURE 1.2 Schematic of effects on obese phenotypes of parabiosis of *ob*, *db* and wild-type mice. From these studies Coleman inferred that it *ob* might encode a secreted molecule, for which *db* was the receptor. Figure adopted from *Molecular physiology of weight regulation in mice and humans* (Leibel, 2008).

It was known that the *ob* and *db* loci are fully-penetrant autosomal recessive mutations that produce seemingly identical phenotypes such as obesity, hyperphagia, hyperglycemia, and hyperinsulinemia. Since these two genes are located on separate chromosomes (*ob* on chromosome 6 and *db* on chromosome 4), the implication was that the observed phenotype was secondary to some yet unidentified single primary defect. Parabiosis of *db* and normal mice resulted in hypoglycemia and death from starvation in the normal mouse, but no apparent change in its *db* partner. In contrast, parabiosis of *ob* and normal mice produced drastic a weight loss in the *ob* mouse. From these results, Coleman postulated that the *db* mutant produces but does not respond to a satiety factor that is missing in the *ob* mutant (Coleman, 1973). Supporting this viewpoint, parabiosis of normal rats with

rats harboring VMH lesions or genetically obese Zucker rats (the rat version of db), resulted in the normal partner becoming emaciated and showing no interest in food (Harris et al., 1987; Hervey, 1959).

The cloning and characterization of the *ob* gene in mice showed that it encodes leptin (leptos from Greek: thin), a 167-amino-acid protein secreted from adipose tissue (Zhang et al., 1994). In one of the mouse models, C57BL/6J ob/ob[1J], a nonsense mutation in *leptin* results in the synthesis of a truncated and nonfunctional protein. In this mutant, the level of *leptin* mRNA is elevated, suggesting that expression of the gene is under feedback control (Maffei et al., 1995a). Subsequent studies showed that plasma levels of leptin are highly correlated with adipose tissue mass with increased plasma levels in obese humans and several obese mouse models and decreased levels following weight loss (Maffei et al., 1995b). Administration of leptin by intraperitoneal injection or constant subcutaneous infusion results in a dose-dependent decrease in body weight that is restricted to adipose tissue with sparing of lean body mass in both wild-type and *ob/ob* mice, but not *db/db* mice (Campfield et al., 1995; Halaas et al., 1997; Halaas et al., 1995; Pelleymounter et al., 1995). These results indicate that leptin is an afferent signal in a negative feedback loop that regulates adipose tissue mass. Discovery of *leptin* provided the molecular basis for Kennedy and Keesey's adipostatic set-point model of body weight regulation.

1.7 The Central Nervous System in Regulation of Food Intake

Leptin's primary site of action is in the central nervous system (FIGURE 1.3), in particular, the hypothalamus, where a dense population of neurons expressing leptin receptor (encoded by *Lep-R*) reside (Mercer et al., 1996; Vaisse et al., 1996). Using purified leptin protein, expression cloning from a phage library of the choroid plexus isolated Lep-R that binds to leptin (Tartaglia et al., 1995). The *Lep-R* candidate was confirmed by mapping to a *db* mutant, which contains a premature stop codon resulting in abnormal splicing (Chen et al., 1996; Chua et al., 1996; Lee et al., 1996). Positional cloning of *Lep-R* showed that this gene encodes five alternative spliced forms, but only one variant, *Lep-Rb*, is essential for leptin's weight-reducing effect and the function of other forms are quite modest compared to *Lep-Rb* (Li et al., 2013). Taken together with the fact that *db* mice and mice with hypothalamic lesions are leptin resistant (Halaas et al., 1995; Maffei et al., 1995a), these findings support the conclusion that the hypothalamus is an important site of leptin action.

Several hypothalamic nuclei, including the arcuate nucleus, VMH, LH, DMH, and PVN express neuropeptides and neurotransmitters that regulate food intake and energy balance. Neuropeptide Y (NPY) and its receptors respond to the absence of leptin, whereas melanocyte-stimulating hormone (MSH) and its receptors, melanocortin-4 receptor (MC-4R) and agouti-related receptor (AgRP), respond to increased leptin levels (Fei et al., 1997; Friedman, 1997; Mercer et al., 1996). Upon binding to leptin, the leptin
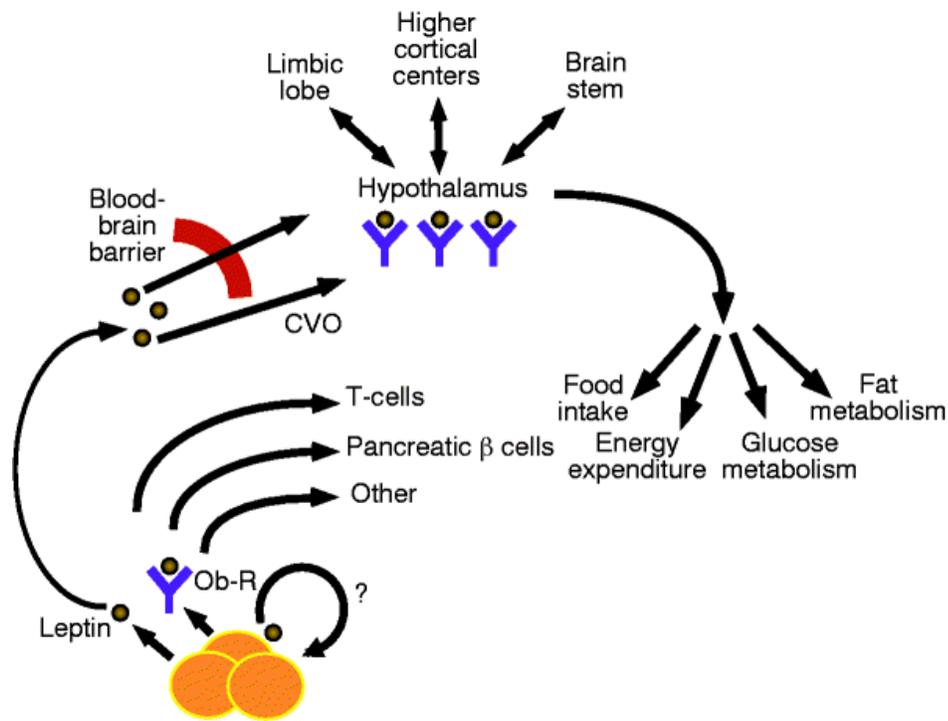
FIGURE 1.3 Summary of leptin signaling pathway. Leptin is the afferent signal in a negative feedback loop that maintains constancy of adipose tissue mass. Leptin is secreted from adipocytes (bottom left) at levels correlated with body adiposity. Increased plasma leptin results in negative energy balance, whereas decreased levels lead to positive energy balance. Leptin acts mainly on the hypothalamus (top), via which leptin acts to decrease food intake and modulate glucose and fat metabolism (right). Figure adopted from *Leptin and the regulation of body weight in mammals* (Friedman and Halaas, 1998).

receptor activates the downstream pathways JAK/STAT3 and PI3-kinase,

both of which are required for proper control of energy metabolism (Bates et

al., 2003; Gao et al., 2004; Hill et al., 2008; Niswender et al., 2001). Chronic

and acute dosing of leptin via intracerebroventricular (ICV) injection reduce

food intake and induce other metabolic effects at doses significantly lower

than peripheral administration (Halaas et al., 1997; Kamohara et al., 1997).

Such leptin-induced anorexia is transient and is attenuated once the adipose

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tissue is depleted, suggesting the presence of yet undiscovered afferent

signals (Halaas et al., 1997; Montez et al., 2005).

1.8 Metabolic and Physiological Effects of Leptin

Animals treated with leptin voluntarily decrease their food intake and

induce weight loss that selectively depletes body adipose stores (Halaas et al.,

1997). Although decreased food intake is a prominent effect of leptin

treatment, the decrease in food intake alone cannot account for the metabolic

changes observed. First, pair-fed *ob/ob* mice that are food restricted to the

level of intake of leptin-treated *ob/ob* mice lose significantly less weight than

the leptin-treated mice (Levin et al., 1996). Second, while leptin-induced

weight loss is restricted to fat mass, starvation causes a loss of both lean

body mass and fat mass at a ratio that maintains body adiposity (Halaas et al.,

1997; Pelleymounter et al., 1995). Third, leptin treatment does not lead to

the compensatory drop in energy expenditure generally evident with

decreased food intake (Halaas et al., 1997). Consistently, leptin-treated *ob/ob*

mice showed a reduced respiratory quotient, indicating a preference for fat

oxidation over carbohydrate oxidation (Hwa et al., 1997). Overall, both leptin

replacement in genetically leptin-deficient animals and pharmacologically-

elevated leptin in wild-type animals within the physiological range have

profound consequences on energy utilization independent of food intake and

change in body weight.

Leptin directly regulates glucose metabolism. This is evident by the antidiabetic effect observed when *ob/ob* mice are treated with a low dose of leptin that does not decrease body weight (Pelleymounter et al., 1995). Treatment of lean animals with leptin leads to a reduction in serum glucose level without changing insulin levels and increases glucose usage during euglycemic clamp experiments (Sivitz et al., 1997). In type I diabetes mellitus (T1DM, also known as insulin-dependent diabetes), since insulin is required for the synthesis and storage of triglycerides in adipose tissue, weight gain cannot occur in untreated T1DM, and the associated loss of body fat is accompanied by markedly reduced plasma leptin levels. Leptin replacement prevents the development of insulin resistance, characteristic of T1DM in both humans and rodents (Defronzo et al., 1982; Ykijarvinen and Koivisto, 1986). Also, the phenotype was associated with normalization of hepatic glucose metabolism and elevated plasma levels of glucagon and corticosterone (German et al., 2010; Yu et al., 2008). Such effects are likely mediated through the CNS, linking the known leptin signaling pathway to control of glucose metabolism and insulin sensitivity. For instance, rescuing Lep-R expression in the arcuate nucleus and pro-opiomelanocortin (POMC) neurons of Lep-R deficient mice normalizes their glucose and insulin levels, improves their hepatic insulin sensitivity, and reduces gluconeogenesis (Berglund et al., 2012; Coppari et al., 2005; German et al., 2009; Huo et al., 2009).

Despite having enormous fat deposits, *ob/ob* mice show many of the abnormalities seen in starved animals, including decrease body temperature, hyperphagia, decrease energy expenditure and physical activity, compromised immune function, and infertility (Coleman, 1978). Low plasma leptin in *ob/ob* mice leads to a state of "perceived starvation," which activates various response programs resulting in obesity under the presence of food (Friedman and Halaas, 1998). The striking similarity in metabolic implications between genetic leptin deficiency and lipodystrophy (loss of adipose tissue), despite being the complete opposites on body adiposity level, suggests that leptin is the key factor in mediating physiological consequences of energy imbalance (Haque et al., 2002). This is supported by the observation that leptin supplement attenuates the physiological responses to food restriction in mice, including changes in gonadal, adrenal, and thyroid axes in male mice, starvation-induced delay in ovulation in female mice, suppression of T-cell proliferation, and increase in circulating fatty acids and ketone bodies (Ahima et al., 1996; Lord et al., 1998; Shimabukuro et al., 1997). These findings suggested that circulating leptin is a key signal to the brain regarding energy stores, and a fall in leptin results in the endocrine, behavioral, and autonomic responses characteristic of starvation. The nature of the efferent signals from the CNS that regulate metabolism in response to leptin are not well characterized, although some evidence suggests that the sympathetic nervous system may play a role (Dunbar et al., 1997; Elmquist et al., 1999; Haynes et al., 1997).

1.9 Leptin and Pathogenesis of Obesity

The role of leptin in the pathogenesis of obesity can be inferred by measurements of plasma leptin in obese animals (Considine et al., 1996; Maffei et al., 1995b). An increase in leptin suggests that obesity is the result of resistance to leptin, while a low or normal level of leptin in context of obesity suggests an underproduction of leptin. These are analogous to insulin production in type II (insulin resistant) and type I (insulin deficient) diabetes.

The molecular basis of known mouse models with monogenic obesity showing Mendelian inheritance have all been uncovered. The corresponding human disorder arising from homologous or similar genetic defects have been identified using a candidate gene approach. These genetic mutations include *leptin* (Montague et al., 1997a; Strobel et al., 1998), *lep-R* (Clement et al., 1998), and *prohormone convertase 1* (Jackson et al., 1997), corresponding to the naturally occurring mouse mutants *ob/ob*, *db/db*, and *fat*. In addition, patients with mutations in *POMC* resulting in impaired melanocortin signaling are also obese (Krude et al., 1998). These mutations are rare and all result in severe obesity in childhood without pleiotropic developmental syndromes. Other human obesity mutations recognized by Mendelian inheritance have been determined for Prader-Willi, Cohen, Alstrom, and Bardet-Biedl syndromes, all of which obesity is one of its pleiotropic features. Using quantitative trait locus (QTL) analysis, no causative genes have been identified for these pleiotropic syndromes (Barsh et al., 2000; Gunay-Aygun

et al., 1997). Consistent with findings in monogenic forms of obesity, human genome wide studies have shown that genetic susceptibility to common obesity is mostly associated with the function of the central nervous system (Locke et al., 2015).

It is striking how monogenic forms of obesity are all caused by either a defect in leptin itself or leptin downstream pathways. Yet, these defects do not account for common obesity associated with industrialized lifestyles, of which the molecular basis remains largely unknown. Obese humans and diet-induced obese (DIO) rodents are hyperleptinemic yet do not showed decreased food intake or increased energy expenditure, suggesting that the pathogenesis of common obesity is leptin resistance (Considine et al., 1996; Van Heek et al., 1997). As such, therapeutic utility of leptin as an anti-obesity drug is limited to the rare cases of leptin deficiency and not the greater obese population (Heymsfield et al., 1999; Paz-Filho et al., 2011).

Animal studies suggest that leptin resistance can be caused by reduced leptin access to its target and/or altered leptin signaling. Zealand obese (NZO) mice, a model with polygenic late-onset obesity, are resistant to peripherally administered leptin but responsive to centrally administrated leptin, suggesting leptin resistance is a result of impaired leptin transport into CNS (Halaas et al., 1997; Igel et al., 1997). Hyperleptinemic obese models A^Y and *db/db* mice have loss-of-function mutations in *MC4R* and *lep-R* respectively, both of which block leptin signaling in the CNS (Halaas et al.,

1997; Ste Marie et al., 2000). Mutations can lead to altered processing of neuropeptides, or downstream intracellular signaling in the hypothalamic nuclei can also cause obesity with high leptin levels (Jackson et al., 1997; Naggert et al., 1995; Prada et al., 2013; Rovere et al., 1996). Study in DIO mice demonstrated that leptin resistance evolves over the course of diet treatment, and that both decreased access of leptin to the hypothalamus as well as defective STAT3 activation by the leptin receptor are involved (El-Haschimi et al., 2000). Furthermore, it has been shown that hyperleptinemia is required for development of leptin resistance (Knight et al., 2010). On the cellular level, chronic overstimulation of the leptin receptor and activation of negative feedback pathways block further leptin signaling through SOCS-3, a protein that directly inhibits leptin signaling (Bjorbaek et al., 1998; Bjorbak et al., 2000). Moreover, expression of a constitutively active form of STAT3, a key mediator of leptin signaling, is sufficient to induce central leptin resistance (Ernst et al., 2009). This mechanism is analogous to blunted insulin signaling under chronic insulin treatment mediated by IRS-1 (White, 2006).

In human studies, several groups have reported *MC4R* mutations (Hinney et al., 1999; Vaisse et al., 1998; Yeo et al., 1998), which remain the most common obesity syndrome found in 3-5% of people with BMI above 40 (Barsh et al., 2000). The studies on *MC4R* demonstrated an overlapping continuum between monogenic and polygenic obesity, as it has been found the case in three other genes, *PCSK1, POMC and BDNF* (Choquet and Meyre,

2011). Various inheritance patterns have been reported for *MC4R* mutations

(Farooqi and O'Rahilly, 2006), indicating that expressivity and penetrance of

the phenotype can be modulated by other genetic and environmental factors.

Together, these suggest that development of leptin resistance is likely

multifactorial, especially within the context of environment-induced obesity.

In humans, although the notion of leptin resistance is only inferred from

elevated leptin in obese individuals, it has been shown that susceptibility to

leptin resistance or leptin production and/or leptin signaling

disproportionate to energy status underlies common obesity. For instance,

lower leptin concentrations than would be expected from BMI is linked to a

predisposition to weight gain (Ravussin et al., 1997; Scholz et al., 1996).

1.10 Alternative to "Set-Point" Model

While the leptin signaling pathway provides that strongest argument

for a feedback system that maintains energy homeostasis based on energy

storage (adipose tissue), development of leptin resistance in common obesity

and increased prevalence of obesity over the past decades suggest certain

shortcomings of our understanding. In other words, if, as Kennedy and

Keesey (Keesey and Hirvonen, 1997; Kennedy, 1953), had put forth, each

body possesses an energy status "set-point" in terms of adiposity, each

individual ought to maintain his or her body weight through a negative

feedback mechanism that offsets short-term energy fluctuation. However,

anthropological studies disprove such a system by demonstrating that modern obesity is dependent on socioeconomic status of an individual (Dykes et al., 2004; Poskitt, 2009) and is often associated with certain major life events such as attending college, marrying, and immigration to Western countries (Cluskey and Grobe, 2009; Oza-Frank and Cunningham, 2010; Sobal et al., 2009). In Western countries, most people gain weight throughout their life, suggesting at least age-dependent effect for an individual's energy "set-point" (Kuczmarski et al., 1994).

As such, a "settling-point" model was proposed from a mathematical model for weight regulation (Payne and Dugdale, 1977), where any imbalance between energy intake and energy requirement results in a change in body weight. Such change, in turn, alters the maintenance energy requirement so as to counter the original imbalance and the system would hence be stabilized. Since there is no actual set-point or feedback signal in the "settling point" model, altered energy status occurs whenever a regulated variable is adjusted to balance change in an unregulated variable. This model provides cogent explanations for the "obesogenic" environment where food intake can be increased by external cues such as large portion size, exposure to high density foods, frequency of dining out, as well as eating with others (O'Keefe and Abuannadi, 2010; Rolls et al., 2007; Thornton et al., 2011). The extent to which energy intake and expenditure are regulated appears to be determined largely by genetic factors (Vogels and Westerterp-Plantenga, 2005; WesterterpPlantenga et al., 1996). In mice with fixed levels of plasma

leptin, nonetheless, gained weight in response to a HFD at a rate that was indistinguishable from wild-type animals on the same diet, and the two groups reached an identical body weight plateau (Knight et al., 2010; Tanaka et al., 2005). If proportional increase in leptin with weigh gain is necessary for establishing energy balance, these animal models with artificial leptin deficiency would be predicted to gain weight more quickly than wild-type controls. The fact that this was not observed indicates that a leptin-independent mechanism specifies the body weight set point in animals fed a high-fat diet. Likewise, leptin infusion fails to prevent weight gain in wild-type animals undergoing HFD treatment (Surwit et al., 2000). In aggregate, these results are consistent with settling-point model. How energy regulation is achieved through balancing various long and short term variables remains an important question.

Despite its shortcomings in explaining development of obesity under over-nutrition, the "set point" model provides a better picture for energy regulation during starvation and deprivation. In the classical Minnesota Experiment, food-restricted individuals over-eat to replenish the body mass (both fat and lean) to pre-restricted level (Dulloo et al., 1996; Speakman et al., 2011), suggesting an active regulatory mechanism in response to being underweight. Even after energy homeostasis, which is substantially perturbed in anorexia nervosa, recovered patients often return to premorbid body weight (Coners et al., 1999; Hebebrand et al., 1997). On the other hand, in both normal and overweight humans, diet-induced weight loss is resisted

by reduced energy expenditure (Dulloo and Jacquet, 1998; Luke and Schoeller, 1992). Perhaps most strikingly, body fat removed by surgery is restored within one year post-operation (Hernandez et al., 2011). In sum, body weight and body adiposity are defended in the face of energy deprivation, since loss of energy storage and intake due to starvation present an immediate risk to survival. Consistently, hypothalamic targets of leptin, including NPY, POMC, and CART neurons, are more responsive to leptin during fasting than in the overfed or obese state (Ahima et al., 1999). Low leptin levels induced by fasting and reduction in energy stores mediate various physiological consequences of energy deprivation (Ahima et al., 1996; Keim et al., 1998). As such, falls in body adiposity and leptin levels serve as signals of negative balance from the "set-point," which activates adaptive response to starvation and defense against further deviation.

1.11 Regulation of Leptin Gene in Adipose Tissue

Acting as a surrogate for fat mass, plasma leptin levels are highly correlated with adipose tissue mass in animals and humans over a very large dynamic range (Frederich et al., 1995; Maffei et al., 1995b). Changes in plasma leptin levels are associated with changes in *leptin* mRNA per adipocyte and the levels of *leptin* mRNA per cell are highly correlated with intracellular lipid content (Couillard et al., 2000; Maffei et al., 1995a). This suggests that regulation of *leptin* is primarily achieved at the transcriptional

level with only a few *in vitro* studies demonstrating increased leptin

secretion with insulin treatment (Barr et al., 1996; Bradley and Cheatham,

1999; Lonnqvist et al., 1997; Moreno-Aliaga et al., 2000). Small amounts of

leptin expression have been reported in brain, pituitary, stomach, mammary

epithelial cells, liver, chondrocytes and muscle, but the physiological

importance of this low level expression, if any, is unclear (Birsoy et al., 2008b;

Rosenbaum et al., 2002). Both quantitative (fat mass dependent) and

qualitative (tissue-specific) regulation of *leptin* gene expression is important

for its physiological role in metabolic regulation, yet little is known since the

cloning of *leptin* in 1994.

Leptin production is regulated by various metabolic stimuli including

positive regulator glucocorticoids (Devos et al., 1995; Slieker et al., 1996) and

proinflammatory cytokines TNF and IL-1 (Grunfeld et al., 1996; Sarraf et al.,

1997), and negative regulators include beta adrenergic agonists and cAMP

(Mantzoros et al., 1996; Slieker et al., 1996). Insulin has also been found to be

capable of modulating *leptin* expression (Wabitsch et al., 1996), particularly

as a suppressor during starvation where a rapid fall in leptin is

disproportional to the fall in adipocyte energy stores (Ahima et al., 1996;

Boden et al., 1996). Despite these external influences, it is likely that cell-

autonomous factors are the major links between adipocyte size and *leptin*

gene expression. Recent studies have shown that individual adipocytes

maintain idiosyncratic levels of fat accumulation and nutrient uptake during

de-differentiation and re-differentiation (Katz et al., 2014; Varlamov et al.,

2015). Which intracellular metabolites, signaling molecules, or transcription factors provide the necessary link is still unclear.

Further studies have been impeded by lack of a tissue culture model for adipocytes, as leptin expression levels in preadipocyte cell lines are 1–2% of that seen in adipose cells *in situ* (Macdougald et al., 1995). Interestingly, cultured adipocytes transplanted subcutaneously can grow into fat pads, which showed a 10-fold increase in *leptin* expression to a level 10–15% of that seen in adipocytes *in situ* (Green and Kehinde, 1979; Mandrup et al., 1997). These results suggest the existence of a key, unidentified, *in vivo* regulatory factor, and it mandates studies of *leptin* expression performed *in vivo*.

The proximal promoter of *leptin*, a 762bp region upstream to the transcriptional start site (TSS) has been characterized by DNA sequence analysis in both humans and mice (de la Brousse et al., 1996; Gong et al., 1996). This region contains several transcription factor binding sites of factors known for adipocyte functions such as the C/EBP family, Sp-1, CREB, and SREBP-1c (He et al., 1995; Hwang et al., 1996; Mason et al., 1998). The promoter of *leptin* is positively regulated through by C/EBPα (He et al., 1995; Miller et al., 1996), and suppressed by PPARg through functional antagonistic interaction between C/EBPa and PPARg (DeVos et al., 1996; Hollenberg et al., 1997). However, a transgene driven by the 762bp proximal promoter showed a lower expression than the normal physiological level and

did not confer adipose tissue specific expression (Chen et al., 1999; Hwang et al., 1996). This suggested that while the proximal promoter contains sequences sufficient to drive leptin transcription using ubiquitous machinery, additional mechanisms involving distal *cis*-elements and novel *trans*-factors are required to confer tissue specific regulation. Suppression of leptin transcription in other tissues remains to be explored.

In adipocytes, leptin expression is proportional to cell size, which in turn is dependent on the amount of triglyceride stores. Several possibilities on cell-autonomous regulation of *leptin* expression have been proposed. It is known that membrane cholesterol concentration is inversely proportional to adipocyte size and that cholesterol regulates glucose metabolism in adipocytes via gene expression modulation (Le Lay et al., 2001). However, manipulation of cholesterol concentration in 3T3-L1 cells, despite alteration of many metabolic genes, does not change *leptin* expression (Le Lay et al., 2001), and that membrane cholesterol composition may simply be the adaptive response to change in cell size and mechanical strain in the cell membrane (Khatibzadeh et al., 2012). Mechanical force itself has been implicated in regulating adipogenesis (David et al., 2007; Sen et al., 2008; Tanabe et al., 2004). Notably, the extracellular matrix (ECM), serving as a scaffold of adipose tissue, is also capable of regulating physiological and pathological responses of fat mass expansion. Up-regulation of many ECM components results in reduced mechanical plasticity of fibrotic adipose tissue and presents a hallmark of metabolically dysfunctional adipose tissue

in a state of obesity (Khan et al., 2009; Sun et al., 2011). The direct

relationship and the signal transduction pathway between mechanical

strains exerted on expanding adipocytes and increased *leptin* expression is

unknown. Alternatively, hypoxic conditions encountered by large adipocytes

with limited oxygen diffusion can directly regulate *leptin* expression through

hypoxic response transcription factor HIF1α (Sun et al., 2013). Hypoxic

stress is also prominent at the tissue level in obesity as capillary density is

lower in the adipose tissue of obese humans compared with lean subjects

(Pasarica et al., 2009; Rajala and Scherer, 2003). The molecular mechanism

coordinating and connecting these different cellular responses to *leptin*

expression remains to be explored. Chapter 3 of this thesis presents the

identification of a distal enhancer sequence of *leptin* gene, and the

identification of its binding protein and subsequent functional analysis is

detailed in Chapter 4.

From the approach of genomic study, transcriptional regulation of

leptin can be considered as specific molecular programs in white adipose

tissue that coordinates as well as responds to physiological and metabolic

changes in animals. As such, comprehensive molecular characterization and

comparison of white adipose tissue from different states of ambient leptin

can elucidate specific cellular programs affected by leptin. Previous study

had utilized a microarray technique to characterize the molecular events in

white adipose tissue associating with alteration in circulating leptin levels

(Soukas et al., 2000). These results uncovered a large number of metabolic,

inflammatory, and structural genes dysregulated with chronic leptin deficiency (ob/ob) as well as the acute responses of fatty acid and triglyceride metabolic pathway following leptin change. Chapter 5 of this thesis presents an updated work of similar approach, which utilized next-generation-sequencing that allows unbiased molecular characterization.

Since plasma leptin level is determined by aggregated production from all adipocytes in an animal, it remains uncertain how *leptin* gene regulation within individual adipocytes rely metabolic signal at an organismal level. Indeed, with the production of various adipokines with hormonal functions, adipose tissues should be considered, as a whole, an endocrine organ in addition to its classical role as a storage organ (Ahima, 2006). Indeed, as organ size control is central to mammalian development, each species also possesses predefined body adiposity. However, prominent heterogeneity in adipocytes from different depots and within each depot complicates studies of adipocyte biology (Guo et al., 2004; Montague et al., 1997b; Montague et al., 1998). In addition, two distinct processes, hypertrophy and hyperplasia, that accompany adipose tissue expansion during normal development and obesity (Jo et al., 2009; Sun et al., 2011), contribute to the complexity of the issue. Therefore, understanding the process of adipogenesis from the perspective of organ size control in multicellular organisms is essential to elucidate regulation of the *leptin* gene.

1.12 Transcriptional Regulation of Adipogenesis

The mechanisms controlling cellularity of adipocytes *in vivo* is best approached by studying adipose tissue development. Adipogenesis is described as a two-step process: generation of committed adipocyte precursors (or preadipocytes) from embryonic stem cells and terminal differentiation of preadipocytes into mature adipocytes (Billon et al., 2007). Differentiation of preadipocytes has been studied extensively *in vitro*, from which several adipogenic transcription factors, such as peroxisome-proliferator activated receptors PPARγ and PPARβ and *CCAAT*-enhancer binding proteins C/EBPα, C/EBPβ, and C/EBPδ, were identified (Chawla et al., 1994; Christy et al., 1991).

Adipogenesis in vitro follows a highly ordered and well characterized sequence of events (Rosen et al., 2000). Initially, there is growth arrest of proliferating preadipocytes, usually achieved in cultured cell lines after contact inhibition. This growth arrest is induced by the addition of pro-differentiation hormones into the cultured models and is followed by one or two additional rounds of cell division known as clonal expansion. This process is followed by expression of the key transcription factors PPARγ and C/EBPα and a second, permanent period of growth arrest. Over the course of terminal differentiation, the preadipocyte converts to a spherical shape, accumulates lipid droplets, and progressively acquires the morphological and biochemical characteristics of the mature white adipocyte. The appearance of PPARγ and C/EBPα activate and/or enhance expression of

most or all of the genes that characterize the adipocyte phenotype, including

glycerophosphate dehydrogenase, *fatty acid synthase*, *acetyl CoA carboxylase*,

malic enzyme, *Glut4*, *insulin receptor*, and *aP2* or *FABP4* (Spiegelman et al.,

1993). At the molecular level, differentiation can be considered as a shift in

gene expression patterns from that of a proliferative, multipotent state to

that which defines the phenotypic appearance of the differentiated stage. In

this sense, changes in transcription factor expression and activity define the

process of differentiation.

It is now well-accepted that the transcriptional network of

adipogenesis involves a sequential activation of C/EBP factors and PPARg

(FIGURE 1.4). First, C/EBPb and C/EBPd mRNA and protein levels rise early

and transiently (Cao et al., 1991; Yeh et al., 1995) and induce the expression

of PPARg through direct transcriptional effects (Fajas et al., 1997; Wu et al.,

1996; Zhu et al., 1995). PPARg is responsible for inducing C/EBPa, which

further reinforces PPARg expression during the differentiation cascade

(Kubota et al., 1999; Rosen et al., 1999; Wu et al., 1999). This positive

feedback feature ensures that the cascade, once initiated, will maintain the

expression of these critical factors as well as the terminal differentiated state.

How PPARg and C/EBPa orchestrate their transcriptional activities remains

uncertain, although it is known that PPARg is sufficient to stimulate most, but

not all, adipocyte markers (Wu et al., 1999) and that PPARg and C/EBPa act

synergistically at many adipogenesis-linked loci (El-Jack et al., 1999;

Lefterova et al., 2008).
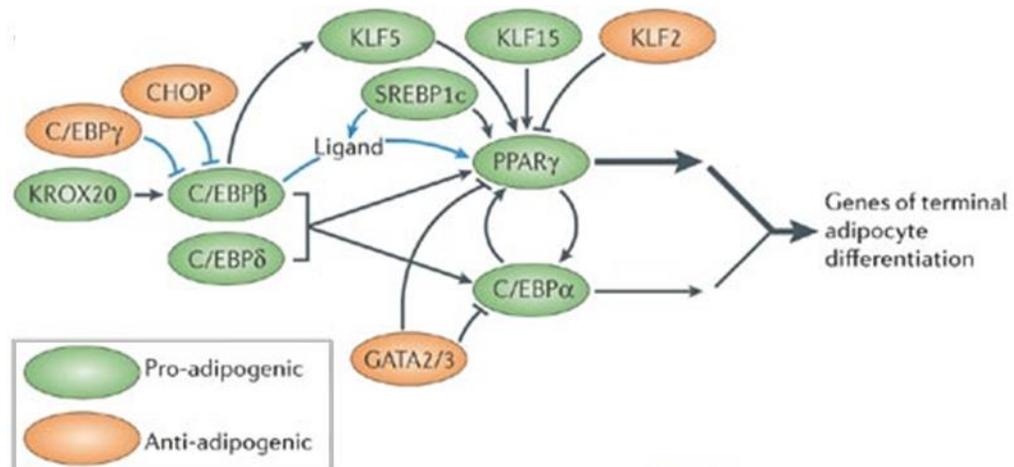
FIGURE 1.4 Transcriptional Regulation of Adipogenesis. The expression of Pparg is regulated by several pro-adipogenic (green) and anti-adipogenic (orange) factors. PPARg itself is activated by an as-yet-uncharacterized ligand. CCAAT-enhancer-binding protein a (C/EBPa) is regulated through a series of inhibitory protein–protein interactions. Figure adopted from *Adipocyte differentiation from the inside out* (Rosen and MacDougald, 2006).

1.13 Adipose Tissue Development *in vivo*

Histological and microscopic observations performed on human and porcine embryos indicate that white adipose tissue forms early in life. Developmental history of adipose tissue starts with the sites of fat deposition in the embryo called the primitive organs of the white adipose tissue (Han et al., 2011; Wassermann, 2010). The primitive organs are characterized by a network of capillaries and cells with the appearance of fibroblasts except for the presence of small fat droplets in cytoplasmic vacuoles (Wassermann, 2010).

In humans, lipid-filled adipocyte precursors are first visible around gestational week 14 in structures called fat lobules (Poissonnet et al., 1984). These primitive adipose tissue depots progressively increase in both size and number until gestational week 23, when fat lobule number begins to plateau. In the following weeks, fat lobule growth is almost exclusively accomplished through hypertrophy of existing adipocytes (Poissonnet et al., 1983; Poissonnet et al., 1984). In mice, the appearance of cells expressing adipogenic markers in subcutaneous compartments is observed relatively late in embryogenesis, with overt lipid filling of white adipocytes being initiated at birth (Birsoy et al., 2011). Visceral adipose depots form after birth during the first week of life (Han et al., 2011). Studies in rats show increases in both adipocyte number and size early in life, followed by a plateau in adipocyte number by 3 months of age, with WAT mass increasing thereafter resulting from increased adipocyte size (Greenwood and Hirsch, 1974; Hirsch and Han, 1969). A recent study using inducible, irreversible labeling of mature adipocytes via the *adiponectin* promoter (termed AdipoChaser mice) supports these findings (Wang et al., 2013b).

The developmental origin of adipose tissue in different depots has not been characterized, with the exception of depots in the head region, which have been shown to derive from the neural crest (Billon et al., 2007). It is generally considered that adipocytes are of mesenchymal origin based on the ability of mesenchymal cells and fibroblastic cell lines to form adipocytes in culture (Rosen and MacDougald, 2006; Taylor and Jones, 1979). Further,

recent studies using transgenic mouse reporter showed that mesenchymal cells expressing *pref-1* form early precursors of adipocytes *in vivo* (Hudak et al., 2014). Other studies have suggested that white adipocytes are derived from endothelial (Tran et al., 2012) or hematopoietic lineage (Crossno et al., 2006; Sera et al., 2009). However, conflicting results of these studies indicate that the appearance of endothelial lineage markers in mature adipocytes was due to proximity of adipocytes and vascular structures (Berry et al., 2014; Berry and Rodeheffer, 2013b), and that hematopoietic linage likely originated from contamination of macrophages (Berry and Rodeheffer, 2013b; Koh et al., 2007; Tchoukalova et al., 2012). Lastly, based on the observation that differentiating preadipocytes reside within vascular compartments of adipose tissue, it was proposed that a subset of pericytes are adipocyte precursors (Cinti et al., 1984). Lineage tracing using pericyte marker *PdgfRβ-cre:R26R-LacZ* mice showed that all mature adipocytes stain positive for β-galactosidase, and PdgfRβ-expressing cells can form adipocytes when transplanted into mice (Tang et al., 2008). Developmental origin of adipocytes may reflect the interplay between vascular growth and adipogenesis *in vivo* (Fukumura et al., 2003; Rupnick et al., 2002).

Several studies have isolated different cell subpopulations from total stroma vascular fraction (SVF) of adipose tissue in an effort to identify adipocyte precursors. Using flow cytometry and fluorescence-activated cell sorting (FACS), several recent studies were able to isolate populations of live adipocyte progenitors with adipogenic properties *ex vivo* in culture and
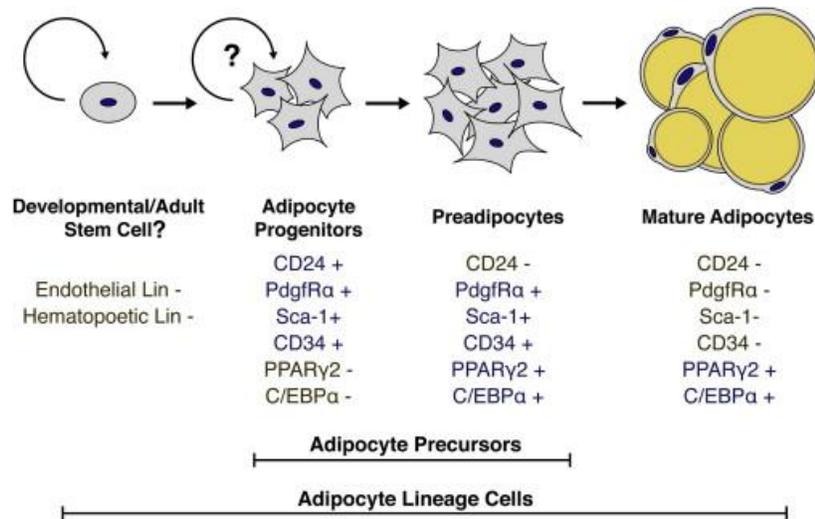
FIGURE 1.5 Model of in vivo adipogenesis. A schematic representation of the adipocyte cellular lineage is shown. The defined lineage, cell surface, and transcriptional markers for each cell type are shown below each listed cell type. Figure adopted from *Weighing in on Adipocyte Precursor* (Berry et al., 2014).

in vivo following transplantation (Lee et al., 2012; Rodeheffer et al., 2008; Tang et al., 2008). This leads to the identification of a population characterized by the cell surface marker profile CD45−;CD31−;Ter119−;CD29+;CD34+;Sca-1+;CD24+ (hereafter CD24+), which is highly adipogenic in culture and capable of considerable expansion prior to differentiation, leading to reconstitution of an entire functional adipose depot with correction of hyperglycemia and hyperinsulinemia when transplanted into lipodystrophic AZIP mice (Rodeheffer et al., 2008). Further analysis revealed that the CD24+ adipocyte progenitor loses CD24 expression to commit into CD24− preadipocytes, which express early adipogenic factors *PPARg* and *C/EBPa* but not *AdipoQ* or *Perilipin* (FIGURE 1.5). Interestingly, adipogenic capacity of CD24+ progenitors is dependent of

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adipose tissue microenvironment (Berry and Rodeheffer, 2013b), suggesting

certain non-adipocyte autonomous factors contribution to regulation of

adipogenesis *in vivo*. Chapter 6 of this thesis describes a novel blastocyst

complementation technique to elucidate cell-autonomous versus non-cell-

autonomous mechanisms that participates in regulation of whole body

metabolism and adiposity.

1.14 Disease of Adipogenesis: Lipodystrophy

While the disproportional gain of fat mass is described by obesity,

disproportional loss of adipose tissue is termed lipodystrophy.

Lipodystrophy is a heterogeneous acquired or inherited disorder

characterized by the partial or general loss of adipose tissue (Prieur et al.,

2014). Based on linkage analysis of candidate gene screening, the molecular

basis of lipodystrophy primarily involves genes that regulates adipocyte

differentiation, nutrient uptake (particular fatty acid), and/or lipid droplet

(its main component triacylglycerol) formation (Huang-Doran et al., 2010).

The genetic forms of lipodystrophy are very rare, and a large number of

patients carry no mutations in known genes. In contrast, the most common

form of lipodystrophy is induced by protease inhibitors used to treat HIV,

affecting up to 50 % of HIV patients (Asterholm et al., 2007).

Loss of adiposity results in decreased adipokine secretion,

particularly leptin, and excess lipid deposit in other tissues such as the liver

with the development of a severe metabolic syndrome, including insulin

resistance, hyperglycemia, and dyslipidaemia in both humans and mouse

models (Reue and Phan, 2006; Savage, 2009; Schott et al., 2004). Despite

their opposite phenotypic appearance in adiposity, obesity and

lipodystrophy syndrome share paradoxical similarity in these metabolic

complications, suggesting common factors on the pathogenesis of these

diseases (TABLE 1.1). In turn, the "lipid overflow" hypothesis proposes that,

since the capacity of adipose tissue to accommodate excess energy in the

form of triglyceride is finite, excessive lipid load, typically in obesity, leads to

ectopic lipid accumulation and insulin resistance (Unger, 2003). Insulin

resistance as a result of such "lipotoxicity" is most prominent in the liver,

skeletal muscles, and pancreas (Huang-Doran et al., 2010). Lipodystrophy is

an extreme example of reduced adipose tissue "capacity" and therefore is

characterised by severe ectopic lipid accumulation and insulin resistance

(Savage, 2009). Moreover, abnormal secretion of adipokines and hormones

TABLE 1.1 Phenotypic Comparisons among Lipodystrophy, Obesity, and Leptin-Deficiency.

	Lipodystrophy	**Obesity**	**Ob/Ob**
Fat mass	↓	↑	↑
Leptin	↓	↑	↓
Adiponectin	↓	↓	↓
Inflammatory cytokines	↑↓	↑	↑
Metabolic complications	↑	↑	↑

from dysfunctional adipose tissue can lead to an altered energy balance and loss of dynamic metabolic regulation (Asterholm et al., 2007). Consistently, leptin replacement therapy improves both insulin sensitivity and lipid metabolism in patients and mouse models with generalized lipodystrophy (Colombo et al., 2002; Ebihara et al., 2007; Gavrilova et al., 2000; Javor et al., 2005).

Several mouse models of lipodystrophy have been constructed. These models not only contribute to understanding disease etiology, but also to providing different perspectives on the study of normal adipose tissue physiology. The induced lipodystrophy mouse model *FAT-ATTAC* (Pajvani et al., 2005) make it possible to characterize the physiological functions of adipocytes and their secreted factors by acute fat loss without the confounding effects of long-term ectopic lipid accumulation in liver and muscles. Earlier constitutive loss of adipocytes during development, including *PPARg2*-knockout mice, *flp* mice with *lipin* mutation, and *AZIP-F1* (Barroso et al., 1999; Moitra et al., 1998; Peterfy et al., 2001; Zhang et al., 2004), can be achieved by blocking adipogenesis. These animals seem to have much more profound consequences on systemic energy homeostasis compared to a late onset loss, suggesting the important role that adipose tissue plays during the early developmental stages. More refined time courses of fat loss during development are needed to understand the effects of adipose tissue on the developmental aspect of metabolic regulation. In addition, the ability to precisely block adipogenesis *in utero* or in the early

postnatal period will be useful in understanding adipogenesis *in vivo*. The

blastocyst complementation technique described in Chapter 6 of this thesis

presents a novel genetic approach to study adipogenesis *in vivo.*

CHAPTER 2: METHODS AND MATERIALS

2.1 BAC Modificaiton

Recombineering was performed as previously described (Gong et al., 2003) on a *leptin* gene containing BAC (RP24-69D4) and with primer sequence included in supplementary to produce the -22kb to +18kb and -22kb to +8.8kb leptin-luciferase reporter construct and the subsequent modified construct with 32 bp deletion. Sequences of cloning primers are included in TABLE 2.1. Genomic sequence and coordinates were based on NCBI37/mm9 mouse genome. Modified BAC were purified using CsCl gradient followed by restriction digestion using NotI (NEB) to remove the cloning backbone. Linearized BAC was separated by gel purification, followed by electro-elution and spot dialysis to remove debris and contaminants that might impede embryo manipulation.

2.2 Animal Experiments

Generation of transgenic mice: Leptin-luciferase reporter BACs were used to generate transgenic animals in either C57BL/6J or FVB N/J mice (Jackson Lab) using common pronuclear injection techniques (Birsoy et al., 2008a; Nagy, 2003).

Generation of chimeric mice: AZIP(FVB) chimeras were generated by injecting CAG-driven tubulin-YFP-ES-cells (B6-Tyr[c]; Gene Targeting Resource

TABLE 2.1 BAC Modification Primers.

150kb kb GalK primer For	5'CCTGTCTGAAATGCTGGTCCATGGAGACTTGTTCCCGGG TGAGGTTCCACCTGTTGACAATTAATCATCGGCA
22kb GalK primer Rev	5'TGTTTTTTGAAATGTATTTAAATATTTATTTATTTTATGT ATATGAGTATCAGCACTGTCCTGCTCCTT
150-22kb 5' deletion oligo	5'CCTGTCTGAAATGCTGGTCCATGGAGACTTGTTCCCGGG TGAGGTTCCATACTCATATACATAAAATAAATAAATATTT AAATACATTTCAAAAAACA
150-22kb 3' deletion oligo	5'TGTTTTTTGAAATGTATTTAAATATTTATTTATTTTATGT ATATGAGTATGGAACCTCACCCGGGAACAAGTCTCCATGG ACCAGCATTTCAGACAGG
8.8kb GalK primer For	5'TGCTGGAGACCCCTGTGTCGGTTCCTGTGGCTTTGGTCC TATCTGTCTTATGTTCAACCTGTTGACAATTAATCATCGGC A
18kb GalK primer Rev	5'CGGTACCCAATCAATGGTCATGGCTTTCTACGATGTTTG AGTGTCTACAGACATCTCTCAGCACTGTCCTGCTCCTT
8.8-18kb 5' deletion oligo	5'TGCTGGAGACCCCTGTGTCGGTTCCTGTGGCTTTGGTCC TATCTGTCTTATGTTCAAGAGATGTCTGTAGACACTCAAA CATCGTAGAAAGCCATGACCATTGATTGGGTACCG
8.8-18kb 3' deletion oligo	5'CGGTACCCAATCAATGGTCATGGCTTTCTACGATGTTTG AGTGTCTACAGACATCTCTTGAACATAAGACAGATAGGAC CAAAGCCACAGGAACCGACACAGGGGTCTCCAGCA
32bp GalK primer For	5'AGGTTAATCTTTGAAGTCCCTAAAGATTTGAACTTTCCG CAGAATTGGCTGCAGCGTCCCTGTTGACAATTAATCATCG GCA
32bp GalK primer Rev	5'TGGTGGCTTCCTGCTGTGGCCTCTTCTTCCCTCCCCTCCC CAGCACAGCTTCTTGCTTTCAGCACTGTCCTGCTCCTT
32bp 5' deletion oligo	5'AGGTTAATCTTTGAAGTCCCTAAAGATTTGAACTTTCCG CAGAATTGGCTGCAGCGTCAAGCAAGAAGCTGTGCTGGG GAGGGGAGGGAAGAAGAGGCCACAGCAGGAAGCCACCA
32bp 3' deletion oligo	5'TGGTGGCTTCCTGCTGTGGCCTCTTCTTCCCTCCCCTCCC CAGCACAGCTTCTTGCTTGACGCTGCAGCCAATTCTGCGG AAAGTTCAAATCTTTAGGGACTTCAAAGATTAACCT
32bp deletion sequencing primer For	5'-AGTTGACCTGTGCTTCCATACCCT
32bp deletion sequencing primer Rev	5'-TGTTCTCTTATATCCGCCCTGGGT
4bp mutation oligo For	5'TAAAGATTTGAACTTTCCGCAGAATTGGCTGCAGCGTCT AGTGGGTTAGAGTCTAAcgtaAGTAGAGCAGAAGCAAGAA GCTGTGCTGGGGAGGGGAGGGAAGAAGAGGCCACAGC
1bp mutation oligo For	5'TAAAGATTTGAACTTTCCGCAGAATTGGCTGCAGCGTCT AGTGGGTTAGAGTCTAATTcGAGTAGAGCAGAAGCAAGA AGCTGTGCTGGGGAGGGGAGGGAAGAAGAGGCCACAGC

Center at Rockefeller) into 3.5-day blastocysts from breeding of wild-type

FVB females (Jackson) to AZIP(FVB) transgenic males (Moitra et al., 1998).

Since both the blastocyst and the ES-cells have white coat color, AZIP(FVB)

chimeras are completely albino. Degree of chimerism was determined by the

percent of YFP-genotype cells in blood DNA samples. AZIP(B6) chimeras

were generated by injection B6-Tyr^c ES-cells into 3.5-day blastocysts from

breeding of wild-type C57BL/6J females to AZIP(B6) transgenic males.

AZIP(B6) transgenic mice were generated by backcrossing AZIP(FVB) male

to wild-type C57BL/6J females for more than 7 generations. Chimerism of

AZIP(B6) chimeras were scored by coat color and averaged from two

independent observers as well as quantifying the percentage of Tyr^c allele

versus Tyr wild-type allel using Taqman qPCR (Applied Biosystem). AZIP

transgenic mice are genotyped according to previous protocol (Moitra et al.,

1998). Primer sequences are detailed in TABLE 2.2.

TABLE 2.2 Genotype and ChIP primers

CRE For	5'-CAC GAC CAA GTG ACA GCA AT
CRE Rev	5'-AGA GAC GGA AAT CCA TCG CT
gLUC For	5'-GTT GTT CCA TTC CAT CAC GG
gLUC Rev	5'-TCC TCT GAC ACA TAA TTC GCC
gAZIP For	5'-CTG TGC TGC AGA CCA CCA TGG
gAZIP Rev	5'-CCG CGA GGT CGT CCA GCC TCA
gNFYAf_For	5'-GTA AGT CAG GCT CCA GGG
gNFYAf_Rev1	5'-AGG CAA GGC AGA TTT AGG AAG GTC
gNFYAf_Rev2	5'-GGG TTG TCA GGA TGT TCG CAG
YFP_For	5'-GCG AGG GCG ATG CCA CCT AC
YFP_Rev	5'-GCG AAG CAC TGC AGG GCG TA
FABP4_For	5'-AAT GTC AGG CAT CTG GGA AC
FABP4_Rev	5'-GAC AAA GGC AGA AAT GCA CA
chip_leptin5e_For	5'-CGC AGA ATT GGC TGC AGC GT
chip_leptin5e_Rev	5'-GGT GGC TTC CTG CTG TGG CC
chip_ins_For	5'-GGA CCC ACA AGT GGA ACA AC
chip_ins_Rev	5'-GTG CAG CAC TGA TCC ACA AT

Housing condition for AZIP mice: Due to cold sensitivity of neonates, breeding pairs consist of AZIP male and wild-type females are housed in non-ventilated cages and in elevated temperature (27°C-30°C). AZIP-pups were weaned around 4 weeks old, upon which the animals are transferred to standard housing condition.

In vivo Luciferase imaging: Measurement of luciferase reporter activity in Leptin-BAC transgenic mice was performed with Xenogen IVIS Lumina imaging system (Caliper), 10 mins after ip-injecting 150mg/kg of luciferin (PerkinElmer) in 150uL 1XDPBS prepared freshly.

Generation and experiments of adipocyte-specific knockout NF-YA: Adiponectin-CRE mice were purchased and genotyped according to standard protocol (Jackson Lab). NFYA-fl/fl strain and its genotyping were described previously (Bhattacharya et al., 2003). NFYA-fl/fl mice were backcrossed to C57BL/6J background for at least 8 generations prior to all experiments. Leptin at 350ng/hour (Amgen) or PBS was delivered for 14 days by subcutaneously implanted pre-equlibrated osmotic pumps, model 2002 (Alzet). Insulin (Alpco), serum Leptin (R&D Systems), and serum total Adiponectin (Alpco) were measured by ELISA according to the manufacturers' protocols using serum samples collected by retro-orbital bleeding using EDTA coated capillaries (Drummond). Blood glucose was measured by tail vein sampling using a Breeze2 glucometer (Bayer). High fat diet treatments used 58Y1 60 kcal% fat (TestDiet). Body adiposity was

measured by DEXA scan using Lunar PIXImus2 Densitometer and analyzed with manufacture software (GE Medical Systems). Glucose tolerance test (GTT) was performed by IP injecting 10% glucose aqueous solution calculated to 1mg/g of body weight following a 12 to 16 hour overnight fast. For triglyceride quantification in liver, fresh liver tissue with known weight (between 60 to 80mg) was homogenized using Polytron in 5%NP-40/water on ice, followed the assay protocol outlined in Triglyceride Quantification Kit (Abcam).

Metabolic profiling: Energy expenditure and oxygen consumption were measured using Oxymax Comprehensive Lab Animals Monitoring System (Columbus Instrument). Core body temperature was measured using IPTT-300 transponder probes and DAS-8001Console reader (Bio Medics Data Systems). The probes were implanted into the peritoneal space.

All animal surgeries were performed under Isothesia (Henry Schein) delivered via a flow regulator. All animal experiments were performed incompliance with regulation and approved by institutional board at The Rockefeller University.

2.3 Biochemical Assays

In vitro Luciferase assay: Various tissues (spleen, muscle, stomach, brain, heart, liver, kidney, intestine, inguinal adipose tissue, epididymal

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adipose tissue) were dissected and collected from transgenic mice and flash-frozen with liquid nitrogen. The tissues are homogenized in cell lysis buffer (Promega) with a polytron homogenizer. Luciferase activity in tissue lysate was measured using the Luciferase Assay kit (Promega) and normalized to protein content with a BCA kit (Pierce).

Western Blot: White adipose tissue and liver tissue were collected and minced with razor blade and heated in 95C in loading buffer for 5min. Antibodies against NF-YA H209 (Santa Cruz) and b-Actin 13E5 HRP conjugated (Cell signaling) were used.

2.4 Molecular Experiments

EMSA and supershift assays: EMSA was performed by incubating 6 μg of adipose tissue nuclear extracts from 12-16 weeks old Lep ob/ob mice (Jackson Lab) in a 20 μl reaction volume with 10 mM HEPES pH 7.9, 4% glycerol, 80 mM KCl, 1 mM MgCl2, 2 μg poly (dI-dC), 3 μg BSA, with 20,000 cpm of the 32P-labeled DNA probe for 20 min at room temperature. Samples were then loaded onto a 4-6% polyacrylamide gel, run at 150V for 4 hours, dried, detected overnight in a phosphor screen (GE healthcare) and read in an Amersham Biosciences Typhoon 9400 imager. For supershift assays 2 μg antibody was added after 20 min of incubation with the probe and incubated another 20 min before loaded onto the gel. Antibodies were NFY-A (H209), NFY-B (FL207), C/EBPα (14AA), C/EBPβ (Δ198) from Santa Cruz

Biotechnology, Inc. The sequence of the wild type 32 bp probe is

TAGTGGGTTAGAGTCTAATTGGAGTAGAGCAG (individual sequences of

mutated oligos are shown in TABLE 2.3).

CHIP assay and luciferase reporter assay: Adipose tissues were

harvested from 8 to 10 weeks old C57BL/6J male mice of either wild-type or

Lep ob/ob genotype (Jackson Lab). Tissues were washed in cold 1XPBS and

minced with razor blade on ice, followed by cross linking in 1%

formaldehyde/PBS for 20min and quenching with glycine for 5min at room

temperature. The tissues were washed with 1XPBS three times and flash-

freezed in liquid nitrogen. Frozen cross linked tissues were thawed in buffer

A (20mM Tris, pH7.9, 25% glycerol, 0.1mM EDTA, 0.5% TX-100, 0.5% NP40,

0.5mM PMSF) and homogenized with a douncer on ice. The lysate was span

at 2000xg for 5min to collect the nuclei, which were suspended in CHIP

buffer (50mM HEPES/KOH, pH7.5, 140mM NaCl, 0.1% Na-deoxycholate, 1%

TX-100, 1mM EDTA, 0.1% SDS) with complete protease inhibitor cocktail

(Roche). The nuclei were sonicated at 50% amplitude for 15min on ice. The

1mL of supernatant was used to perform one IP reaction using ProteinG

Dynal beads (Invitrogen), preincubated with 2ug antibody against NF-YA

(H209; Santa Cruz) for 2 hrs. After overnight incubation at 4C on a rotator,

the reaction was washed with high salt buffer (20mM Tris pH8.1, 500mM

NaCl, 2mM EDTA, 1% TX-100, 0.1% SDS) three times and EB buffer once.

Reverse cross link was performed using TE with 1% SDS at 65C for 15min on

a thermomixer. Sample was treated with RNase and protrease K (Sigma),

TABLE 2.3 Gel shift and super shift assay oligonucleotides.

32bp wt F	TAGTGGGTTAGAGTCTAATTGGAGTAGAGCAG
32bp wt R	CTGCTCTACTCCAATTAGACTCTAACCCACTA
32bp mut1F	TAGTGGGTTAGAGTCcAATTGGAGTAGAG
32bp mut2R	CTGCTCTACTCCAATTgGACTCTAACCCA
32bp mut2F	TAGTGGGTTAGAGTCTcATTGGAGTAGAG
32bp mut2R	CTGCTCTACTCCAATgAGACTCTAACCCA
32bp mut3F	TAGTGGGTTAGAGTCTAcTTGGAGTAGAG
32bp mut3R	CTGCTCTACTCCAAgTAGACTCTAACCCA
32bp mut4F	TAGTGGGTTAGAGTCTAAcTGGAGTAGAG
32bp mut4R	CTGCTCTACTCCAgTTAGACTCTAACCCA
32bp mut5F	TAGTGGGTTAGAGTCTAATcGGAGTAGAG
32bp mut5R	CTGCTCTACTCCgATTAGACTCTAACCCA
32bp mut6F	TAGTGGGTTAGAGTCTAATTcGAGTAGAG
32bp mut6R	CTGCTCTACTCgAATTAGACTCTAACCCA
32bp mut7F	TAGTGGGTTAGAGTCTAATTGcAGTAGAG
32bp mut7R	CTGCTCTACTgCAATTAGACTCTAACCCA
32bp mut8F	TAGTGGGTTAGAGTCTAATTGGcGTAGAG
32bp mut8R	CTGCTCTACgCCAATTAGACTCTAACCCA
32bp mut9F	TAGTGGGTTAGAGTCTAATTGGAcTAGAG
32bp mut9R	CTGCTCTAgTCCAATTAGACTCTAACCCA
32bp mut10F	TAGTGGGTTAGAGTCTAATTGGAGcAGAG
32bp mut10R	CTGCTCTgCTCCAATTAGACTCTAACCCA

purified with PCR purification kit (Qiagen), and analyzed with qPCR (see supplementary for primer sequences). For Luciferase reporter assay, 115bp leptin enhancer site (GAGAACACTTAACAGCAAAGGTTAATCTTTGAAGTCCCT AAAGATTTGAACTTTCCGCAGAATTGGCTGCAGCGTCTAGTGGGTTAGAGTCTA ATTGGAGTAGAGCAGAAGCAAG) was PCR amplified using -22kb to +8.8kb leptin BAC as template and primers 5'-gcggcgcg <u>CTCGAG</u> AAC ACT TAA CAG CAA AGG TTA ATC and 5'-cgcccggc <u>AGATCT</u> CTT GCT TCT GCT CTA CTC CAA TTA GA. The PCR product was cloned into pGL4.15 (Promega) between XhoI and BglII sites. Expression plasmids of NF-YA, NF-YB, NF-YC, and CEBPa in pCMV-SPORT6 vector as well as vector plasmid were commercially available

(Open Biosystem). The constructs were transfected into 293T cells using Lipofectamine 2000 (Invitrogen). The assay was performed using Duo Luciferase Reporter Assay System (Promega).

Gene expression analysis: To extract RNA from adipose tissues, approximately 70mg of adipose tissue was harvested and flash-frozen in liquid nitrogen. The frozen tissue was homogenized with a Polytron homogenizer for 30 sec in 700uL of Trizol Reagent (Invitrogen) on ice. After removing debris with centrifugation, the lysate was added to a column of Direct-zol RNA MiniPrep (Zymo) and processed according to the manufacture's manual. cDNA was synthesized using qScript cDNA SuperMix (Quanta Bioscience). Quantitative PCR was done using Quantitect SYBR Green PCR Kit (Qiagen) and 7500 Fast Real-Time PCR (Applied Biosystem) with primers specific for each gene of interest (TABLE 2.4).

2.5 Tissue Culture Experiments

Gene knockdown and differentiation in 3T3-L1: Knockdown of NFYA in 3T3-L1 cells was achieved by lentiviral vector-mediated shRNA expression with MISSION shRNA plasmids 439 (CCGGAGCAAGTTACAGTCC CTGTTTCTCGAGAAACAGGGACTGTAACTTGCTTTTTTG) and 441 (CCGGCCA AACCAAGCTGACGAAGAACTCGAGTTCTTCGTCAGCTTGGTTTGGTTTTTG) (Sigma). Lentivirus was generated in 293T cells using 2nd generation packaging system (Addgene) and Lipofectamine 2000 (Invitrogen). Viral

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TABLE 2.4 qPCR primers for gene expression analysis

Gene	Forward	Reverse
NFYA	5'-ATC AAC AGG CCA ACC CAT CA	5'-CTG GAT CTG TCC AGG AGG CA
HPRT1	5'-TCA GTC AAC GGG GGA CAT AAA	5'-GGG GCT GTA CTG CTT AAC CAG
AdipoQ	5'-GCC GTG ATG GCA GAG ATG GCA C	5'-GGA AGC CCC GTG GCC CTT CAG
Leptin	5'-GCA AGA AGA AGA AGA TCC CAG G	5'-CAG ATA GGA CCA AAG CCA CAG
Adipsin	5'-CAT GCT CGG CCC TAC ATG G	5'-CAC AGA GTC GTC ATC CGT CAC
Resistin	5'-AAG AAC CTT TCA TTT CCC CTC CT	5'-GTC CAG CAA TTT AAG CCA ATG TT
TNFa	5'-TCC CAG GTT CTC TTC AAG GGA	5'-GGT GAG GAG CAC GTA GTC GG
FABP4	5'-AAG GTG AAG AGC ATC ATA ACC CT	5'-TCA CGC CTT TCA TAA CAC ATT CC
PPARg	5'-GCA TGG TGC CTT CGC TGA	5'-TGG CAT CTC TGT GTC AAC CAT G
CEBPa	5'-CAA GAA CAG CAA CGA GTA CCG	5'-GTC ACT GGT CAA CTC CAG CAC
CEBPd	5'-CGA CTT CAG CGC CTA CAT TGA	5'-CTA GCG ACA GAC CCC ACA C
CD36	5'-ATG GGC TGT GAT CGG AAC TG	5'-GTC TTC CCA ATA AGC ATG TCT CC
Glut4	5'-GTG ACT GGA ACA CTG GTC CTA	5'-CCA GCC ACG TTG CAT TGT AG
Ehhadh	5'-ATG CTG AGT ATC TGA GGC TG	5'-GGT CCA ACT AGC TTT CT GGA G
Hadha	5'-ACT ACA TCA AAA TGG CT CTC AG	5'-AGC AGA AAT GGA ATG CGG ACC
Ctsk	5'-GAA GAA GAC TCA CCA GAA GCA G	5'-TCC AGG TTA TGG GCA GAG ATT
PGC1a	5'-GAT GCG CTC TCG TTC AAG AT	5'-GGT GTC TGT AGT GGC TTG AT
UCP1	5'-GCT TTG CCT CAC TCA GGA TT	5'-TAA GCC GGC TGA GAT CTT GT
Pref1	5'-AGT GCG AAA CCT GGG TGT C	5'-GCC TCC TTG TTG AAA GTG GTC A
Klf4	5'-ACA CCT GCG AAC TCA CAC AG	5'-ACT TCT GGC ACT GAA AGG GC

supernatants were supplemented with 8 μg/ml polybrene and added to cells

for infections for 36 hours. Cells were selected with 3 μg/ml puromycin

(Sigma) for 48 hours, expanded and seeded for differentiation experiments.

3T3-L1 cells were maintained in DMEM with 10% heat inactivated NCS

(Invitrogen) in 5% CO_2. Differentiation was performed as previously

described (Zebisch et al., 2012). Oil red staining was performed as previously

described (Zebisch et al., 2012). Non-differentiated controls were maintained in DMEM with 10% FBS. RNA extraction was performed using RNeasy (Qiagen) following manufacturer's instruction.

2.6 Statistical Analysis

Two-ended, unpaired Student T's test was used. Error bars indicated standard error of mean. Unless specifically mentioned, * indicated $p<0.05$, ** indicated $p<0.01$, *** indicated $p<0.001$, and **** indicated $p<0.0001$. Outliers were detected by comparing to corresponding Thomspon Tau values.

CHAPTER 3: TRANSCRIPTIONAL REGULATION OF LEPTIN BY DISTAL ELEMENTS

3.1 Introduction

Leptin is an adipocyte hormone that functions as the afferent signal in a negative feedback loop that maintains homeostatic control of adipose tissue mass. When weight is lost, leptin level falls thus stimulating appetite and altering metabolism and neuroendocrine function leading to weight gain (Coll et al., 2007). Conversely, in leptin responsive individuals, weight gain results in an increase in leptin levels and a reduction of food intake and body weight (Friedman-Einat et al., 2003; Jequier, 2002). Leptin production by adipocytes is highly correlated to body adiposity (Frederich et al., 1995; Maffei et al., 1995b) and is capable of exerting profound effects on both energy intake and energy expenditure (FIGURE 3.1). A fuller understanding of the elements of this system could have important implications for the pathophysiology and treatment of obesity.

Particularly, the changes in plasma leptin levels are associated with changes in *leptin* mRNA per adipocyte, and the levels of *leptin* mRNA per cell are highly correlated with intracellular lipid content (Couillard et al., 2000; Maffei et al., 1995b; Marikovsky et al., 2002; Motyl and Rosen, 2012). These observations have raised the possibility that the regulatory elements of the *leptin* gene consist of a putative lipid sensing mechanism that regulates *leptin* expression in response to changes in the amount of intracellular lipid and adipocyte size. However, the nature of such a mechanism is not known. To

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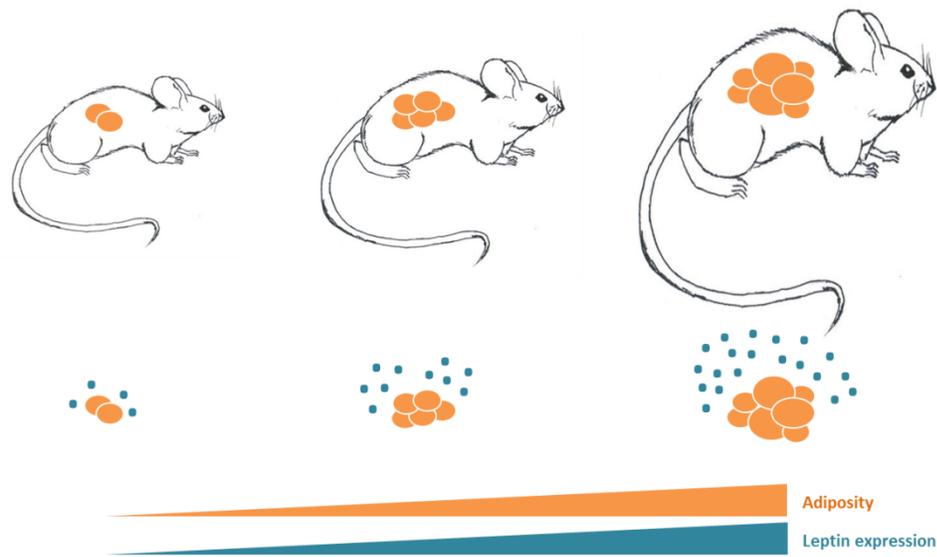
FIGURE 3.1 Plasma Leptin is Highly Correlated with body adiposity.
In leptin responsive individuals, weight gain results in an increase in leptin levels and a reduction of food intake and body weight. By this mechanism, body weight is maintained within a relatively narrow range. Plasma leptin thus serves as a surrogate for fat mass. The changes in plasma leptin levels are associated with changes in *leptin* mRNA per adipocyte and the levels of *leptin* mRNA per cell are highly correlated with intracellular lipid content.

elucidate this, we undertook a similar approach to that used to identify a cholesterol sensing pathway. In Brown and Goldstein's seminal work on the regulation of cholesterol metabolism, the identification of the *sterol response element* (*SRE*), a key *cis*-element, led to the identification of SREBP (SRE binding protein) and the cholesterol sensor SCAP (SREBP cleavage-activating protein) that controls a set of cleavages that leads to the transport of this transcription factor to the nucleus (Brown and Goldstein, 1999; Lee et al., 1994; Nohturfft et al., 2000). As such, we focus our endeavor on dissecting the transcriptional regulation of the *leptin* gene, although there is no

evidence to exclude other means of gene expression regulation such as post-transcriptional modification.

Analysis of quantitative expression of the *leptin* gene necessitates that studies be conducted *in vivo* because cultured adipocytes express orders of magnitude lower amounts of *leptin* mRNA compared to adipcoytes *in vivo* (Macdougald et al., 1995; Mandrup et al., 1997). A previous study from our laboratory showed that a leptin-luciferase BAC reporter construct extending from from -22kb to +150kb (RP2469D; mm9 chr6: 28987901-29158589) showed faithful qualitative and quantitative expression *in vivo* with increased reporter expression in *ob/ob* adipocytes and decreased expression in adipocytes after fasting (Birsoy et al., 2008b). This finding was consistent with data from another report showing that this BAC and a second clone extending from -160kb to +18kb (mm9 chr6:28854232-29028379) showed fat-specific reporter expression, although in the previous report, studies of reporter expression in *ob/ob* and fasted mice were not performed (Wrann et al., 2012). This part of the thesis presents the follow-up study on a series of modified leptin-luciferase BAC reporter mice to identify *cis-* and *trans-* elements regulating *leptin* transcription.

3.2 Results and Discussion

In aggregate of previous leptin-BAC reporter mice, it is inferred that DNA sequences between -22kb to +18kb (mm9 chr6:28987900- 29199010) are likely to be sufficient for *leptin* transcriptional regulation (FIGURE 3.2A). To test this directly, we generated a BAC-transgenic luciferase reporter mouse carrying a single 40kb leptin-luciferase BAC that extends from -22kb to +18kb, the region of overlap between the aforementioned BAC clones (FIGURE 3.2B). Using the same strategy as in a previous study (Birsoy et al., 2008b), luciferase was cloned into the *leptin* start codon in the second exon of the *leptin* gene together with a polyadenylation signal (this construct is used to generate all subsequent constructs presented in this chapter) so that luciferase is expressed under the control of the *leptin* promoter.

Two out of two founders expressed luciferase specifically in adipose tissue as shown using *in vivo* luciferase imaging and by analyzing luciferase activity from tissue lysates under chow-fed *ad libitum* (one representative animal shown as "fed" in FIGURE 3.2B). In addition, we did not find luciferase expression in the GI tract, muscle, brain or any other visceral organs in which low levels of *leptin* gene expression have previously been reported (Birsoy et al., 2008b; Rosenbaum et al., 2002). Quantitative regulation of luciferase expression was assayed by crossing the reporter mice to *ob/ob* animals, which increases endogenous *leptin* expression (Note, the *ob* mice that were used carry a mutation of the *leptin* coding sequence but still show marked induction of *leptin* gene expression), and by food restricting wild-type mice
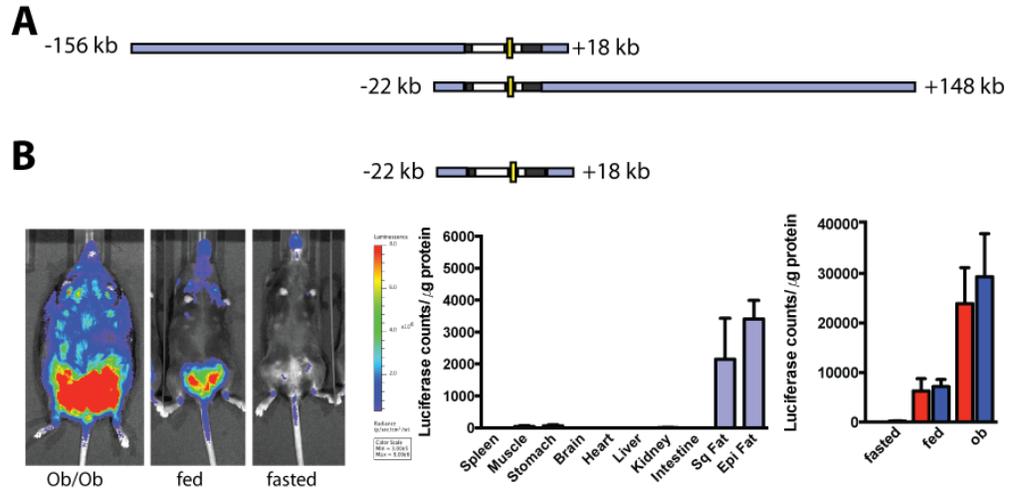
FIGURE 3.2 Analysis of Leptin-Luciferase BAC-transgenic animals. (A). A luciferase construct including a poladenylation signal was cloned into the Leptin initiation codon of two nested leptin-BAC clones by homologous recombination in bacteria, -156kb to +18kb (RP2433803; chr6:28854232-29028379) and -22kb to +148kb (RP2469D4; chr6: 28987901-29158589) and both recapitulate *leptin* expression *in vivo*, suggesting that the essential regulatory element of leptin must be contained in the 40kb region -22kb to +18kb. (B). Transgenic founders carrying a single clone of -22kb to +18kb leptin-luciferase BAC, the region of overlap between the two BACS in (A), showed adipose tissue specific luciferase expression using *in vivo* imaging (one representative mouse out of 2 founder mice as "fed"). The level of luciferase with metabolic status where fasting reduced Luciferase expression by 145.4-folds in eWAT (p=0.09) and 35.6-folds (p=0.0054) in iWAT and crossing to ob/ob genotype increase expression by 3.3-folds in eWAT (p=0.02) and 4.7-folds in iWAT (p=0.0083) (labeled as "fasted" and "ob/ob" respectively in B). The numbers of animals used are 4 for fasted, 7 for fed, and 4 for ob/ob. Luciferase assays from tissue lysate confirm that luciferase expression is qualitatively and quantitatively regulated similarly to *leptin*.

for 48 hours to lower endogenous *leptin* expression (representative images

are shown and labeled as "ob/ob" and "fasted" respectively in FIGURE 3.2B).

Luciferase expression was increased 3.3 fold in eWAT, p=0.02, and 4.7-fold in

iWAT, p=0.0083, in ob/ob background (n=4) and decreased 145.4-fold in

eWAT, p=0.094, and 35.6-fold in iWAT, p=0.0054, after fasting(n=4); both

results are compared to 7 animals from transgenic line -22kb to +18kb that

are fed *ab libitum*. These data confirmed that all of the *cis*-elements required for qualitative and quantitative expression are within this 40 kb interval and provided a starting point for the identification of *cis*-elements and *trans*-factors responsible for this regulation.

We then generated a comprehensive set of 5' and 3' deletions, the data from which revealed that there were redundant *cis*-elements upstream and downstream of the *leptin* transcription start site. Thus in order to further study *leptin* gene regulation, we needed to subdivide the *leptin* gene into separate 5' and 3' reporter constructs. A manuscript on the complete series of leptin-luciferase BAC transgenic mice study is in preparation and partial data is available in previous thesis work (Birsoy, 2009).

To further study the 5' cis-element without the complication from 3' element, we then generated a leptin-luciferase BAC that extends from -22kb to +8.8kb BAC clone (chr6:28987900- 29019010, ending at exon 2 of the leptin gene, thus excluding any element 3' to *leptin* gene). All of the founders (5 out of 5) of this new -22kb and +8.8kb leptin-luciferase BAC were found to express luciferase specifically *in vivo*. Quantitative regulation of *leptin* expression was confirmed by crossing the transgenic animals to *ob/ob* where we found a 1.5-fold increase in luciferase in eWAT, p=0.46, and a 3-fold increase in iWAT, p=0.0013 (n=3 for *ob/ob*). Fasting resulted in a 17-fold decrease in eWAT, p=0.019, and 6.5-fold decrease in iWAT, p=0.0002, *leptin* expression (n=6 for starved), compared to 8 wild-type *ab libitum* fed animals

(FIGURE 3.3A). Individual founders showed similar expression pattern (FIGURE 3.3B). These data confirm that sequences between -22kb to +8.8kb can, or in another word the 5' upstream of leptin is sufficient to, recapitulate qualitative and quantitative expression of the leptin gene *in vivo*.

We next performed a homology search of sequences within this -22kb and +8.8kb interval and identified an 115bp sequence (mm9 chr6:28993746-28993860) that is highly conserved among all mammals including mice (FIGURE 3.4). We found that 84.4% of the 115bp sequence is identical between human (GRCh37/hg19 genome) and mouse (NCBI37/mm9 genome) and there is 73.9% sequence identity between platypus (WUGSC 5.0.1/ornAna1 genome) and mouse. Gel shift assays using a series of 5 overlapping 32 bp oligonucleotides spanning this 115bp region assays were performed using nuclear extracts from adipose tissue of *ob/ob* mice and other tissues. One of these 32 bp oligonucleotides showed a clear gel shift activity using nuclear extract prepared from adipose tissue, as well as liver and spleen (FIGURE 3.5A; data for liver and spleen not shown). This 32 bp segment included a *CCAAT*-box on the minus-strand which is known to be a binding site for a number of transcription factors including the CEBP and the NF-Y transcription factors. Further, gel shift assays with mutant oligos
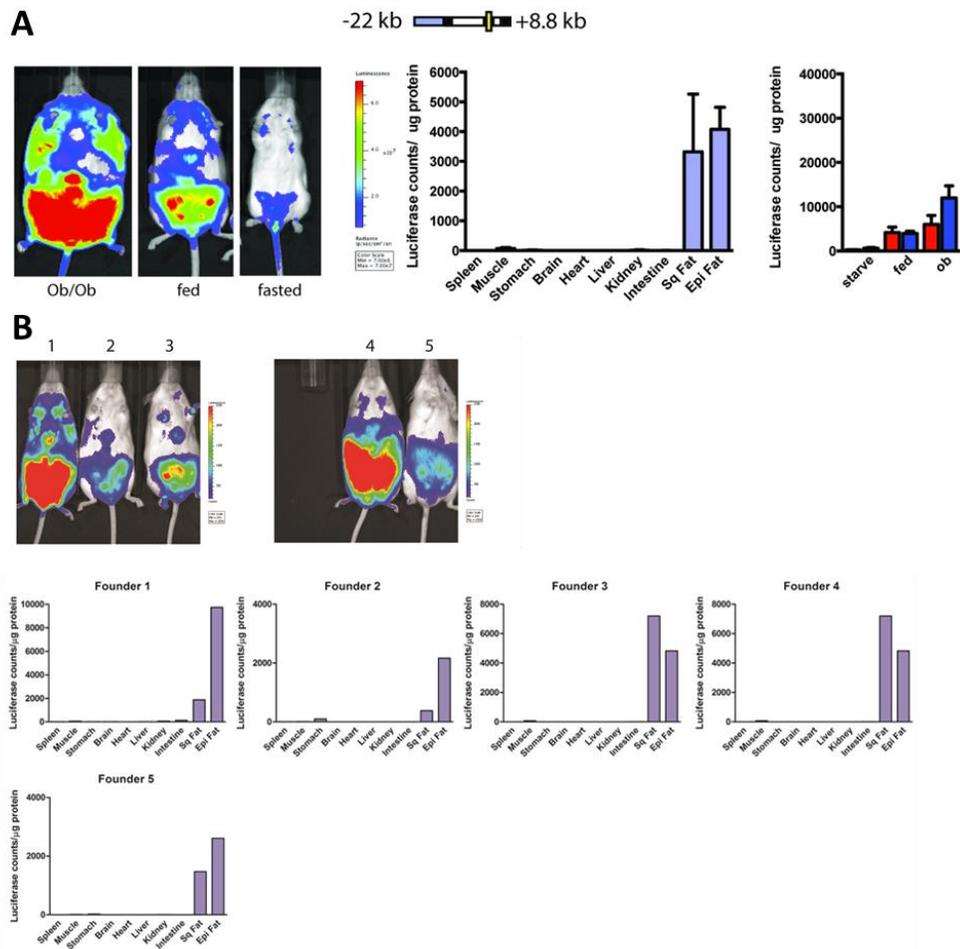
FIGURE 3.3 Analysis on -22kb to +8.8kb leptin-luciferase BAC-transgenic animals. (A). A leptin-Luciferase BAC extending from -22kb to +8.8kb (5 founders) also recapitulates leptin expression *in vivo*, showing that the included sequences along are sufficient for *leptin* regulation. Schematic maps of the three reporter transgenes are shown over the respective animals with the yellow bands indicating *luciferase*, the black segments indicating exons of *leptin*, the white segments indicating introns of *leptin*, the blue segments indicating genomic sequence framing *leptin*, and the red band indicating an internal deletion. All heat maps are scaled to minimum 0 and maximum 2000 luminescence counts in the manufacturer's program. (B). Individual founders of -22kb to +8.8kb, 5 mice, all showed adipose tissue specific leptin expression. Each tissue of individual transgenic founders (5 mice) of -22kb to +8.8 kb construct is shown. Luciferase activities are normalized to total protein amount as measured by BCA assay. Schematic maps of the two reporter transgenes are shown over the respective panels.
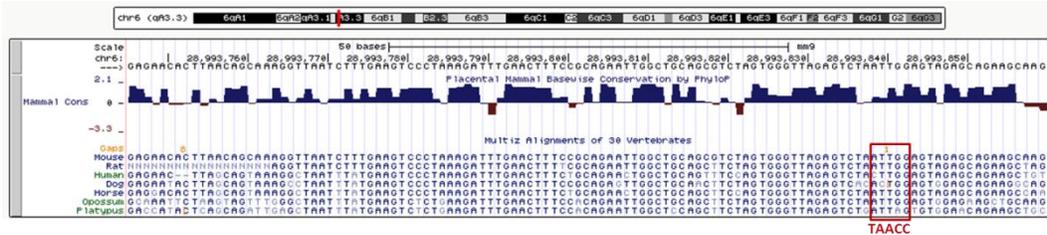
FIGURE 3.4 Conserved Non-Coding Region Upstream of *leptin*. The figure includes an 115bp region at -16.5kb of leptin (mm9 chr6:28993746-28993860) that is highly conserved among all mammals including mice. We found that 84.4% of the 115bp sequence is identical between human (GRCh37/hg19 genome) and mouse (NCBI37/mm9 genome). Genome information extracted from UCSC Genome Browser.

showed that base changes within the *CCAAT* sequence abrogated this DNA-protein interaction (FIGURE 3.5B).

To assess whether it was NF-Y or another DNA binding protein that was responsible for the activity, the gel shift assays were repeated in the presence of anti-NFYA (H209, Santa Cruz) and anti-NFYB (FL207, Santa Cruz). These data revealed a clear super-shift in the presence of the antibody which is directed against two subunits of the factor, NF-YA and NF-YB (FIGURE 3.5C). In contrast, antibodies to two other *CCAAT*-box binding protein C/EBPA or C/EBPB (14AA and Δ198 respectively, Santa Cruz), both of which are known to play a role in adipocyte function, did not result in a super-shift. To confirm that NF-Y binds to the *CCAAT* sequence of the leptin gene *in vivo*, chromatin immunoprecipitation (ChIP) assays from adipose tissue nuclear extract were performed. The ChIP assay revealed a 2.7-fold enrichment (p=0.02) of the NF-Y binding sequences from *ob/ob* inguinal

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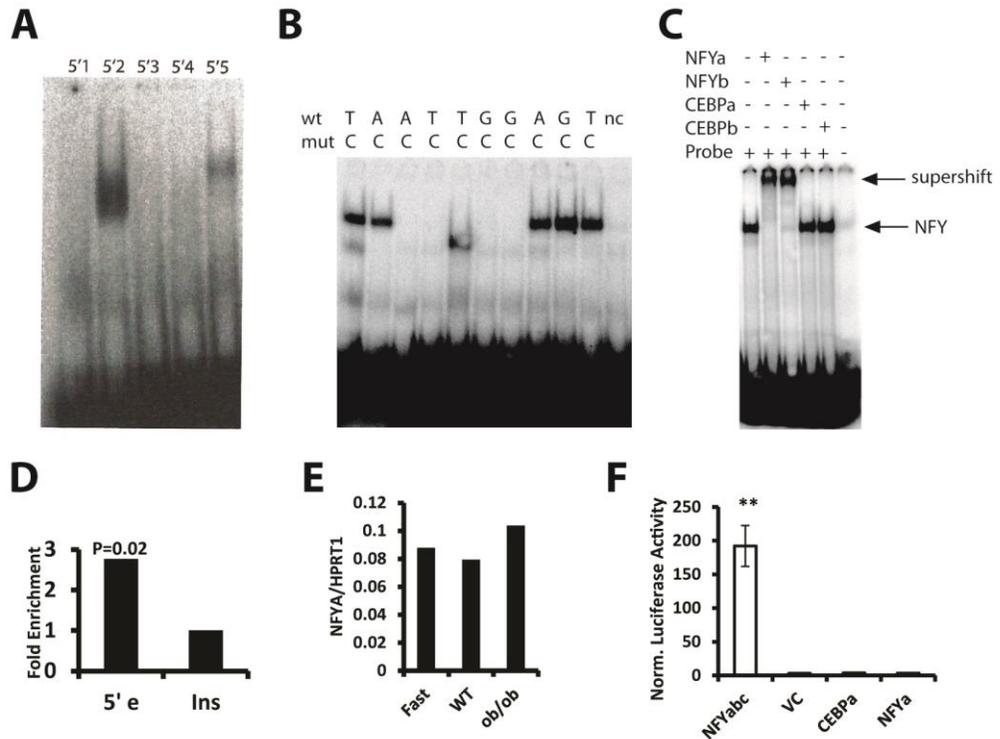
FIGURE 3.5 Identification of an NF-YA binding site between -16.401kb to -16.370kb. A series of overlapping radioactive oligonucleotide probes were used to perform gel shift assay using adipose tissue nuclear extract from *ob/ob* mice in this region to identify enhancer site. Two of the five probes showed DNA-binding activity in (A) and the 5'5 site containing a 32bp sequence from -16.401kb to -16.370kb (mm9 chr6: 28993820- 2899385) was further analyzed. (B). A series of oligos of the 32 bp sequence of 5'5 site with single mutations (from "wt" sequence to "mut" in figure) identified a core binding sequence of a DNA binding protein compatible with NF-Y binding. (C). Super-shift assays shown in were performed for possible CCAAT binding proteins NF-YA (H209), NF-YB (FL207), C/EBPa (14AA), or C/EBPb (Δ198) to gel shift reaction with wild-type oligonucleotide. A super-shift using the NF-YA (H209) and NF-YB (FL207) antibodies is indicated with an arrow. Antibodies against CEBPs did not cause a super-shift. (D). CHIP assay in mouse subcutaneous white adipose tissue using anti NF-YA shows binding at the identified 32bp enhancer sequence (labeled 5'e) in 10 week old *ob/ob* mice. Fold enrichment is normalized to insulin (labeled Ins), a locus that is not expressed in adipose tissue. (E). *NF-YA* mRNA levels as normalized against *HPRT1* expression are indistinguishable in fast, fed, or *ob/ob* adipose tissue. (F). An *ex vivo* luciferase assay was performed using a luciferase construct driven by an 115bp region encompassing the 32bp sequence co-transfected with NF-YABC subunits (lane NF-Yabc) showed a 60-fold increase in luciferase activity compared to vector-only control (VC), while co-expression of the reporter with CEBPA, or NFYA alone did not increase activity.

adipose tissue using the anti-NFYA antibody (H209, Santa Cruz) compared to DNA sequences from the insulin gene (FIGURE 3.5D). Insulin was used as the control region because the gene is not expressed in adipose tissues. We next assayed the levels of NF-Y mRNAs and did not find a change in mRNA levels among *ob/ob*, and wild-type mice fed *ab libitum* and fasted (FIGURE 3.5E).

A functional analysis of the transcriptional activity NF-Y was demonstrated by co-transfecting plasmids expressing three subunits of NF-YA, NF-YB, and NF-YC together with a luciferase reporter cloned downstream of the 115bp homologous sequence of *leptin* enhancer. We found a 60-fold increase in luciferase expression when the NF-Y plasmids were co-transfected compared to vector control (FIGURE 3.5F). Consistent with the super-shift data, expressing C/EBPA failed to increase *luciferase* expression in the same assay, nor did NF-YA alone consistent with previous data showing that all three NF-Y subunits are required for its transcriptional activity. Together, these experiments showed that NF-Y binds to the CCAAT-box 16.5kb upstream of the leptin TSS and can increase the level of expression of a reporter construct. The identified *cis*-element is a true enhancer that can activate transcription independent of their location, distance or orientation with respect to the promoters of genes (Ong and Corces, 2011). Note that since NF-Y was identified using a candidate approach, it is likely that other transcription factors also interact with this enhancer site. Other trans-elements can be identified using the same in vitro

luciferase assay combined with cDNA library of transcription factors, which is an approach under active pursue.

Although the C/EBP family transcription factors are known for inducing preadipocyte differentiation and in modulating gene expression in the fully differentiated adipocyte (Darlington et al., 1998), it is not unexpected that we did not detect bindings of either C/EBPa or C/EBPb at the identified distal enhancer. As mentioned, *leptin* is expressed at much higher levels *in vivo* versus *in vitro* such as in differentiated 3T3-L1 or F442 cells (Macdougald et al., 1995; Mandrup et al., 1997). Since these cultured models express C/EBP factors and their targets in abundance (Soukas et al., 2001), it can be inferred that adipocyte specific expression of leptin is controlled by a different set of transcriptional mechanism from that dictating adipogenesis, at lease in vitro. Nonetheless, since expression of *leptin* serves as a hallmark of adipocyte maturation both *in vivo* and *in vitro* (Hwang et al., 1997; Slieker et al., 1998), the *in vivo* adipogenesis events leading to *leptin* activation during development is worth of further investigation.

To confirm a role for the 32 bp sequence that includes the NF-YA binding site *in vivo* we generated a -22 kb to +8.8 kb leptin-luciferase BAC reporter line in which there was an internal deletion of the 32 bp that included the NFY-A *CCAAT*-sequence. (GTCTAGTGGGTTAGAGTCTAATTGGA GTAGAG found between mm9 chr6: 28993820- 2899385; FIGURE 3.6A). This deletion completely abrogated reporter expression in all 11 transgenic
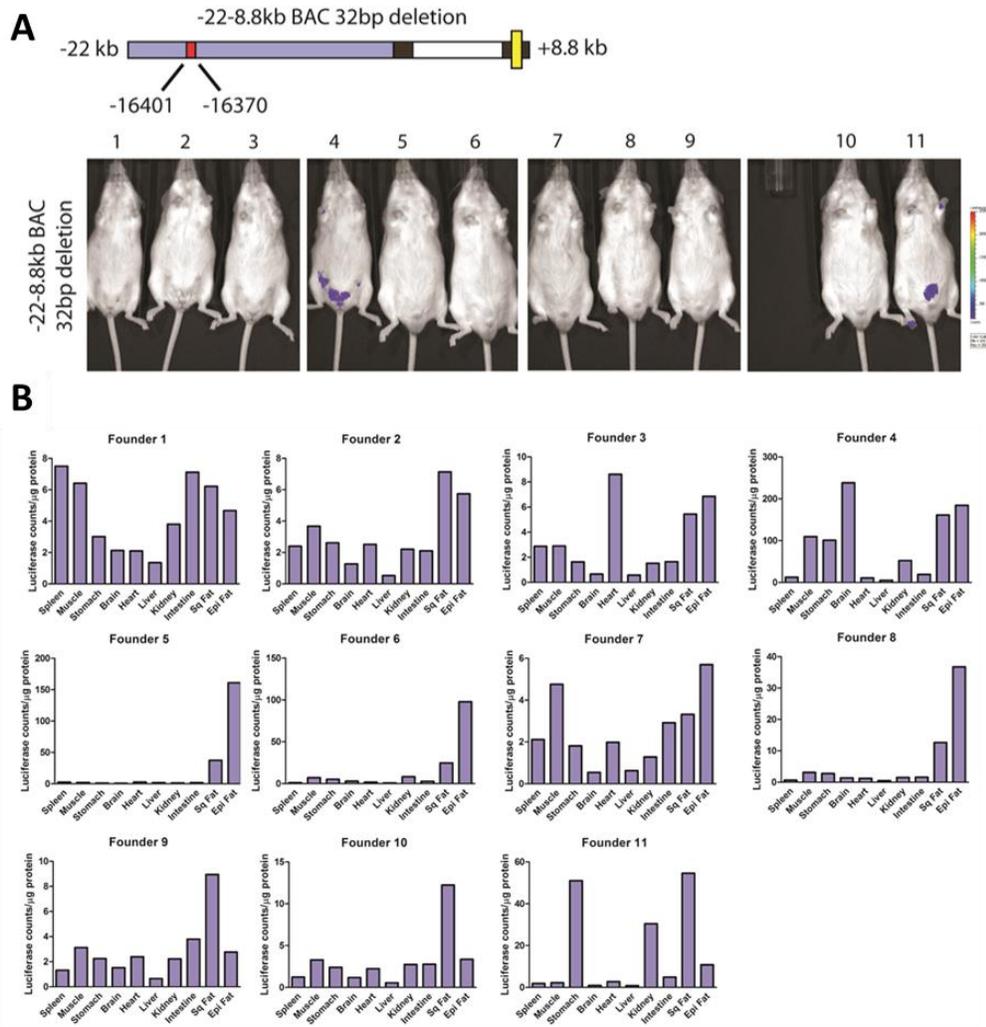
FIGURE 3.6 Deletion of a 32bp sequence abolish *leptin* expression *in vivo*. (A) A 32bp sequence internal deletion of the 32 bp NF-YA binding site (GTCTAGTGGGTTAGAGTCTAATTGGAGTAGAG; mm9 chr6: 28993820-28993851) in the -22kb to +8.8kb BAC (11 mice) abrogated Luciferase expression, confirming that these sequences are essential for leptin expression *in vivo*. (B). Luciferase activity of individual tissues from each founders of the -22kb to +8.8 kb leptin-luciferase gene with the 32 bp deletion are graphed, showing no adipose tissue specificity nor comparable expression level to wild-type construct. Luciferase activities are normalized to total protein amount as measured by BCA assay. Schematic maps of the two reporter transgenes are shown over the respective panels.

founders using *in vivo* Luciferase imagining as well in biochemical assays

from tissue lysates as described above (FIGURE 3.6B). To confirm that the

reverse *CCAAT*-box is essential for the transcriptional activity observed from

the -22 kb to +8.8 kb leptin-luciferase BAC reporter line *in vivo*, we generated

two additional transgenic lines: a 4bp mutation from 5'-ATTGG to 5'-Acgta

(construct NFY-4bp; mm9 chr6: 28993840- 28993843) and a 1bp mutation

to 5'-ATTcG (construct NFY-1bp; mm9 chr6:28993842). For NFY-4bp 5 out

of 5 and for NFY-1bp 4 out of 5 transgenic founders lost leptin expression in

vivo (FIGURE 3.7A and B). These data confirmed that the *CCAAT*-box element

is essential for *leptin* and led us to test the function of NF-YA in adipocytes *in*

vitro and *in vivo* (see Chapter 4).

This finding is in contrast to data from a prior publication by Wrann *et*

al suggesting that a DNA sequences between -5.2kb and the proximal

promoter are responsible for *leptin* expression *in vivo* (Peterfy et al., 2001).

In this prior report, the authors generated the same -22kb to +18kb Leptin

BAC clone (except for using EGFP as oppose to Luciferase), that we used to

recapitulate Leptin expression. They then used a -5.2kb to +18kb BAC clone

and showed that these sequences can also recapitulate fat specific *leptin*

expression though quantitative expression in ob/ob and fasted mice was not

analyzed. The authors thus concluded that the regulatory element controlling

fat specific *leptin* expression must reside within the 5.2kb 5' region. However,

we have found that there are redundant elements controlling *leptin*

expression including either the 32 bp sequence on the 5'side at -16.5kb
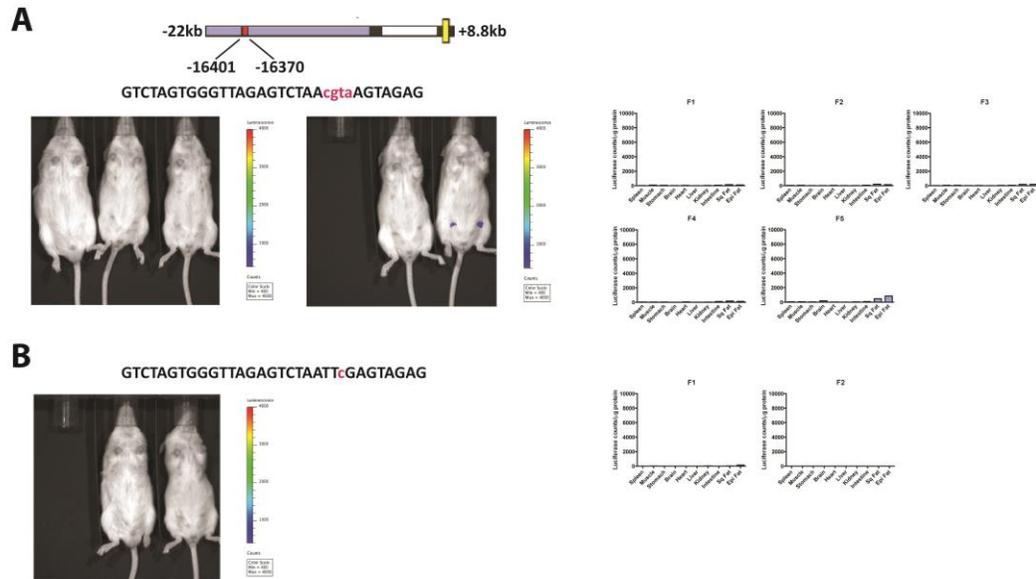
FIGURE 3.7 Point mutations at NFY binding site ablate *leptin* transcription. Two transgenic lines were generated by point-mutagenesis on -22kb to +8.8kb leptin-luciferase BAC (top diagram). The red bar corresponds to the sequences in each line and mutated base pairs are in low case red letters. (A). Four base pair mutation of reverse CCAAT-box from ATTGG to Acgta abolishs leptin expression in 5 out of 5 founders. (B). Single base pair mutation from ATTGG to ATTcG abolishs leptin expression in 2 out of 2 founders. Biochemical assay from tissue lysate of individual founders are on the right.

presented here or a 3' sequences at approximately +14kb (manuscript in preparation). Either of these 5' or 3' sequences is capable of independently conferring fat specific expression of the *leptin* gene (manuscript in preparation). For example, we find that a BAC clone that extends from -16.4kb to +8.8 kb does not lead to fat specific expression of a luciferase reporter thus excluding sequences between -5.2 kb and +1bp as being capable of supporting *leptin* expression. Thus the reason the BAC extending from -5.2kb to +18kb shows fat specific expression is because of sequences at ~ +14 kb, not sequences between -5.2 kb and the transcription start site. The

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findings explain our data showing that there is faithful qualitative and quantitative expression of a 5' reporter extending from -22 kb to +8.8 kb, and that an internal deletion of a 32 bp sequence at -16.7 kb abrogates this expression despite the fact that more proximal promoter elements between -5.2 kb to +1bp are intact.

To investigate *in vivo* function NF-Y binding site, it will be necessary to generate knockout mouse model. With the recent advance in genome editing technique using CRISPR (Wang et al., 2013a), precise deletion or mutation of the sequence of interest can be achieved in one generation. Although the aforementioned redundancy of multiple *cis*-elements could mask the potential effect of knockout, such mouse model will also provide information on broader role of *leptin* regulation *in vivo* that was beyond the scope of luciferase reporting.

Since NF-Y is ubiquitously present in many tissues, adipocyte specific and fat mass dependent expression of *leptin* cannot be explained by the regulatory function of NF-Y factor alone. Recent advances in understanding transcriptional mechanism suggested an important role of repressor in conferring spatial and temporal gene expression (Ong and Corces, 2011). Since *leptin* gene is not expressed until late gestation in mice in differentiated adipocytes, transcription of *leptin* must be repressed in the early developmental stages of adipocytes. Indeed, several studies have suggested that NF-Y is capable of activating as well as inhibitory regulation, either by

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direct interaction with different DNA sequences or by recruiting different co-factors (Peng and Jahroudi, 2002; Zhu et al., 2012). Genome wide study showed that NF-Y functions as "switch" between proliferation and differentiation in stem cell by re-directing the transcriptional profile in global level (Ceribelli et al., 2008; Donati et al., 2008). The precise molecular mechanism of *leptin* transcriptional regulation remains to be explored. Particularly, identification of other *cis*-elements and corresponding *trans*-element will facilitate further investigation on interactions among these factors. One straightforward speculation is that other *cis*-elements are modulated by some yet unknown adipocyte specific factors, while NF-Y serves as co-factor in transcriptional activation. Unfortunately, there is a lack of unbiased method to identify *trans*-factor to a known sequence, and classical biochemical purification of transcription factor requires large amount of material that is impractical to obtain from animal tissue (Yang, 1998).

Recent studies highlight the importance of NF-Y on stem cell proliferation and maintenance, notably in mesenchymal linages including myoblast and hematopoetic stem cell (Bungartz et al., 2012; Farina et al., 1999; Gurtner et al., 2008; Gurtner et al., 2003). Similar to our observation in 3T3-L1 cells where NF-Y expression is reduced during adipogenesis, down-regulation of NF-Y accompanies differentiation in both the hematopoetic and muscle lineage. NFY has also been shown to control tissue specific gene expression by interacting with other cell specific DNA binding proteins. For

instance, a study comparing fetal and adult erythrocytes shows that NF-Y

differentially recruits activators or repressors to induce or inhibit the

gamma-globin gene during development (Zhu et al., 2012). Biochemical

studies also showed that NF-Y displays histone-like DNA binding activities,

and can regulate transcription in a non-sequence specific manner (Nardini et

al., 2013; Oldfield et al., 2014). Further studies of NF-Y and other factors will

be required to dissect how NF-Y controls qualitative and quantitative *leptin*

transcription.

CHAPTER 4: NF-Y KNOCKOUT AS A NOVEL LIPODYSTROPHY MODEL

4.1 Introduction

Identification of NF-Y as a *trans*-element regulating *leptin* gene expression, described in Chapter 3, prompted us to study its function in the 3T3-L1 adipogenesis model and in a knockout mouse model. NF-Y (also known as CBP, CCAAT-box Binding Protein) is a transcription factor composed of three subunits, NF-YA, NF-YB, and NF-YC (also as CBP-B, CBP-A, CBP-C respectively). NF-Y recognizes the CCAAT sequence mostly through the conserved C-terminus of NF-YA, although all three subunits are required for DNA binding activity (Mantovani, 1999). A germ line knockout of NF-YA is embryonic lethal at E8.5, demonstrating that NF-Y is essential for early development (Bhattacharya et al., 2003). In addition, NF-Y is required for stem cell maintenance and for controlling cell type specificity during differentiation, particularly in mesenchymal lineages such as blood cells, myoblasts, and osteoblasts (Bungartz et al., 2012; Goeman et al., 2012; Gurtner et al., 2003; Zhu et al., 2005). NF-Y has been implicated in human diseases including myodystrophy, neurodegenerative diseases, cancer, and cardiovascular diseases (Ly et al., 2013; Tohnai et al., 2014; Yamanaka et al., 2014). From *in vitro* studies, NF-Y binds to the promoters of genes controlling cholesterol and fatty acid synthesis as well as *adiponectin* although its function in adipogenesis and tissue specific gene regulation *in vivo* has not been evaluated (Park et al., 2004; Reed et al., 2008).

In Chapter 3, we showed that a 32bp sequence at -16.5kb of the *leptin* gene is essential for *in vivo* expression of leptin in luciferase reporter mice. A computational analysis identified a CCAAT-box sequence this 32 bp segment and NF-Y was identified as the binding protein using a candidate approach in super-shift assay. ChIP using an anti-NFY antibody confirmed that it binds to this site *in vivo*. A knockdown of NF-YA *in vitro* and a fat specific ablation of NF-YA *in vivo* both decreased adipogenesis and *leptin* gene expression, but the decrease in *leptin* expression appeared, in part, to be a result of a general effect on adipocyte development rather than an exclusive effect on the *leptin* gene. Thus animals with a knockout of NF-YA develop a moderately severe lipodystrophy with low leptin levels and a metabolic phenotype that is remediable with leptin therapy. Overall, these data identify a new etiology for lipodystrophy and further suggest that there could be a large number of other causes for this disorder. The data also provide a framework for future studies aimed at identifying the gene regulatory mechanisms that control *leptin* gene expression.

4.2 Results and Discussion

Preadipocyte culture model 3T3-L1 has been widely used in studies of adipogenesis. Since 3T3-L1 cells can be induced to differentiate into lipid-filled adipocytes *in vitro* in the presence of insulin, dexamethasone and IBMX (FIGURE 4.1A), this model allows precise control over the process of adipocyte differentiation (Zebisch et al., 2012). In wild-type 3T3-L1 cells, the mRNA levels of *NF-YA* were detected early in undifferentiated 3T3-L1 cells and decreased approximately 10-fold after induction of adipocyte differentiation by hormone treatment starting from day3 (FIGURE 4.1B). This pattern is similar to other preadipocyte genes such as *KLF4* and *Pref-1* (FIGURE 4.1C). In contrary, adipogenic transcription factors PPARg and C/EBPa as well as mature adipocyte markers show increase expression following differentiation (FIGURE 4.1D and E).

To assay a role for NF-Y in adipogenesis, we next introduced RNAi constructs, labeled 439 and 441, targeting *NF-YA* into 3T3L1 cells using lentivirus. We first confirmed that NF-YA down-regulation is an adipogenesis specific effect, because un-differentiated cells do not show lowered expression (FIGURE 4.2A). Construct 439 and construct 441 achieved 34% and 62% knockdown of *NF-YA* mRNA levels respectively (FIGURE 4.2B). Despite the incomplete knockdown, both constructs abrogated adipogenesis, with a markedly decreased number of oil-red stained cells after differentiation of cells (stained on day10, 7 days post induction) compared to wild type cells or cells expressing a non-hairpin control (SCRAM) construct.

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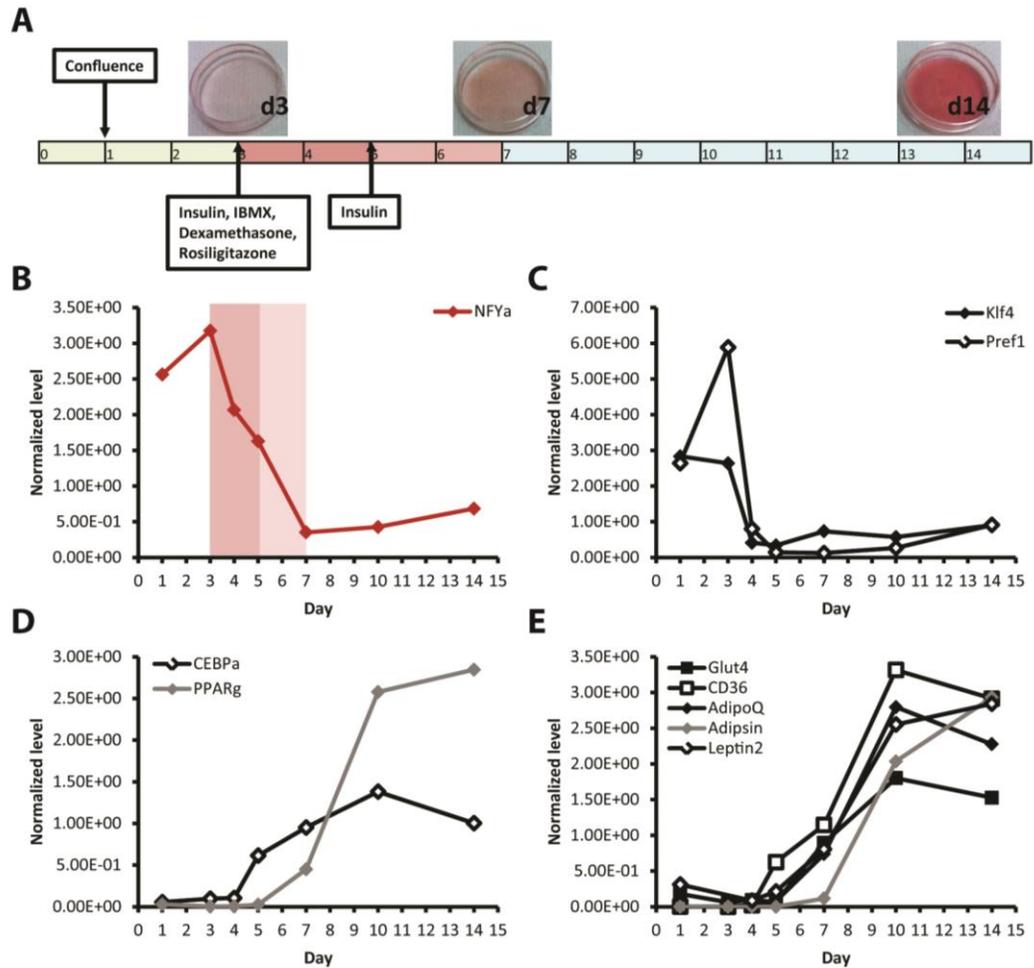
FIGURE 4.1 Differentiation of 3T3-L1 cells. (A). Schematic summary of differentiation protocol of 3T3-L1 cells. The red bars indicates presence of hormonal induction. Top panel depicts oil red staining of cells in three different time points. (B). NF-Ya expression lowers during differentiation and remains low in mature adipocytes. (C). Two other preadipocyte factors Klf4 and Pref1 show similar expression profile as NF-Ya. (D) and (E). Adipogenic factors and mature adipocyte markers are induced by initiation of terminal differentiation and remain expressed in mature adipocytes.

These data show that adipogenesis is quite sensitive to even a modestly

reduced expression level of NF-YA (FIGURE 4.2C top panel). Cells that were

not induced to differentiate showed comparable viability versus SCRAM cells,

showing that the defect did not affect pre-adipocyte viability and rather that NF-YA is essential for adipogenesis (FIGURE 4.2C bottom panel). Consistent with an effect of an *NF-YA* knockdown on adipogenesis, on day14, mRNA levels of adipocyte markers *C/EBPa*, *FABP4*, *LPL*, *leptin*, and *adipoQ* were reduced, further confirming that there is a defect in adipogenesis in the *NF-YA* shRNA targeted cells (FIGURE 4.2D).

Our results showed that *NF-Y* expression reduces with differentiation and that knockdown of *NF-Y* reduces adipocyte differentiation, both of which suggest that it plays a role either to prime pre-adipocytes prior to differentiation or functions early in the process before the point of adipocyte development when it is no longer expressed. Further studies will be needed to distinguish these possible mechanisms. Consistent with the former possibility, NF-Y has been shown to play a role in the proliferation and maintenance of stem cell precursors, although we failed to note any difference in the proliferation or viability of preadipocytes after an *NF-Y* knockdown (Benatti et al., 2008; Bhattacharya et al., 2003; Bungartz et al., 2012). Moreover, since many adipocyte markers including leptin are only expressed in mature adipocytes that are terminally differentiated, the altered expression of a differentiated marker reflects defects during differentiation process to some extent. Consistently, expressions of many mature adipocyte genes are dependent on adipogenic factors PPARg and C/EBPa (Rosen et al., 2000). While C/EBPa was shown to transcriptional activation of *leptin* via
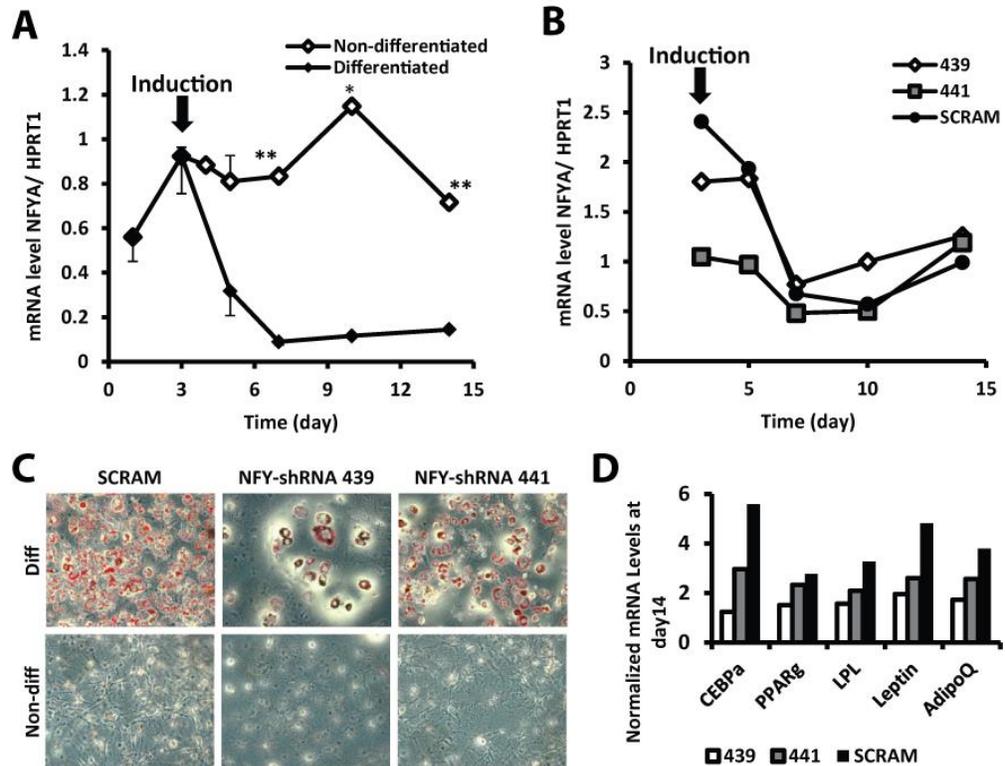
FIGURE 4.2 NF-YA Expression and Function of NF-YA in 3T3-L1 adipocytes *in vitro*. (A). *NF-YA* mRNA is expressed at a high level in undifferentiated 3T3-L1 and its level is decreased during day3 to day7 after differentiation using a standard cocktail of insulin, dexamethasone and IBM treatment after which *NF-YA* remains expressed at a low level in differentiated 3T3 cells (black diamonds), as compared to non-differentiated controls (white diamonds). (B). Two lentiviral mediated shRNA targeting *NF-YA*, denoted 439 and 441, were introduced into undifferentiated 3T3-L1. After puromycin selection, normalized NF-YA mRNA levels were reduced by 34% and 62% in cells expressing 439 (white diamonds) and 441 (grey squares) respectively compared to SCRAM (black circles). After differentiation of 3T3-L1 cells, *NF-YA* levels become comparable in knock down and SCRAM cells. (C) Despite an incomplete knockdown, both lines show reduced adipogenesis after 7 days of differentiation with staining with oil-red for lipid droplets on day10. The bottom panel shows virus-treated, non-differentiated controls with comparable cell numbers in knocked down and non-hairpin control cells. SCRAM indicates scramble, non-hairpin control construct. (D). The mRNA levels of *CEBPa*, *PPARg*, *LPL*, *leptin*, and *adipoQ* in the shRNA knockdown 3T3-L1 were measured and normalized to housekeeping HPRT1). The adipogenic transcription factors *CEBPa* and *PPARg* were lowered by 78%/47% and 45%/16% in the knockdown cells, *LPL* was lowered by 52%/36%, and adipokine genes *leptin* and *adipoq* were lowered by 59%/46% and 55%/32% respectively.

proximal promoter (He et al., 1995; Hwang et al., 1997; Miller et al., 1996), it

the functions of other distal elements that interact with C/EBP factors

remain unknown. Nonetheless, recent genome-wide study uncovered

cooperative binding of PPARg and C/EBPa at many adipocyte genes,

including non-coding regions of leptin, suggesting global transcriptional

regulation in differentiated adipocytes (Lefterova et al., 2008).

Since a total body knockout of *NF-YA* is lethal (Bhattacharya et al.,

2003), we generated an adipocyte-specific NF-Y knockout by crossing the

adipocyte-specific *Adiponectin-CRE* mouse to conditional knockout *NFYA-fl/fl*

mouse to study the function of NF-Y in adipocytes in animals (FIGURE 4.3A).

(In the following sections, we refer to the *Adiponectin-CRE*; *NFYA-fl/fl* mice as

NFY-KO and wildtype control NFYA-fl/fl as WT control). The knockout was

confirmed by genotyping as well as by performing western blots to assay NF-

YA protein expression (FIGURE 4.3A). When fed a chow diet, the NFY-KO and

WT had similar body weights beginning at weaning (3 weeks old) up until 28

weeks old (FIGURE 4.3B). However, on chow diet the NFY-KO mice showed a

significantly decreased adiposity as assessed using DEXA (p=0.002 at at 8

weeks). Furthermore, while WT mice showed an age-dependent increase in

body adiposity between 8 weeks to 28 weeks old, NFY-KO mice actually

showed a progressive loss of body fat, with an adiposity of 9.7% at 28 weeks

of age which is comparable to the fat content of lipodystrophic AZIP mice

(FIGURE 4.3C). The result was confirmed by measuring the weight of

individual epididymal, inguinal, and brown adipose tissues at different ages
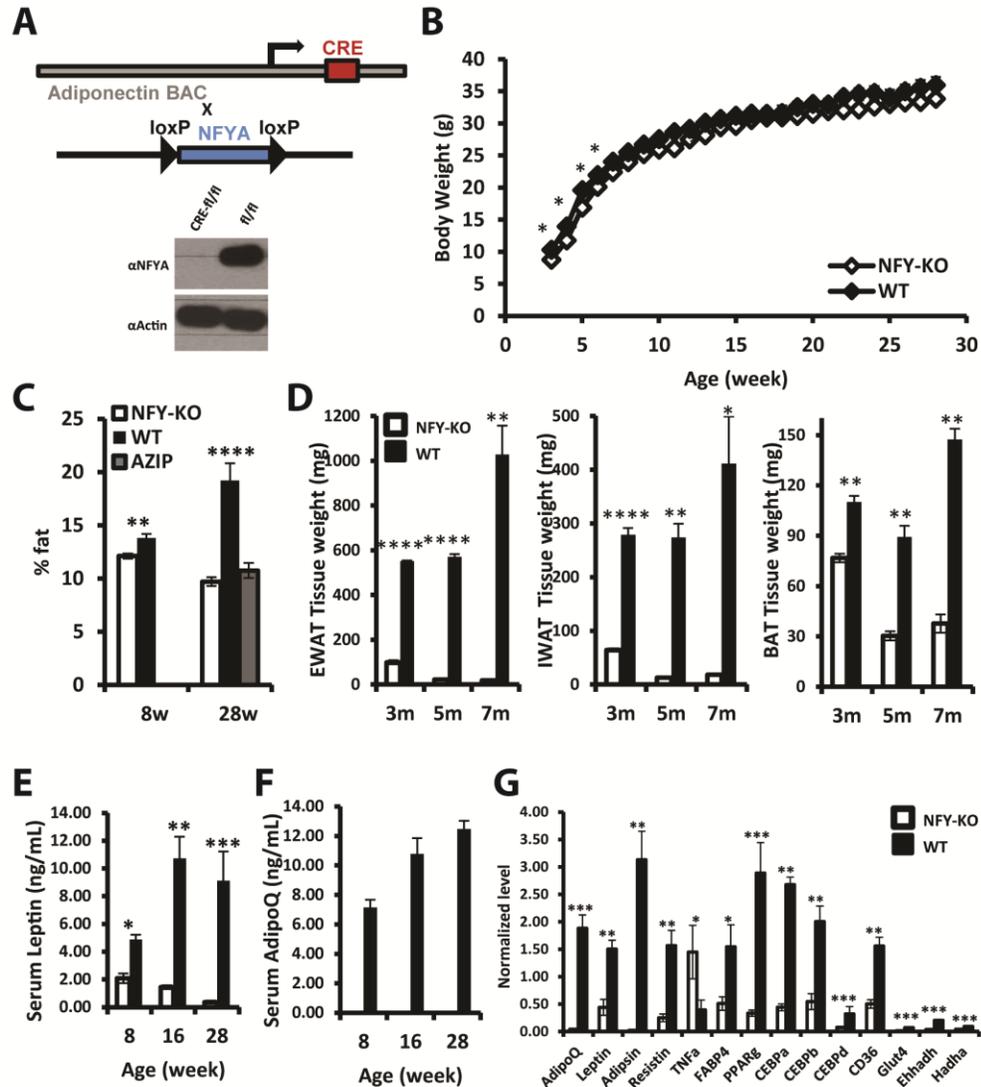
FIGURE 4.3 Generation of an Adipose Tissue Specific NF-Y Knockout.
NF-YA adipocyte specific knockout mice, NFY-KO, were generated by
crossing *Adiponectin-CRE* to *NFYA-flox/flox* mice. CRE-negative NFYA-
flox/flox animals are used as WT control (A). Depletion of NF-YA protein
is confirmed by western blot from adipose tissue. The NFY-KO mice
(white diamonds) have lower body weight compared to the WT control
(black diamonds) from weaning (3 weeks) to 6 weeks, but the difference
becomes insignificant afterwards, n=9 (B). NFY-KO mice (white bars)
show significantly reduced body adiposity (total fat mass divided by total
body weight) by DEXA scan with an age dependent worsening with a % fat
level consistent with lipodytrophy, n=9 (C). At 28 weeks, NFY-KO mice
show 9.7% body adiposity, comparable to the previously published AZIP
strain which shows generalized-lipodystrophic mouse model, at 10.7%

FIGURE 4.3 (cont.) (grey bar, 7 age matched animals). (D) Gross tissue weight of EWAT, IWAT, and BAT of NFY-KO and WT animals of various ages are shown, n=9. Consistent with DEXA result, NFY-KO show significantly reduced amount of EWAT and IWAT, while other visceral organs remain comparable sizes (Table 4.1); BAT is less affected by NF-Y knockout. (E). NFY-KO show lowered levels of serum Leptin with age progressing lowering and (F) non-detectable (ND) level of serum adiponectin at all age groups measured, n=9. (G). Gene expression profiling in epididymal adipose tissues of 3-months old mice show significantly decreased levels of adipocyte marker genes (adipokines *adipoQ*, *leptin*, *adipsin*, *resistin*; adipogenic transcription factor *FABP4*, *PPARg*, CEBPs; adipocyte surface marker *CD36*, and lipid metabolism genes *glut4*, *Ehhadh*, *Hadha*, Ctsk) when expression level is normalized to *HPRT1*, n=2 for WT and n=4 for NFY-KO (G). Inflammatory factor *TNFa* shows increased expression.

level of NF-YA protein, showed no significant abnormality on body weight, body adiposity, plasma adipokines, insulin and glucose levels (data not shown), therefore it is not included in further investigation. It should be noted that the shRNA knockdown in 3T3-L1 resulted in less than 50% reduction in gene expression yet a rather dramatic impairment in adipogenesis. The phenotypic discrepancy between 3T3-L1 knockdown and NFY heterozygous knockout was likely due to the timing of gene ablation. In NFY-KO mice, NF-Y gene ablation was introduced following expression of adiponectin in mature adipocytes, while in 3T3-L1, the gene knockdown was performed prior to terminal differentiation. This result is consistent with the notion that NF-Y's critical role in pre-adipocytes. In another word, using a CRE-line expressing in preadipocyte population (which is not yet available) will provide a comparable in vivo model to the 3T3-L1 experiment.

Consistent with the possibility that a fat specific knockout of NF-YA can cause lipodystrophy, NFY-KO mice showed a 2-fold decrease of plasma leptin at 8 weeks and a 25-fold decrease in leptin level at 28 weeks with undetectable levels of serum adiponectin at all ages tested (FIGURE 4.3E and F). Epididymal adipose tissues were collected from 3 months old mice and mRNA levels of various adipocyte specific genes were measured using qPCR (Figure 4.3G). Consistent with ELISA results, the mRNA levels of *adiponectin* (AdipoQ) and *leptin*, as well as *adipsi*n and *resistin* RNAs were significantly lowered in NFY-KO mice as were the RNAs for other adipose marker RNAs including *FABP4*, *PPARg*, *C/EBPs*, *CD36*, *Enoyl-CoA dehydrogenase* (*Ehhadh*), *hydroxyl-CoA dehydrogenase* (*Hadha*) and *Glut4*. These data showing a
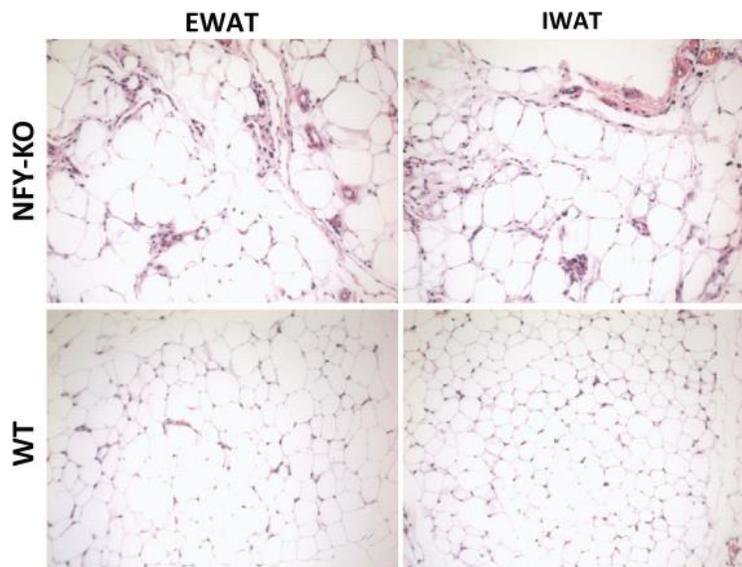
FIGURE 4.4 Histology of adipose tissue in NFY-KO animals. Prominent stromal structures in tissues from knockout animal is characteristic of lipodystrophy. Larger yet fewer adipocytes are present in knockout tissue, suggested possible compensatory growth of remaining cells. However, the mechanism of such growth is unknown. All slides were prepared from 5 months old male mice.

general decrease in the expression of a battery of adipocyte specific genes are consistent with the adipogenesis defect observed in 3T3 cells after an NF-Y knockdown. Histological study of the adipose tissue showed prominent stroma vascular structure characteristics of adipose tissue dysfunction (FIGURE 4.4). The enlarged adipocytes in NF-YA are likely mature cells that were not affected by the knockout and resulted in compensatory growth for loss of total cell number. As such, progressive lipodystrophic model like this one may be useful to study the dynamics between hypertrophy and hyperplasia processes.

NF-Y, also known as CBP or CCAAT-binding protein, binds to the most prevalent enhancer sequences in the mammalian genome, CCAAT, and has been shown to be essential for a range of cellular functions including cell cycle, proliferation, and as mentioned stem cell maintenance (Bhattacharya et al., 2003; Bungartz et al., 2012; Dolfini et al., 2012a; Dolfini et al., 2012b; Mantovani, 1999; Zhu et al., 2005). A whole-body knockout a NF-Y is embryonic lethal due to a failure of cell proliferation during in utero development, and that other pluripotent stem cells such as mesenchymal stem cells and hematopoietic stem cells requires NF-Y to maintain their population (Bhattacharya et al., 2003; Bungartz et al., 2012; Dolfini et al., 2012a; Tohnai et al., 2014). In adipocytes, prior studies have only showed that NF-Y can regulate adipocyte specific genes such as *adiponectin* and fatty acid synthetic pathway *in vitro* (Park et al., 2004; Reed et al., 2008), suggesting a role in mature adipocytes. Our results suggest that NF-Y also

plays a role in pre-adipocyte (and/or an adipocyte stem cell) maintenance and/or commitment to adipogeneis, leading to the development of an age-progressive fat loss by and lowering of serum leptin in NFY-KO mice.

The NFY-KO animals also showed an age dependent hyperglycemia and hyperinsulinmia. At 28 weeks old, NFY-KO mice showed 1.5-fold higher blood glucose (p=0.001) and 10-fold higher insulin levels (p=0.01) compared to WT (FIGURE 4.5A). Glucose tolerance tests (GTT) were next performed showing that the NFY-KO had higher blood glucose levels at all of the time points during the 120 minute assay (Figure 4.5B). The NFY-KO mice also showed reduced glucose clearance with an area under curve (AUC) of blood glucose 1.3-fold greater than WT control group (p=0.007; FIGURE 4.5C). During GTT, the NFY-KO mice showed an ∼ 4-fold higher insulin level at 30 minutes after glucose injection (p=0.03), suggesting insulin resistance contributed to the reduced glucose clearance (FIGURE 4.5D). Finally, the NFY-KO mice had hepatic steatosis with a 2-fold higher of triglyceride content in liver (p=0.009, FIGURE 4.5E).

Leptin replacement has been shown to markedly improve the metabolic abnormalities of lipodystrophy in animals and humans and a leptin response can be considered a *sine que non* for this condition. To test the effect of leptin, we treated NFY-KO animals with 350ng/hour of recombinant leptin via osmotic pumps for 14 days (red graph in FIGURE 4.6). Control NF-Y KO mice received PBS (black graph in FIGURE 4.6). Leptin treatment of 20

week old NFY-KO mice normalized blood glucose at day 10 and day 14

(p=0.006 and p=0.05 respectively) compared to control mice (FIGURE 4.6A).

Leptin treatment lowered insulin level to wild-type levels (wide-type data

not shown) (p<0.01 after day 4, FIGURE 4.6B). Leptin treatment also

corrected the glucose tolerance during a GTT with a marked lowering of

blood glucose (p=0.006) and the AUC (P=0.04) (FIGURE 4.6C and D). After 14

days of treatment, the liver triglyceride content of NFY-KO was lowered by

50% (p=0.002; FIGURE 4.6E) and the total wet weight of liver was lowered

by 40% (p=0.001; FIGURE 4.6F). Cessation of leptin treatment led to a

worsening of glucose tolerance and heaptic steatosis (data not shown).

We next analyzed the effect of a high fat diet on these animals by

feeding NFY-KO and WT mice a 60% fat diet (HFD) starting at 4 weeks old.

On this diet NFY-KO mice failed to gain body weight with a statistically

different body weight compared to WT mice beginning at 7 weeks of age

(p<0.01 and $p<10^{-5}$ after 11 weeks, Figure 4.7A). Similarly, DEXA

measurements for body adiposity after 6 month of HFD showed that while

WT showed a marked increase of adiposity of 40%, NFY-KO had an adiposity

of 9% which is the same adiposity that is seen when these mice are on a

chow diet ($p<10^{-16}$; FIGURE 4.7B). Consistent with their low adiposity, serum

leptin remained low, between 2 to 10 ng/ml, in NFY-KO mice (FIGURE 4.7C).

The hyperglecemia and hyperinsulinemia were similar in HFD and CHOW fed

NFY-KO mice, suggesting that a lack of adipose tissue caused the metabolic

complications and that the diet per se did not contribute to the phenotype of

the knockout mice (FIGURE 4.7D).
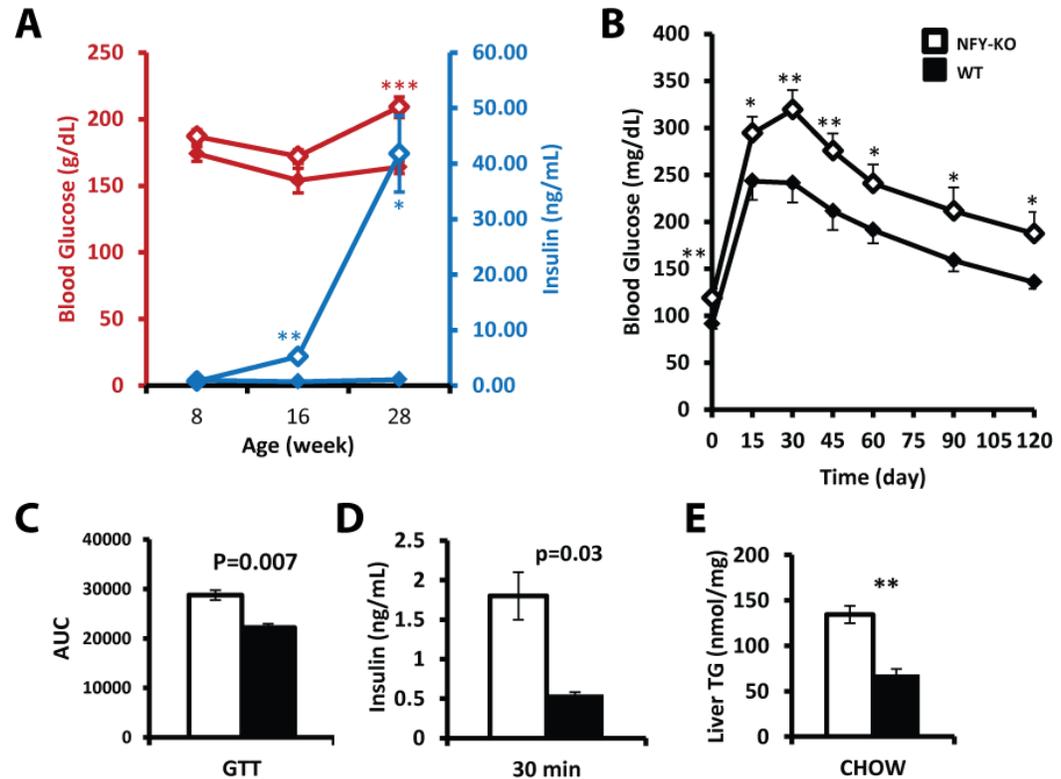
FIGURE 4.5 Mice with an Adipose Tissue Specific NF-Y Knockout Develop Lipodystrophy. (A). NFY-KO mice develop hyperglycemia (red graph) and hyperinsulinemia (blue graph) that progresses with age, n=9. At 5 month old, NFY-KO mice show significantly higher increase of blood glucose level. (B) The knockout caused an abnormal glucose tolerance test (GTT) compromised glucose clearance with (C) a significantly increased area under curve (AUC) after IP injection of 1mg glucose/g body weight given at time 0, n=16. (D). At 30min after GTT, NFY-KO animals show 3.5-fold higher insulin level compared to WT, n=7 (E). The knockout mice had hepatic steatosis with a 2-fold increased triglyceride levels in liver tissue of NFY-KO, n=9. NFY-KO is indicated by white and WT is indicated by black in each result.
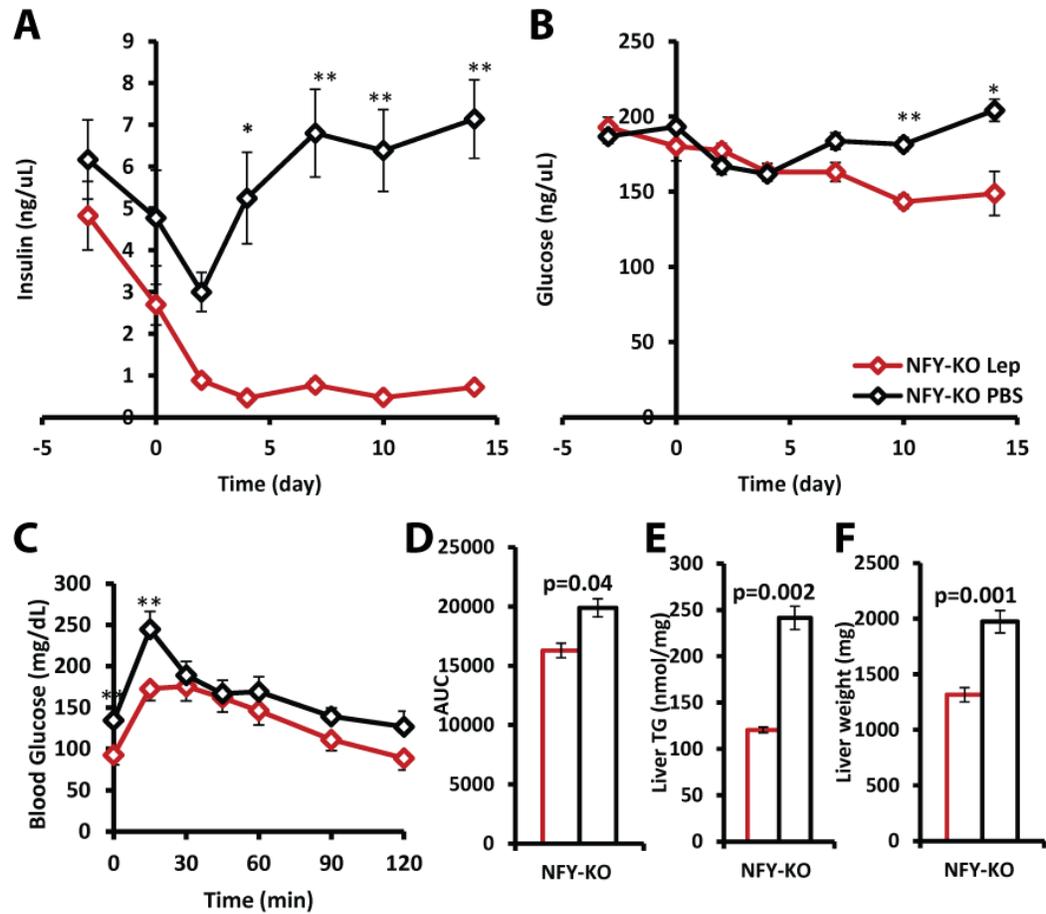
FIGURE 4.6 Leptin treatment ameliorates lipodystrophy phenotypes in 5-month-old NFY-KO mice. (A). 14 days of 350ng/hour of Leptin treatment via osmotic pump (red graph from day0 to day14 in figure; black graph indicates PBS treated NFY-KO animals) normalizes insulin level and (B) blood glucose in NFY-KO mice. The difference in insulin levels become significant after the initial response to surgery (after day 4) and the blood glucose difference becomes significant after day10 of leptin treatment. (C) .The GTT is normalized by Leptin at day14, with smaller AUC (D). Liver tissues harvested from animals sacrificed on day14 showed reduced liver triglyceride content (D left) as well as smaller overall liver weight (D right) after Leptin treatment. The numbers of animals shown are 10 for Leptin treatment and 7 for PBS control and age-matched 5 months old.

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To understand the mechanism of HFD-resistance in NFY-KO mice, we measured the energy expenditure of NF-Y KO compared to WT mice using calorimetry. Under chow diet, the NFY-KO animals showed decrease respiratory exchange ratio (RER), suggesting that these animals utilize primarily fat energy source as well as maintain a lower basal metabolism (FIGURE 4.8A). Consistently, the NFY-KO animals have lowered core body temperature (34.3°C compared to 34.8 °C in WT control animals, p=0.02; FIGURE 4.8B). However, these differences were not due decreased food intake (data not shown). These result suggested that defect in adipose tissue is the primary cause of low adiposity as oppose to increase energy expenditure or physical activity.

In addition to reducing *leptin* expression, a knockdown of NF-Y *in vitro* and a fat specific knockout *in vivo* also led to a general effect on adipocyte development. This raises the possibility that NF-Y might play a general role early in adipocyte development and functions later in development to regulate the *leptin* gene. Indeed, the data are consistent with the possibility that, together with other factors, NF-Y directly contributes to the regulation of *leptin* gene expression and that the identification of factors interacting with NF-Y could help elucidate the mechanisms controlling *leptin* expression. A recent study showed that NF-Y knockout in neural cells resulted in progressive neural degenerative disease via accumulation of dysfunctional ER membrane (Luo et al., 2008; Yamanaka et al., 2014). Recent studies reveal that ER stress occurs in adipocytes in obesity and diabetes

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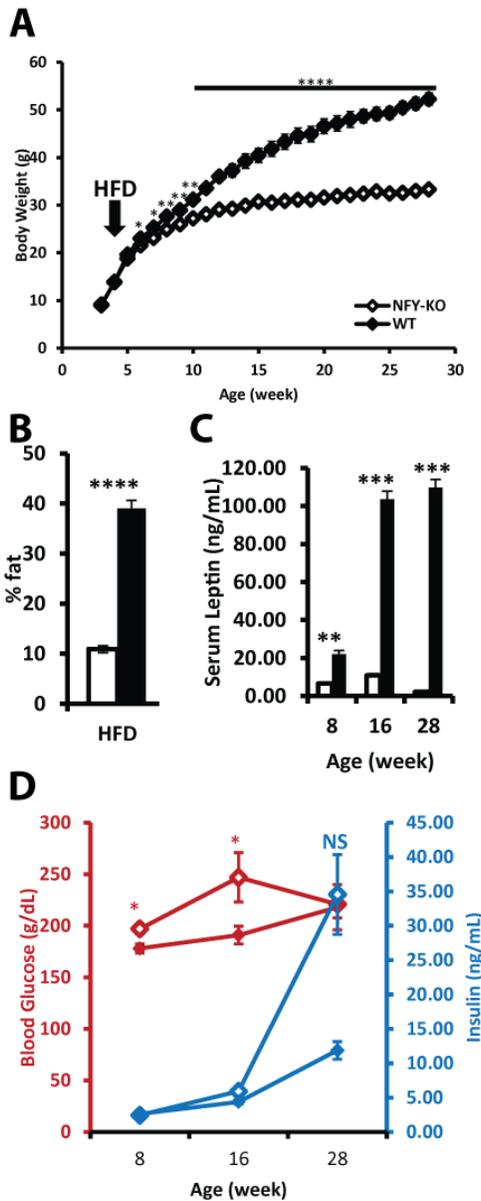
FIGURE 4.7 NFY-KO mice are resistant to HFD. (A) The average body weight of NFY-KO and WT animals treated with HFD (60% fat) starting from 4 weeks old to 28 weeks old is shown, n=9. NFY-KO mice remain a lower body weight compared to WT and the difference is significant after 6 weeks old. (B). After 6 months of HFD, the 28 weeks old NFY-KO mice show significantly lowered body adiposity compared to matched WT control mice, n=9. (C). NFY-KO mice did not show an increase in serum Leptin levels compared to WT animals on the high fat diet (D) Blood glucose and insulin levels of various age groups are shown, n=9. At 28 weeks old hyperinsulinemia and hyperglycemia are present in both NFY-KO and WT mice.

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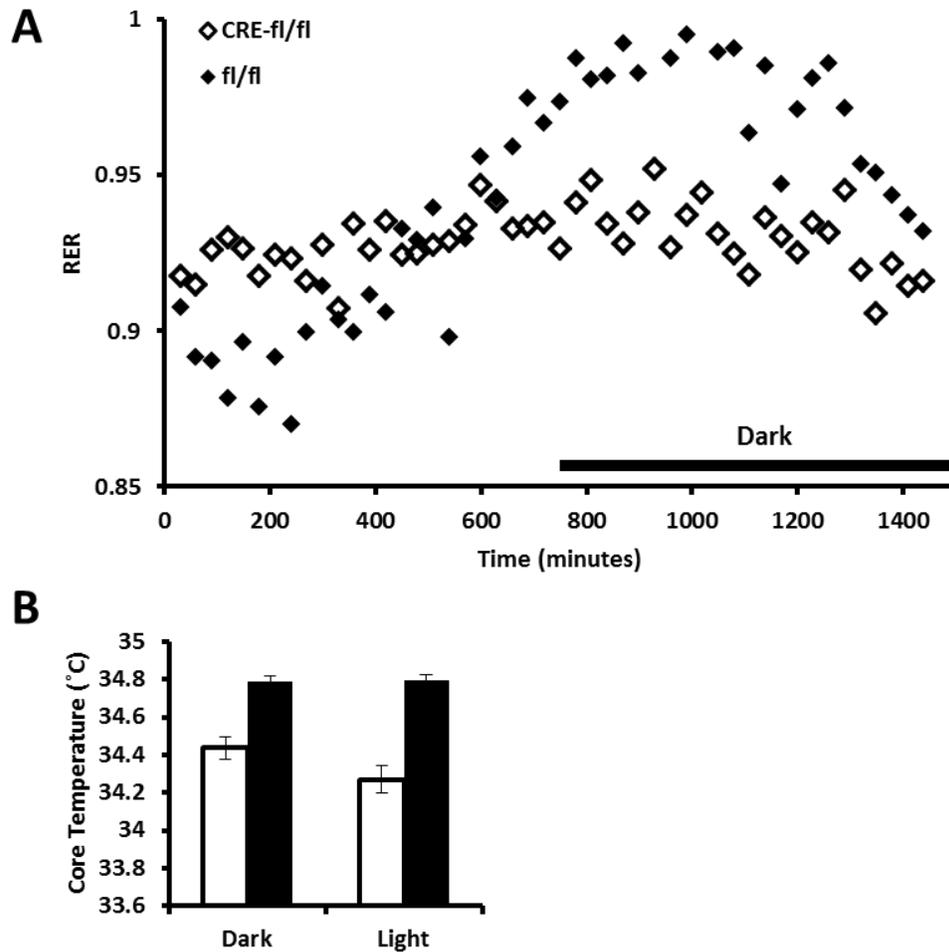
FIGURE 4.8 Calorimetric measurements of NFY-KO mice under chow diet. (A)Oxymax data of NFY-KO and WT animals under chow diet, n=4. NFY-KO animals show significantly lowered RER (respiratory exchange ratio) during dark cycle, indicating that these animals utilize fat energy. (B) During both dark and light cycles, the core temperature of NF-Y-KO mice are lowered for about 0.5 ℃, p=0.02 for both condition, n=8.

(Gregor and Hotamisligil, 2007; Ozcan et al., 2004); this would be a possible mechanism by which NF-Y regulates leptin expression under different metabolic states of adipose tissue. However, NF-Y's general effect on adipose cell development with lower levels of expression of adipocyte specific genes appears to mask a specific effect on the *leptin* gene.

One caveat of the *in vivo* studies is that the *adiponetin-CRE* line that

was used is not expressed until somewhat later in fat cell development

(Eguchi et al., 2011; Lee et al., 2013; Wang et al., 2010), and thus cannot be

used to assess the role of NF-Y at early developmental stages (Jones et al.,

2005). It is thus interesting that a knockout of a gene in adipocyte precursors

under the control of a promoter that is only expressed at later developmental

times (the *adiponectin* promoter) can have a dramatic effect on adipocyte

mass with an age- dependent worsening of lipodystrophy between 4 and 28

weeks. One possible explanation is that an NF-Y knockout also has an effect

on mature adipocytes that leads to reduced replenishment of adipocytes as

they turnover (Arner and Spalding, 2010a). This would explain the

worsening of the lipodystrophy with age compared to the normal trend of

mice to gain fat mass as they get older. It has been previously suggested

using a model of inducible lipodystrophy mouse models that total pool of

adipocytes is finite (Pajvani et al., 2005; Wojtanik et al., 2009). As such, if

there were a defect in adipogenesis and/or a faster turnover of the knockout

adipocytes in NFY-KO mice, the adipose tissues would be depleted over time

manifesting in a progressive lipodystrophy similar to that which we

observed. It should also be noted that when *adiponectin-CRE* mice are used to

generate *PPARg* and *Raptor* adipocyte specific knockout mice, the knockout

animals are lean, resistant to a high fat diet, but metabolically healthy and do

not show signs of lipodystrophy (Jones et al., 2005; Polak et al., 2008). The

phenotypic difference between these mouse models and ours suggests that

NFY could play a prominent role to specifically control *leptin* expression, in addition to its more general role in fat cell development. NFY could also play a functional role in preadipocytes, thus a knockout could exacerbate depletion of adipocytes by disruption of the generation and/or maintenance of a preadipocyte pool. This possibility is supported by our observations in 3T3-L1 preadipocytes, as well as other studies where NF-YA was shown to be crucial for cell proliferation and stem cell maintenance (Bhattacharya et al., 2003; Bungartz et al., 2012; Dolfini et al., 2012a).

Thus one possible mechanism for the lipodystrophy in NF-Y KO mice could be that there is a population of adipocyte precursors that expresses *adiponectin*, and that NF-Y ablation in this population compromises the ability of animals to replace adipocytes over time. Indeed, recent studies report expression of late adipogenic genes including *FABP4* and *adiponectin* in a population of preadipocytes in the stromal vascular fraction where adipocyte precursors reside (Berry and Rodeheffer, 2013b; Shan et al., 2013) . In addition, *adiponectin* expression can be detected during early development at E14 (Birsoy et al., 2011; Wang et al., 2013b). The possibility that lipodystrophy is a result of gene ablation in preadipocytes is also supported by a previous published lipodystrophy mouse model in FPLD (Dunnigan's familial partial lipodystrophy). The FPLD mouse was generated by expressing a dominant negative form of *LMNA*, the major component of nuclear lamina, using the *aP2* promoter (Wojtanik et al., 2009). The FPLD mice showed progressive fat loss and metabolic complication manifesting

lipodystrophy in human patient with the same genotype. Further, primary culture isolated from FPLD mice showed impaired adipogenesis *in vitro*. The FPLD mice and NFY-KO mice are phenotypically similar mouse models in that both are expressed later in development but also develop a more profound defect in adipogenesis than would be expected from an effect only on mature adipocytes.

The importance of the early stages of adipogenesis is also highlighted by data from a patient with congenital generalized lipodystrophy who had a *de novo*, homozygous point mutation in the promoter of *c-fos* (Knebel et al., 2013). In 3T3-L1 cells, *c-fos* expression peaks at early times during hormone induction, which then modulates expression of other downstream proadipogenic factors during differentiation (Distel et al., 1987; Rangwala and Lazar, 2000). Genome wide *in silico* analysis showed co-localization of FOS and NF-Y, but whether this interaction has biological relevance in adipose tissue will require further investigation (Fleming et al., 2013). The importance of early transcriptional events is also supported by our findings for NF-Y which is also expressed only early in adipogenesis and can cause lipodystrophy in homozygous knockout mice. It is also possible that other mutations in NF-Y can act in a dominant negative manner as it has been shown that a dominant negative form NF-Y suppresses *c-jun* activity, a transcription factor that is also important for early adipogenesis (Nabokina et al., 2013; Rangwala and Lazar, 2000; Tiwari et al., 2012).

Several possible mechanisms for the pathologic loss of adipose tissue and lipodystrophy have been identified in both human and mouse models though none have appeared to have a specific role on leptin production. For instance, lipodystrohpy can be caused by an autosomal recessive mutation in *AGPAT2*, an acyltransferase highly expressed in adipose tissues and important for triglyceride synthesis, which leads to an imbalance in fat storage and lipolysis (Luckman et al., 1999; Rosenblum et al., 1996; Rosenblum et al., 1998). A dominant mutation of *PPARg* can cause human lipodystrophy, as the mutant PPARg showed reduced transcriptional activity with a reduced rate of adipogenesis (Rosenbaum et al., 1996; Zhang et al., 2014). The fld mice (fatty liver dystrophy), a spontaneous form of lipodystrophy in mice, results from a point mutation in *lipin-1*(Peterfy et al., 2001). *Lipin-1* is a phosphatase that plays a role in triglyceride metabolism and transcriptional co-activation of PPARg during adipogenesis (Reue and Dwyer, 2009). Both dominant and recessive mutation of LMNA (the aforementioned FPLD mouse) causes lipodystrophy potentially due to its involvement in nuclear lamina that regulates nuclear trafficking and transcriptional activity (Schmidt et al., 2001; Wojtanik et al., 2009). Further studies will be needed to determine whether NF-YA interacts with any of these or other factors that play a role in adipocyte development and/or that can contribute to the development of lipodystrophy. It should be noted that the lipodystrophy can also be acquired resulting from what appears to be an immune mechanism or more recently in HIV patients on triple therapy that

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includes protease inhibitors (Schott et al., 2004). Lipodystrohy can be the result of defective adipogeneis and/or an increase rate of loss of of mature adipocytes which then secondarily lead to a reduced leptin level. This condition is distinct from leanness, in which adipose tissue mass is reduced in the absence of metabolic disorders. Lipodystrophy is caused by a relative deficiency of leptin. Thus while fat cell transplants from wild type to lipodystrophic mice can correct this condition, transplants of leptin deficient adipose tissue cannot (Tran and Kahn, 2010). The fat loss in the NF-Y KO mice while significant is not as severe as it is in other etiologic forms (such as AZIP model) raising the possibility that the NF-Y KO could have a disproportionate effect to decrease leptin production.

In summary of Chapter 3 and Chapter 4, In addition to establishing a role for NF-Y in adipocyte development in vivo and as a potential cause of lipodystrophy, these data also provide a framework for dissecting the regulation of the leptin gene. In unpublished studies we have identified other factors that interact with the *leptin* gene raising the possibility that *leptin* is regulated by a transcription complex of which NF-Y is a component, potentially through both sequence specific and non-specific mechanisms. The elucidation of the underlying transcriptional mechanisms by which NF-Y controls leptin gene expression could thus lead to the identification of a lipid sensing mechanism in fat cells that modulates adipocyte function and leptin production in response to changes in adipocyte lipid content.

TABLE 4.1 Weight of different organs of NFY-KO and WT control at various ages.

	Testicles		Kidneys		Liver		Spleen	
	KO	WT	KO	WT	KO	WT	KO	WT
3 mon	214±12	211±36	419±26	406±31	1408±94	1282±116	110±22	86±7
5 mon	239±10*	213±8	439±8	422±5	1972±100**	1441±30	121±17	116±12
7 mon	212±25	234±15	572±66*	422±24	1970±510	1573±106	208±63	94±3

	Pancreas		Lung		Heart	
	KO	WT	KO	WT	KO	WT
3 mon	250±41	193±8	193±8*	167±8	215±27	242±26
5 mon	398±92*	185±12	222±21	171±17	260±6**	186±7
7 mon	508±140	251±23	367±85*	176±9	251±19*	185±23

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CHAPTER 5: ES-CELL COMPLEMENTATION

5.1 Introduction

Based on the lipostatic viewpoint of energy homeostasis, adiposity

level serves as a gauge for body energy availability. By modulating leptin

production, adipose tissue signals the central nervous system to adjust food

intake and energy expenditure, which are physiological and behavioral

responses aiming to maintain adiposity homeostasis. As such, adipose tissue

size control is central to energy homeostasis in animals. The collection of

studies on adipose tissue development in humans and rodents suggests a

framework of coordinated hypertrophy (increase in cell size) and

hyperplasia (increase in cell number) in determining body adiposity. Under

healthy conditions, total adipocyte number in an animal remains close to

constant after the adolescence period (Greenwood et al., 1979; Greenwood

and Hirsch, 1974; Knittle and Hirsch, 1968) and the average size of

adipocytes also stabilizes in early adulthood (Hemmeryckx et al., 2010; Stiles

et al., 1975). This leads to the hypothesis that the plateau in adipocyte

number in adult animals is determined and controlled by their

developmental program. Restoration of adipose tissue following lipectomy

(surgical removal of adipose tissue) in rodents and humans further suggests

that total adiposity is regulated (Espejel et al., 2010; Hernandez et al., 2011;

Huang-Doran et al., 2010; Reyne et al., 1983). Experiments that studied

adipose tissue cellularity in juvenile rats demonstrated compensatory

growth to generate the same total number of adipocytes as control animals,

which was not observed when the same manipulation was performed on adult rats (Faust et al., 1977a, b). Nutritional manipulation by altering litter size during early development leads to a decrease in adipocyte numbers (Knittle and Hirsch, 1968), yet starvation and refeeding of non-obese adults do not affect the total adipocyte number (Hirsch and Han, 1969; Yang et al., 1990). Additionally, it was recently shown that mature adipocytes turn over at a constant, albeit slow, rate in both adult humans and mice (Spalding et al., 2008; Wang et al., 2013b). In sum, these results suggest developmental programming in determining adipocyte cell number, which is maintained through a fine balance of adipogenesis and adipocyte death. The mechanism involved in regulation of adipocyte cell number and coordination of adipocyte hypertrophy together in determining total adiposity is largely unknown.

The plasticity of adipose tissue is most prominent in the case of obesity. Several studies have investigated the relative contribution of hypertrophy and hyperplasia to achieve massive adipose tissue accumulation in obesity, with varying results. The presented data consistently showed significant contribution of hyperplasia in early-onset obesity, prior to the developmental establishment of adipocyte number (Lemonnier, 1972; Salans et al., 1973), however, it remains in debate whether hyperplasia also contributes to late-onset obesity. Nonetheless, given the upper limit on adipocyte size (Leonhardt et al., 1972), which would limit adipose tissue expansion by hypertrophy alone, it is assumed that hyperplasia must

accompany hypertrophy at least in cases of extreme obesity. Mathematic

modeling on total fat mass versus mean adipocyte volume supports this

conclusion (Jo et al., 2009; Spalding et al., 2008). Additionally, the extent of

hyperplasia contribution to adipose gain appears to be dependent on many

factors including sex, genetic background, genetic versus dietary causes of

obesity, as well as timing of the assay and the specific adipose depot of

question. A recent study using *AdipoChaser* mice demonstrated that the

appearance of new adipocytes in visceral adipose tissue under HFD much

precedes that in the subcutaneous compartment (Lee et al., 2012; Wang et al.,

2013b), while another study showed that HFD induced a higher adipogenic

capacity in subcutaneous rather than visceral adipose tissue (Joe et al., 2009).

Expansion of adipose tissue in response to HFD is critical to the metabolic

health of animals. Under positive energy balance, adipose tissue not only

stores energy for future use but also ensures that the storage is in a safe form.

However, since the capacity of adipose stores is finite, sustained energy

balance results in failure of adipose tissue to accommodate surplus energy as

fat and results in lipotoxicity in other peripheral tissues. Such an

"expandability" model (Virtue and Vidal-Puig, 2008) is supported by studies

of human and mouse models on the opposite ends of the obese spectrum.

Both lipodystrophy (with a very limited adipose capacity) and morbid

obesity (with a very large capacity yet deficit compared with chronic energy

surplus) represent conditions of stressed and unhealthy adipose tissue, thus

various metabolic conditions ensue. Thus, it is possible to ameliorate insulin

resistance in the face of obesity by increased capacity of adipose tissue, which has been demonstrated by crossing adiponectin-overexpression mice to *ob/ob* (Kim et al., 2007).

The mechanism by which obesity affects adipogenesis *in vivo* is also not clear, although the observation of similar turnover rates of adipocytes in obese and non-obese adults suggested that the state of obesity alters the adipocyte number set point (Spalding et al., 2008). Several HFD feeding studies have demonstrated that the appearance of new adipocytes coincides with existing adipocytes reaching their maximal size (Faust et al., 1978; Joe et al., 2009; Wang et al., 2013b). Although it has been shown that paracrine activity of mature adipocytes can affect adipogenesis (Janke et al., 2002; Marques et al., 1998), the details of these processes are not well understood. Adipose tissue, as a whole, is an essential regulatory component in energy homeostasis acting as both an energy reservoir and an endocrine organ. Moreover, it appears that adipocyte cellularity is predictive of whole-body metabolic health in obesity, in which hypertrophy is an independent risk factor for insulin resistance and T2DM regardless of BMI (Arner et al., 2010; Lonn et al., 2010; Weyer et al., 2000). Altogether, studying the mechanisms controlling adipocyte hypertrophy and hyperplasia is instrumental to our approach in viewing adipose tissue as a metabolism organ as well as understanding pathogenesis of obesity and metabolic diseases.

This chapter describes a series of experiments employing a novel blastocyst complementation assay to elucidate the molecular mechanisms that control adipose tissue deposition in mice *in vivo*. Specifically, wild-type embryonic stem cells (ES cells) are injected into the blastocysts of *AZIP* lipodystrophic mice (FIGURE 5.1). When pluripotent wild-type ES cells are injected into *AZIP* blastocysts, the resulting chimeras (AZIP-chimera) are expected to have restored adipose tissue and rescued AZIP phenotypes. As the *AZIP* blastocyst is defective in adipogenesis, such a developmental defect is complemented by the progeny of pluripotent ES-cells. By necessity, the adipocytes in *AZIP*-chimera are exclusively derived from injected ES cells. Since the ES cells are incorporated into the embryo and will contribute to all lineages including adipocyte precursors, this method allows us to observe the earliest developmental processes of adipogenesis *in vivo*. To date, there is no other tool capable of specific manipulation of early adipogenesis *in vivo*, since the nature of these stages is largely unknown.

Similar blastocyst complementation assays have provided unique tools to test the ability of a mutant to form the cell type or organ in question. These are effective *in vivo* systems not only to study the developmental processes but also to study the effect of a genetic defect on adult tissues. Some examples include studies on lymphocytes (Chen et al., 1993), ocular lens (Liegeois et al., 1996), liver (Espejel et al., 2010), pancreas (Jonsson et al., 1994), muscle (Cote et al., 1999), and CNS (Low et al., 2009). Analysis of cell lineage in chimeric tissues allows the distinction of cell-autonomous and
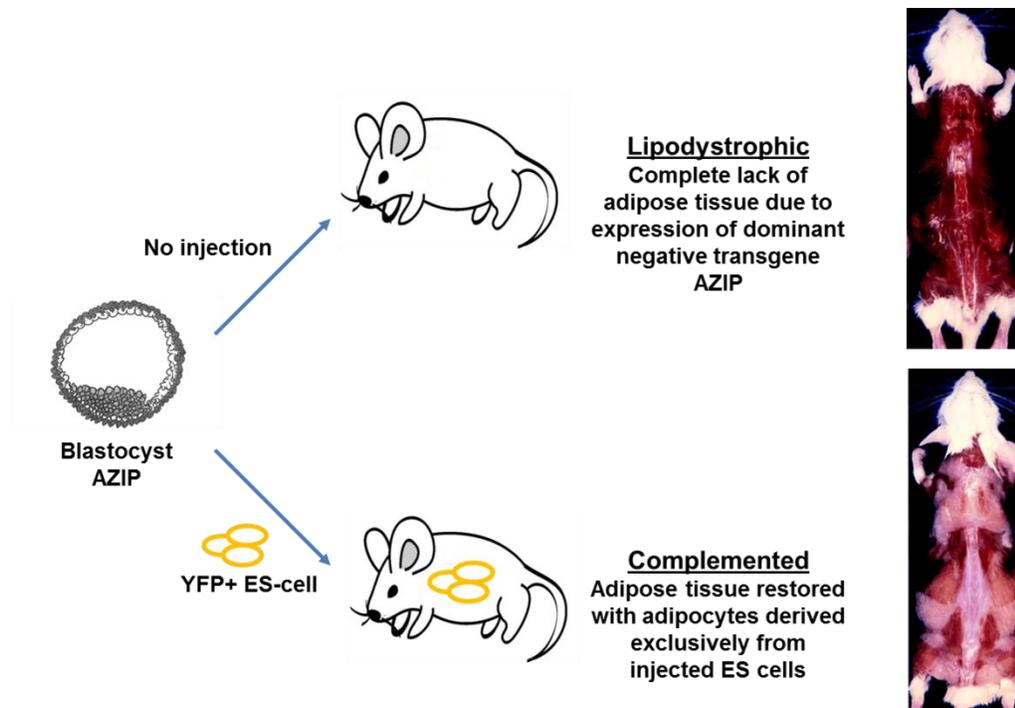
FIGURE 5.1 Generation of AZIP-chimera by ES-cell complementation into *AZIP* blastocysts. AZIP transgenic animals possess lipodystrophic phenotype with complete lack of white adipose tissue (see right side photo). Multipotent wild-type ES-cells are injected into *AZIP* blastocysts, which will complement the lipodystrophic phenotype and restore adipose tissue exclusively derived from ES-cells. Photograph of animals adapted from *Life without white fat: a transgenic mouse* (Moitra et al., 1998).

non-cell-autonomous effects of the mutant. A prominent example is the study

on developmental determinant of pancreas and liver sizes (Stanger et al.,

2007). In such case, wild-type ES cells were injected into blastocysts of

apancreatic *Pdx1*-deficient mice. The resulting chimeras are restored with a

pancreas. However, the size of the restored pancreas is limited by the

number of injected progenitor cells. In contrary, liver size is not limited by

the number of progenitor cells and other intrinsic factors dictate its final size

(Stanger et al., 2007). In a preliminary study, we determined the relationship

between the number of progenitor cells and whole body adipose content and showed that adult adiposity under normal conditions is independent of progenitor number. This is consistent with various prior studies using individual animals that adiposity is maintained by some intrinsic mechanisms during external perturbations. Notably, HFD can be viewed as an external challenge under which adipose tissue responds with hypertrophic and hyperplastic expansion. Using AZIP-chimeric mice with varying chimerism, this part of the thesis discusses how predisposition of limited progenitor affects adipose tissue expansion during HFD exposure.

Previous examples of a loss-of-function study of *PPARg* in adipogenesis utilized a similar approach. Homozygous knockouts of *PPARg* do not survive past embryonic day 10 due to a defect in placental development (Barak et al., 1999; Kubota et al., 1999). To circumvent this problem, one approach was to create an aggregation of chimeras derived from wild-type ES cells and homozygous *PPARg* knockout ES cells (Rosen et al., 1999). The result demonstrated that *PPARg* is required for adipogenesis in vivo by showing exclusion of knockout cells from adipose tissue, but not several other tissues. The alternative approach was to create chimeric embryos using wild-type tetraploid cells, which allow rescue of the placental defect, and *PPARg* knockout ES cells, which are the sole contributor to the embryo proper (Barak et al., 1999). Unfortunately, this resulted in only one live birth with a brown adipose tissue defect and died shortly after birth. In the second part of our study, we demonstrate the utilization of our ES-

complementation model in the study of adipogenesis *in vivo* by first

performing the proof-of-principle experiment with *PPARg* knockout cells.

5.2 Results and Discussion

AZIP (FVB) chimeras were generated by injecting $B6\text{-}Tyr^c\text{-}YFP$ mouse ES-cells into 3.5-day blastocysts from breeding of wild-type FVB females to AZIP(FVB) transgenic males. Since both the blastocyst and the ES-cells (both harboring homozygous Tyr^c alleles) have white coat color, AZIP(FVB) chimeras are completely albino. Within the AZIP positive group, 11 males carried YFP genotype, which indicated these are AZIP(FVB) chimeric mice with composed of both *AZIP*-positive cells and *YFP*-positive cells. Alternatively, the B6-ES cell derived cells can be distinguished from FVB-blastocyst derived cells at agouti locus, where FVB strain carries the alleles for *agouti A/A* and B6 strain carries *nonagouti a/a*. However, we did not employ this genotyping strategy, nor did we compare measurements for chimerism using different loci. In practice, degree of chimerism of these AZIP-chimeric mice was determined by the percent of *YFP*-genotype cells comparing to a genomic locus common to both strains, *fabp4*, in DNA sample extracted from whole blood samples. This approach was rationed that, since adipose tissue are always 100% ES-cell contributed (see below) and that different developmental lineages may demonstrate variability in ES-contribution (Kusakabe et al., 1988), a tissue type that is closely related to adipose tissue should be used as a surrogate for chimerism in preadipocyte population. In addition, this surrogate tissue is best collected via simple, non-invasive biopsy. Since adipocytes are generally considered mesenchymal linage along with muscles, endothelial cells, osteoblasts, and blood cells, we

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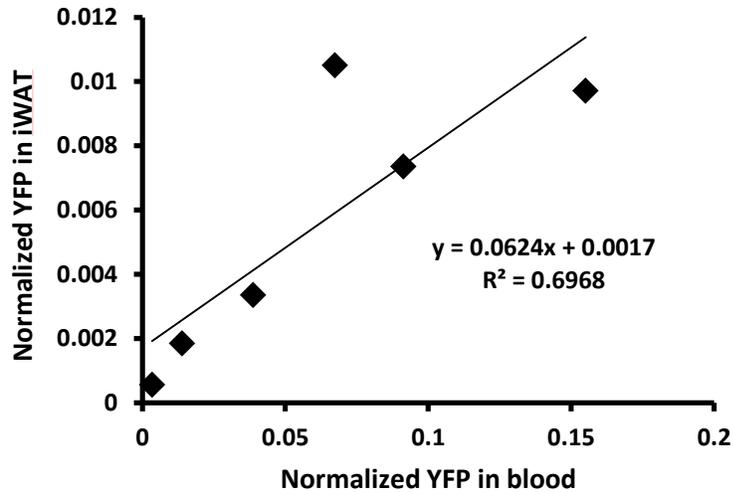
FIGURE 5.2 Correlation between degrees of chimerism measured in genomic DNA of subcutaneous adipose tissue versus whole blood of WT-chimera. Chimerism is defined as amount of *YFP* genes (carried by ES-cells) out of amount of *FABP4* gene (carried by both ES-cell and blastocyst).

hypothesized that DNA extracted from whole blood could represent the genotype composition of DNA from adipose-lineage tissues. This hypothesis was tested by comparing DNA samples of wild-type chimeric animals (i.e. chimeric mice produced by combing wild-type ES cell and wild-type blastocyst), which are chimeric for every tissue including adipose tissue and blood (FIGURE 5.2). Our hypothesis is confirmed, because the percentage of YFP-cells in adipose tissue in wild-type chimeras highly correlated (R^2=0.6968) to the percentage measured in whole blood samples. Using this method, we measured the degree of chimerism of 11 AZIP(FVB) chimeric male mice, and summarized the result in TABLE 5.1. Sectioning of the
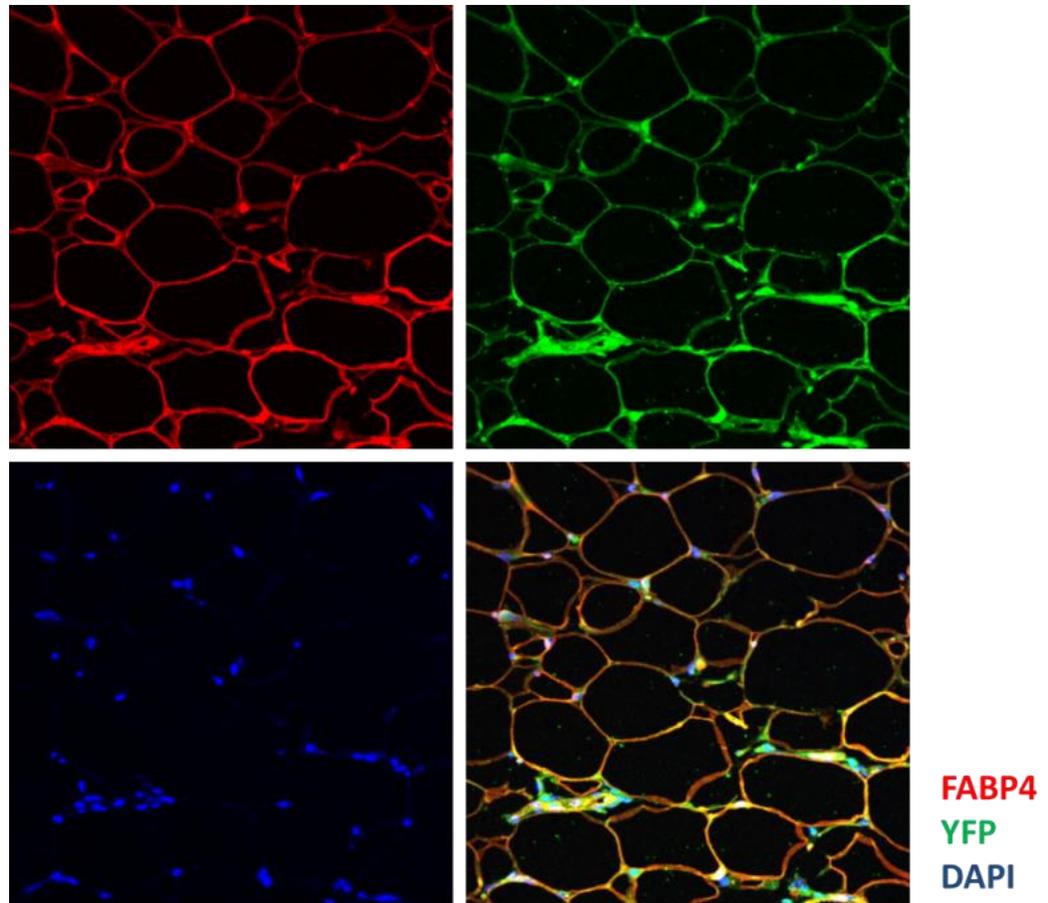
FIGURE 5.3 Immunohistology of adipose tissue from one AZIP-chimeric animal. The tissue is fixed in 4% PFA and paraffin embedded before sectioning. FABP4 staining indicates white adipocytes, which colocalized with YFP staining for ES cells.

adipose tissue collected from one of the chimeras showed 100% YFP mature adipocytes (FIGURE 5.3).

Lack of adipose tissue in AZIP transgenic mice results in severe metabolic complications, including hyperglycemia, hyperinsulinemia, hyperlipidemia, and liver steatosis (Moitra et al., 1998). Moreover, it has been shown that surgical transplantation of adipose tissue (Colombo et al., 2002; Gavrilova et al., 2000; Tran and Kahn, 2010) and isolated adipocyte

progenitor cells (Rodeheffer et al., 2008) rescue these metabolic phenotypes, primarily by restoration of adipokine secretion (Colombo et al., 2002). In the AZIP-chimeric animals, adipose tissue was restored by providing ES-cells capable of adipogenesis *in vivo*. As such, the lipodystrophic defects caused by *AZIP*-transgene can be complemented and the metabolic phenotypes can be rescued if the ES-cells developed into functional adipocytes in the chimeric mice. At 8 weeks of age, all 11 out of 12 AZIP(FVB) chimeric mice showed normalized blood glucose from 580±12 mg/dL in AZIP(FVB) transgenic animals that are not complemented (n=10; labeled "uncomp.") to 166±6 mg/dL in complemented chimeric animals (n=11), p=3x10E-7 (FIGURE 5.4A). Age matched wild-type chimeric mice have blood glucose 167±7 mg/dL (n=9), which showed no significant difference with the complemented group. One additional chimeric mouse with 2% chimerism was hyperglycemic and leptin deficient, suggesting that the ES-cell did not complement adipocyte development in this animal. Compared to hyperinsulinemia AZIP(FVB) mice, which have plasma insulin of 9.3±0.4 ng/mL (n=5), AZIP(FVB) –chimera have significantly lowered insulin level of 3.5±0.9 ng/mL (n=11; p=2x10E-4). Wild-type chimeric mice have 0.8±0.2 ng/mL plasma insulin (n=7), which is slightly lower than AZIP-chimeric group with p=0.05 (FIGURE 5.4B). Euglycemia accompanied by potential hyperinsulinemia in complemented animals suggested partial insulin resistance and only partial rescue of AZIP phenotypes by wild-type ES cells. Body adiposity as measured by DEXA and serum leptin levels do not correlate with degree of chimerism, ranging from
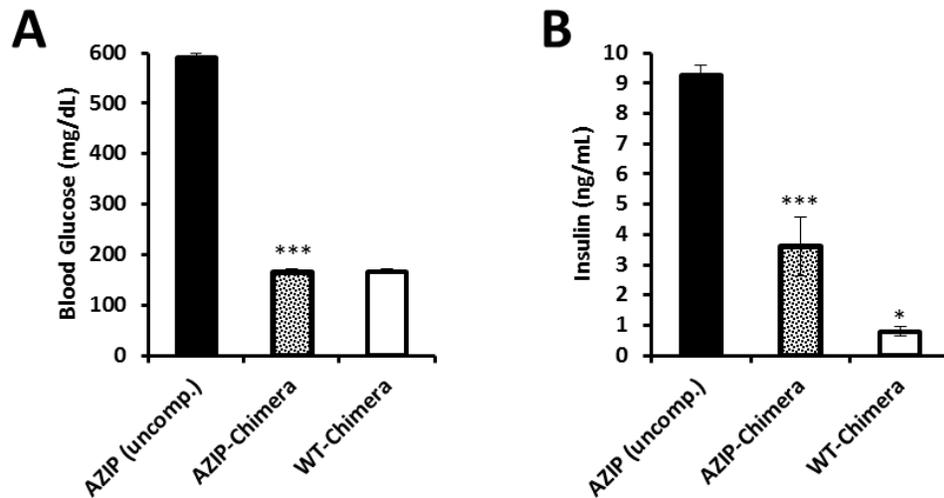
FIGURE 5.4 Metabolic phenotypes of AZIP(FVB)-transgenic animals.
Hyperinsulinemia and hyperglycemia are rescued by YFP-labled ES-cell complementation in AZIP(FVB)-chimeras. All animals depicted are live born from blastocyst injection and categorized into three groups: AZIP(uncomp.) as AZIP animals without ES-cell contribution, AZIP-chimera as animals carrying both AZIP-cells and YFP-cells, and WT-chimera as animals carrying wild-type FVB cells and ES cells. (A). Blood glucose in AZIP-chimera is significantly lowered from 580 mg/dL in AZIP(uncomp.) to 166 mg/dL ($p=3x10E-7$). WT-chimeras have similar blood glucose level of 167 mg/dL. (B). Insulin in AZIP-chimeras is lowered from 9.3 ng/mL in AZIP(uncomp.) to 3.5 ng/mL ($p=2x10E-4$), yet this level is slightly elevated compared to 0.8 ng/mL in WT-chimera ($p=0.05$). n=10 for AZIP(uncomp.), n=11 for AZIP-chimera, and n=9 for WT-chimera.

1% to 97% in both AZIP- and WT-chimeras (FIGURE 5.5A and B). Moreover,

body adiposity measurements correlate with leptin levels in both groups of

chimeras, where the degrees of correlation are identical between the groups

(FIGURE 5.5C). The three groups of animals showed no difference in total

body weight from 4 to 13 weeks old (FIGURE 5.5D). This suggested that body

adiposity and leptin level are controlled by adipocyte autonomous factors
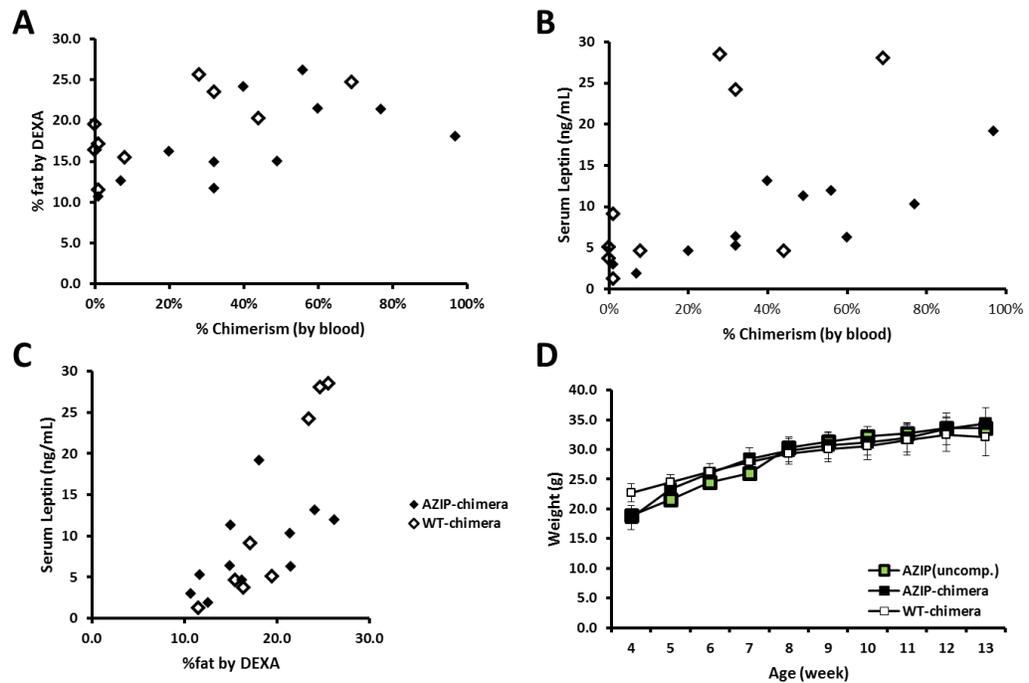
FIGURE 5.5 Relationships between degrees of chimerism, body adiposity, and serum Leptin levels in AZIP(FVB)-chimeric animals. Wild-type chimeric animals, which are chimeras that do not carry AZIP transgenes and therefore are chimeric at every tissues, are plotted in white diamonds, while AZIP-chimera are depicted in black diamonds. Degree of chimerism does not affect serum Leptin levels (A) nor body adiposity (B). Leptin level is correlated with body adiposity in chimeric animals (C), as would be expected in normal animals. (D). Both groups of chimeric animals showed no difference in body weight compared to AZIP(uncomp.) mice (green squares) generated from microinjection.

and that limitation on progenitor number per se does not affect adiposity under normal condition.

Similar insulin resistance phenotype was observed in AZIP-animals underwent either transplantation of wild-type adipose tissue or leptin replacement (Colombo et al., 2002). On the other hand, ob/ob mice treated with leptin showed complete normalization on insulin level. Together these

results demonstrated that while lack of adipocytes and leptin are the primary defect to insulin resistance, additional factors of adipose tissue that are missing in AZIP and/or other tissues remain important in glucose metabolism. In AZIP-chimeric mice, the only cell type completely derived from ES cells is mature adipocytes. As such, it is concluded that cell-autonomous factors of mature adipocytes cannot fully rescue the metabolic defects caused by AZIP transgene. Non-cell-autonomous factors such as AZIP-associated defect in the stroma vascular fraction (SVF) of adipose tissue and in liver and muscle tissues can affect glucose metabolism. For instance, it has been shown that the residual adipose tissue of AZIP-mouse, consisted of primary SVF, presents a hyperadipogenic niche (Berry and Rodeheffer, 2013b; Birsoy et al., 2008a). Moreover, it has been shown that certain population of isolated wild-type preadipocytes showed varied adipogenic capacity when transplanted into AZIP or wild-type mice (Berry and Rodeheffer, 2013a), suggesting non-adipocyte autonomous factors in SVF that can alter the cellularity and functioning of whole adipose tissue, despite all the mature adipocytes are wild-type. Since AZIP-transgene is expressed under adipocyte specific *aP2*-promotor, it is also unclear whether the phenotype is a result of leaky expression. Previous study has demonstrated the promiscuity of *aP2*-promotor in other tissues and especially during development (Lee et al., 2013), therefore it is possible that early developmental events contribute to the observed metabolic phenotypes in adult animals.

The AZIP(FVB) blastocyst complementation experiment provided the preliminary validation of the method. However, we experienced difficulties when manipulating the AZIP(FVB) blastocysts, which developed poorly *in vitro* after isolation at morula stage. We suspected that FVB-background was not ideal for embryo manipulation and it was not compatible with B6-backgroung of ES-cells (Schuster-Gossler et al., 2001). Additionally, B6 is the common strain employed in HFD study and it the best characterized in metabolism (West et al., 1992). Thus, to study effect of HFD on adipose tissue accumulation in chimeric animals, we generated a separate set of chimeric mice using AZIP(B6)-blastocyst and *B6-Tyr^c-YFP* ES-cell. These chimeric animals carried inbred B6 genetic background in all cells, thus also avoided potential complication due to background variability.

We analyzed 17 AZIP(B6)-chimeras and compared the results to 17 AZIP(B6) transgenic mice and 19 WT(B6)-chimeras generated as littermates from blastocyst injection experiments. While AZIP(B6) transgenic mice showed lowered body weight at weaning (3 to 4 weeks old) compared to both chimeric groups, no difference was detected among the three groups in adult animals (FIGURE 5.6). At 12 weeks old, AZIP-chimeras showed 10.0±0.6% body adiposity measured by MRI, which was indistinguishable from that of AZIP-uncomplemented animals at 8.9±0.3% but significantly lowered compared to WT-chimeras at 16.9±0.4% (p=10E-4; FIGURE 5.7A and B). The same comparisons were observed for adiposity measured at 24 weeks old, where AZIP-chimeras showed 11.0±0.9%, AZIP showed 9.2±.4%,
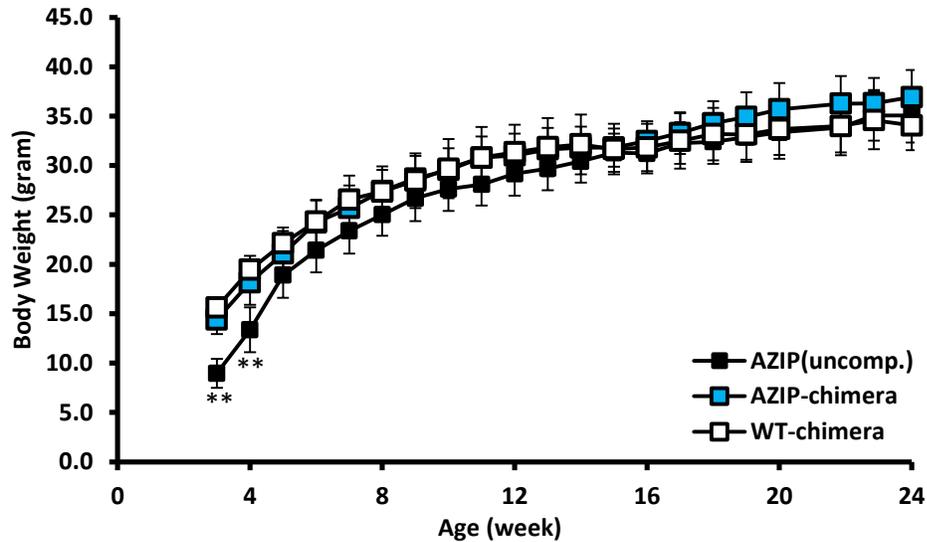
FIGURE 5.6 Body weight of animals from AZIP(B6) blastocyst complementation. AZIP(uncomp.) showed lowered body weight between 3 and 4 weeks (p=0.008 and 0.01 respectively), but no difference is detected among all groups in other age measured. n= 17 for AZIP(uncomp.) showed in black squares, n=17 for AZIP-chimera showed in blue squares, and n=19 for WT-chimera showed in white squares.

and WT-chimeras showed 20.9±0.1%, n=15, 13, 18 respectively due to mortality (FIGURE 5.7C and D). Difference between AZIP-chimeras and WT-chimeras remained significant with p=2x10E-4. Scattered plot of adiposity versus degree of chimerism showed that AZIP-chimeras are leaner than WT-chimeras across the board (FIGRUE 5.7B and D). Consistently, at 12 weeks old, AZIP-chimeras have lowered serum leptin levels at 5.25±0.97ng/mL compared to that of WT-chimeras at 10.45±1.49ng/mL (p=6x10E-3). Nonetheless, AZIP-chimeras showed significant rescue of leptin deficiency phenotype of AZIP-uncomplemented mice, in which serum leptin levels were below detection limit (FIGURE 5.7E and F). In both AZIP- and WT-chimera
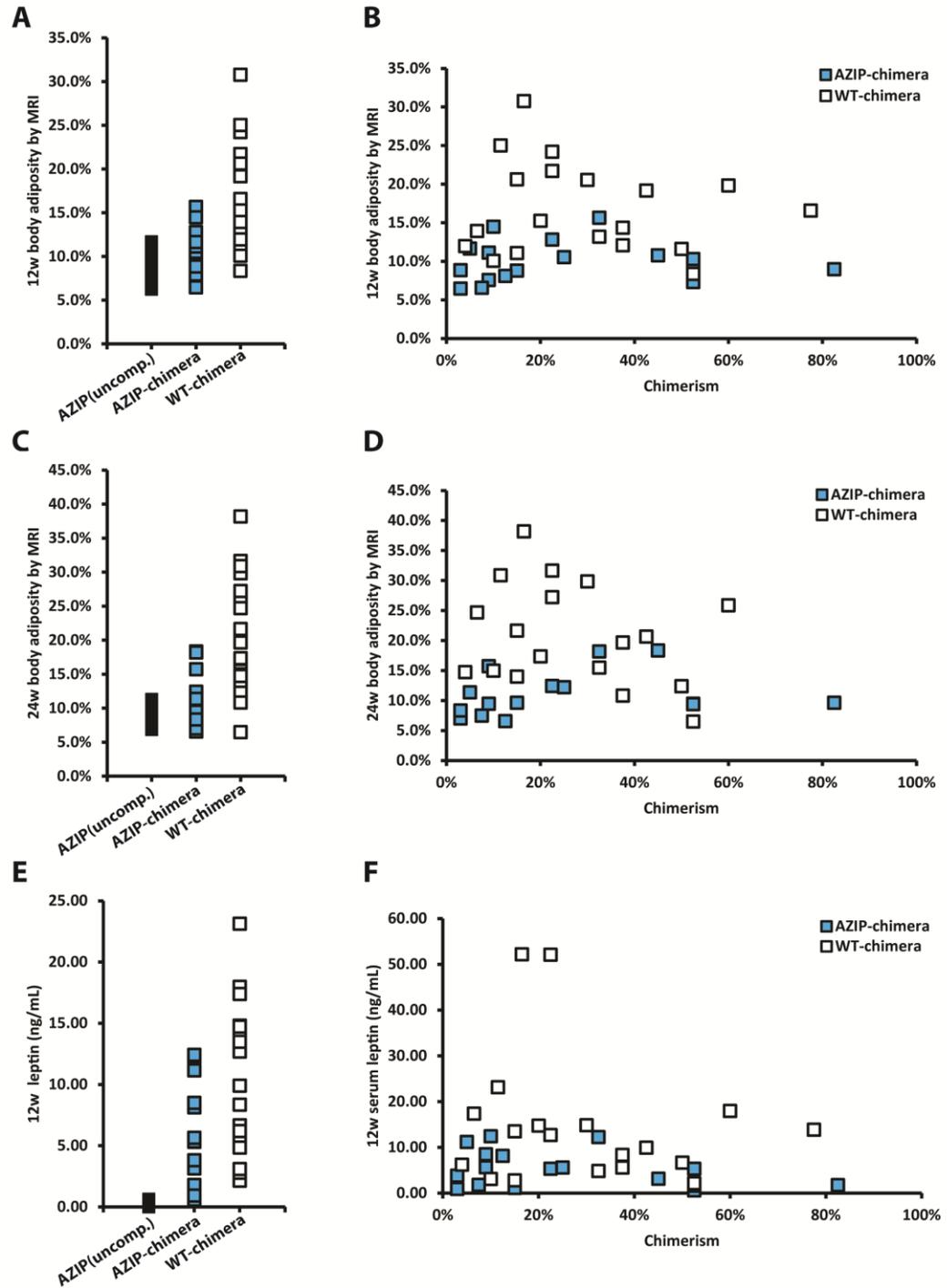
FIGURE 5.7 Body adiposity and serum leptin of AZIP(B6) animals. All animals presented are pups of blastocyst complementation. (A). Body adiposity at 12 weeks old measured by MRI, where AZIP(uncomp.), AZIP-chimera, and WT-chimera have 8.9%, 10.0%, and 16.9% adiposity respectively. AZIP(uncomp.) and AZIP-chimera are indistinguishable while both are significantly lowered than WT-chimera (p=10E-4). (B). Adiposity at 12 weeks old versus chimerism in

FIGURE 5.7 (cont.) AZIP-and WT-chimeras. AZIP-chimera show lower adiposity across all range of chimerism. (C). and (D). Body adiposity at 24 weeks old. (E). Serum leptin in three groups of animals. AZIP(uncomp.) has undetectable level of leptin. AZIP-chimera has 5.25 ng/mL leptin, which is lowered compared to that of WT-chimeras at 10.45 (p=6x10E-3). (F). Serum leptin versus chimerism in chimeric animals shows that AZIP-chimeras have lowered leptin across all range of chimerism.

leptin level does not correlate with chimerism. Two WT-chimeras with high leptin levels were excluded based on outlier tests.

Next, we examined whether metabolic complications in lipodystrophic AZIP-transgenic mice can be rescued by complementing adipogenesis defect with ES-cells. Hyperinsulinemia at 12 weeks old due to adipose tissue defect in AZIP-transgenic mice was rescued in AZIP-chimeras, from 111±11ng/mL to 8±4ng/mL (p=5x10E-12). Insulin level in AZIP-chimeras is slightly elevated compared to WT-chimeras at 1.84±0.24ng/mL, p=10E-2 (FIGURE 5.8A). Insulin level was inversely correlated with chimerism in AZIP-chimeras but not WT-chimeras (FIGURE 5.8B). Moverover, insulin level was also inversely correlated with body adiposity in AZIP-chimeras but not WT-chimeras, suggesting that the incompletely rescued metabolic phenotype was secondary to adiposity defect (FIGRUE 5.8C). On the other hand, no significant difference in blood glucose at 12 weeks old was detected among groups, primarily because AZIP(B6) animals, as previous reported (Colombo et al., 2003; Haluzik et al., 2003), were not hyperglycemic to start with (FIGURE 5.8D). Liver steatosis prominent in AZIP(B6) mice, as measured by total liver weight (Colombo et al., 2002), was rescued by
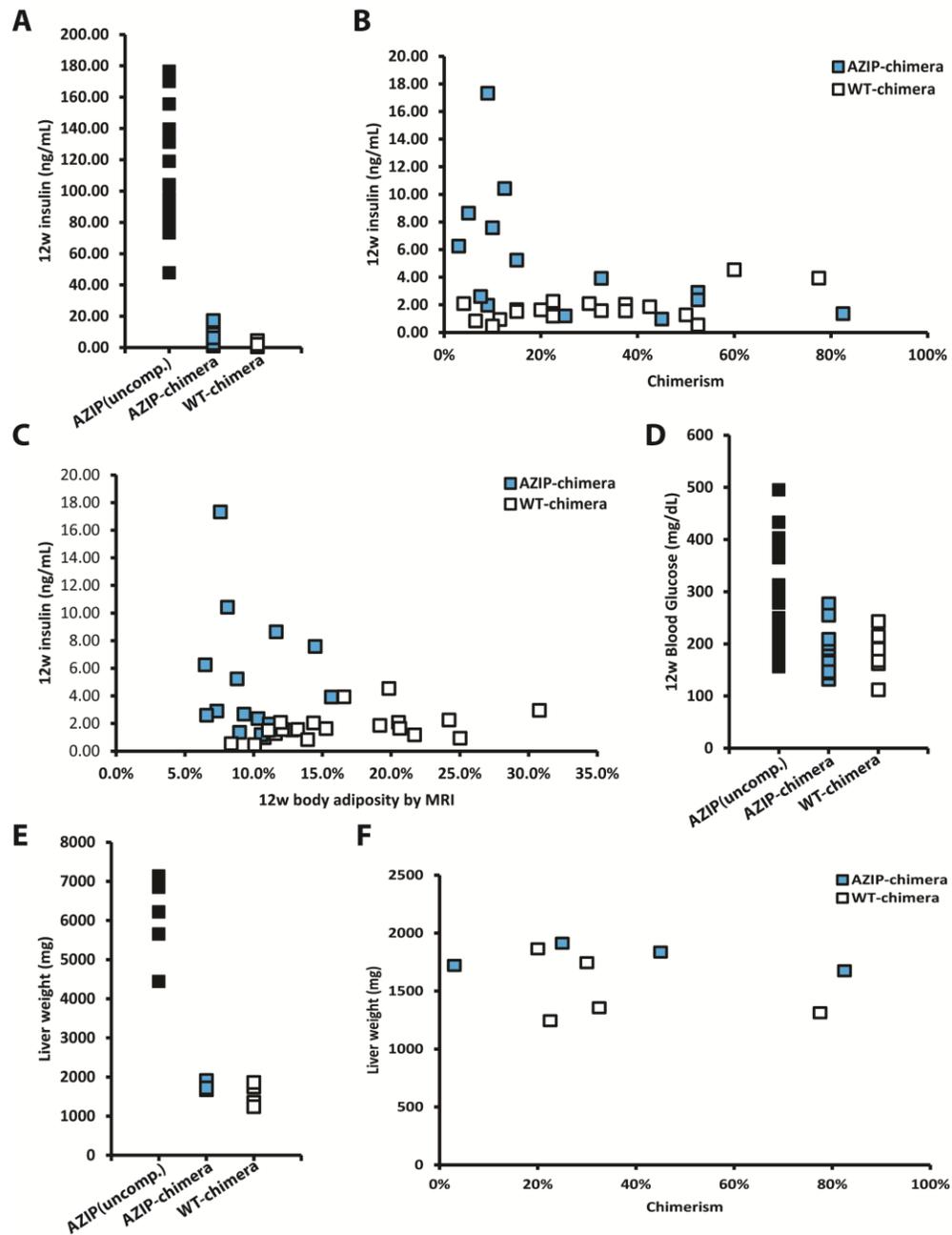
FIGURE 5.8 Metabolic phenotype in AZIP(B6) animals. (A). Insulin levels at 12 weeks old. AZIP(uncomp.) mice are hyperinsulinemic at 111 ng/mL, while insulin level is significantly lowered in AZIP-chimeras at 8 ng/mL (p=5x10E-12), which remain slightly elevated compared to WT-chimera at 1.8 ng/mL (p=10E-2). (B). And (C). Insulin level is inversely correlated with chimerism and body adiposity. (D). Blood glucose in each group of animals at 12 weeks old. Despite having elevated insulin levels, AZIP-chimeras showed wild-type level of blood glucose. (F). And (G). Liver steatosis as measured by total liver weight. Liver weight is comparable in AZIP-and WT- chimeras across all range of chimerism.

complementation, where liver of AZIP-chimera weighed 1786±48mg (n=4) and liver of AZIP-uncomplemented mice weighted 6058±479mg (n=5), p=10E-4. Liver of WT-chimeras weighted 1505±125mg, with no distinguishable difference compared with AZIP-chimeras independent of chimerism (FIGURE 5.8E and F).

The observations that AZIP-chimeras remained slightly lipodystrophic and leptin deficient suggested that partial adipose tissue defect was the primary cause of insulin resistance. This is consistent with observation in human patients where metabolic complication is in general correlated with extent of adipose tissue loss (Huang-Doran et al., 2010). In AZIP-chimeric model, since all mature adipocytes are derived from wild-type progenitors, this may reflect non-adipocyte autonomous factors that contributed to dysfunction of adipose tissue. For instance, insulin resistance in adipocytes can lead to defective adipogenesis and lipid accumulation, which can lead to lipotoxicity in other tissues despite only moderate lipodystrophy (Laustsen et al., 2002; Rosen and Spiegelman, 2000). Impaired glucose metabolism in adipose tissues, such as in case of early stage T2DM, is primary to development of insulin resistance in muscles and liver (Abel et al., 2001; Shepherd and Kahn, 1999). By this mechanism, it is postulated that AZIP-chimeric mice became moderately insulin resistant despite having adipocytes restored. Whether AZIP-transgene, which is expressed in varying degree in other tissues of the chimeras, directly affects other tissues remains unclear. Although previous report suggested PPARg down-regulation in liver

of AZIP-transgenic model as a mechanism of liver insulin resistance (Gavrilova et al., 2003), it cannot be deduced from such case if it originated from a liver-autonomous defect. Alternatively, reduced plasma leptin itself can result in hyperinsulinemia, because leptin has been shown to negatively regulate insulin secretion as part of the adipoinsular axis (Kieffer and Habener, 2000; Kulkarni et al., 1997).

Next, we place AZIP- and WT-chimeric animals on HFD treatment to assay whether limited adipocyte progenitors in AZIP-chimeric mice limits diet induced obesity. After 6 weeks of HFD starting from 6 weeks old, AZIP-chimeras showed averaged 47.3% of weight gain (n=14), which is significantly lower than 78.8% weight gain of WT-chimeras (n=11; p=5x10E-3). Comparing to WT-chimeras, AZIP-chimeras with low chimerism gained less weight (FIGURE 6.9A and B). A subset of these animals that were littermates were further studies on change in body adiposity and insulin levels after HFD. Body adiposity elevated by 5.7% in AZIP-chimeras (n=7), which is significantly lower than the elevation of 21.2% in WT-chimeras (n=10; p=8x10E-5). Similar to total body weight, low-chimerism correlated with less adipose tissue accumulation in AZIP-chimeras but not in their WT-counterparts (FIGURE 6.9C and D). Insulin resistance developed more readily in AZIP-chimeras than WT-chimeras, where insulin levels were 3.17±0.95ng/mL and 0.95±0.25ng/mL respectively (p=10E-2) while glucose levels were comparable (FIGURE 6.9E, F and G). Interestingly, serum leptin correlates with body adiposity measured after 6 weeks of HFD in both AZIP-

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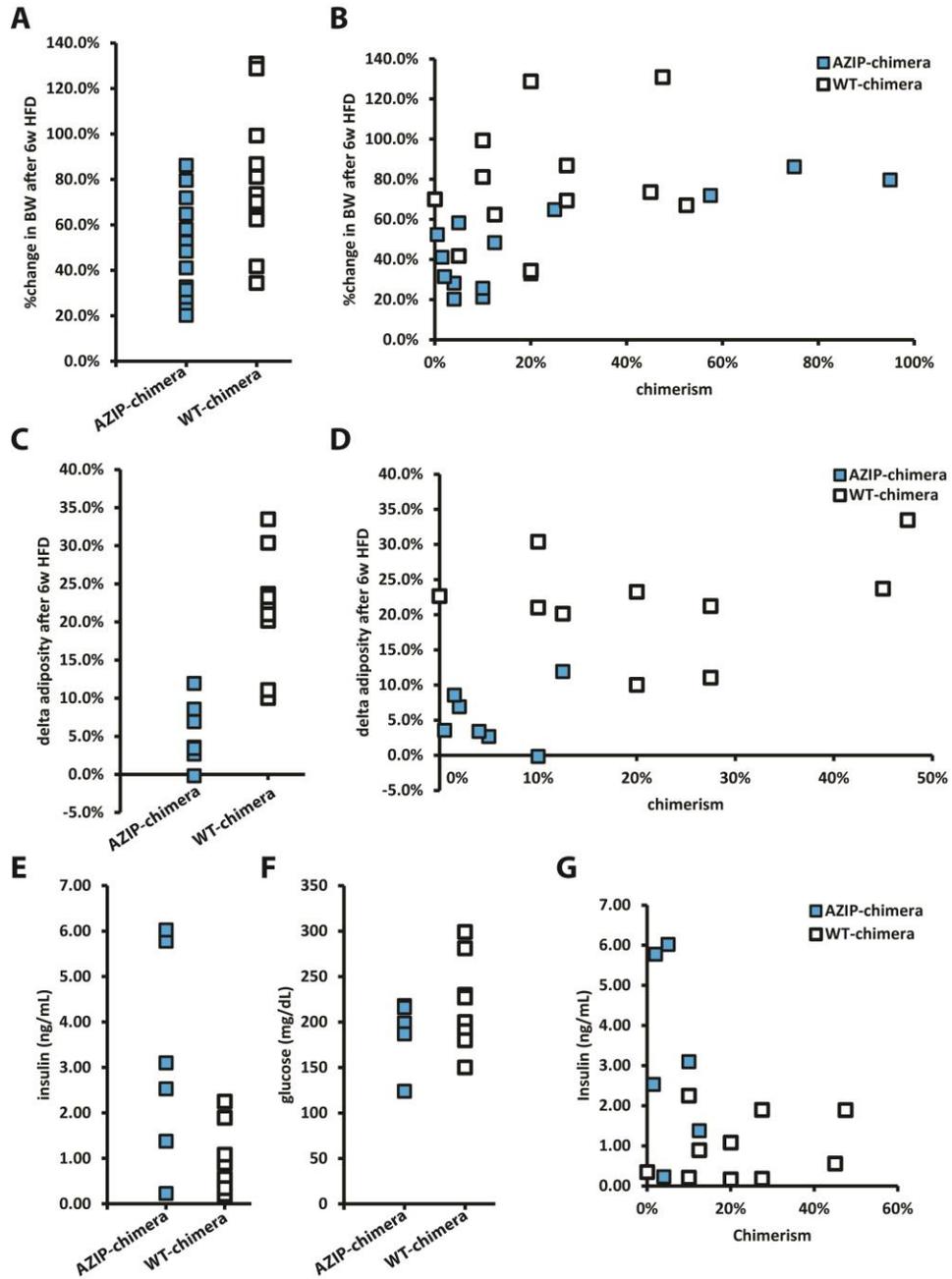
FIGURE 5.9 AZIP(B6)-chimeras are resistant to obesity but not metabolic complication of HFD. (A). Percent of total weight gain after 6 weeks of HFD (from 6 to 12 weeks old). AZIP-chimeras on average gained 47% weight while WT-chimeras gained significantly more, 79% (p=3x10E-3). (B). Analysis on percent weight gain versus chimerism shows that, at lower chimerism, WT-chimeras gained more weight than AZIP-chimeras. n=14 for AZIP-chimera and n=11 for WT-chimera. (C). Analysis of a subset of littermate chimeras on body adiposity change after 6 w of HFD. AZIP-chimeras (6%) accumulated less fat mass compared with WT-chimeras (21%), p=8x10E-5. (D). Change in adiposity

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FIGURE 5.9 (cont.) versus chimerism shows that AZIP-chimeras with low chimerism is defective at accumulate fat mass. (E). Insulin levels of HFD-treated chimeras shows AZIP-chimeras are more susceptible to development of insulin resistance. (F). Blood glucose of HFD-treated chimeras shows no difference in glucose level in AZIP-and WT-chimera. For figure (C) to (F), n=7 for AZIP-chimeras, and n=10 for WT-chimeras.

and WT-chimeras (FIGURE 5.10). In summary, the result suggested that leptin production is a cell-autonomous property of adipocytes, adipose tissue expansion and metabolic complications as a result of adipose tissue defect are not adipocyte-autonomous.

There are two possible explanations for the apparent resistance to HFD observed in AZIP-chimeric animals. Since adipose tissue expansion is accompanied by hypertrophy and hyperplasia (Arner and Spalding, 2010b), defects in either or both processes can lead to failure in adipose tissue accumulation. If AZIP-chimeric adipose tissue is insulin resistant as previously discussed and since insulin signaling is required for lipid accumulation in adipocytes (Yu et al., 2008), AZIP-chimeras will not become obese on HFD through hypertrophic adipose expansion. Alternatively, defect in hyperplasia can be explained by limited adipocyte progenitor imposed by ES-cell complementation. Since it has been shown that the pool of adipocyte is finite in adult animals (Pajvani et al., 2005; Wojtanik et al., 2009), it can be inferred that variation in ES-cell versus AZIP-cells makeup in this pool affects adipogenic capacity of the animal. This is consistent with the observation where higher degree of chimerism conferred to greater increase of body weight after HFD. Consistently, AZIP-chimeras developed greater insulin
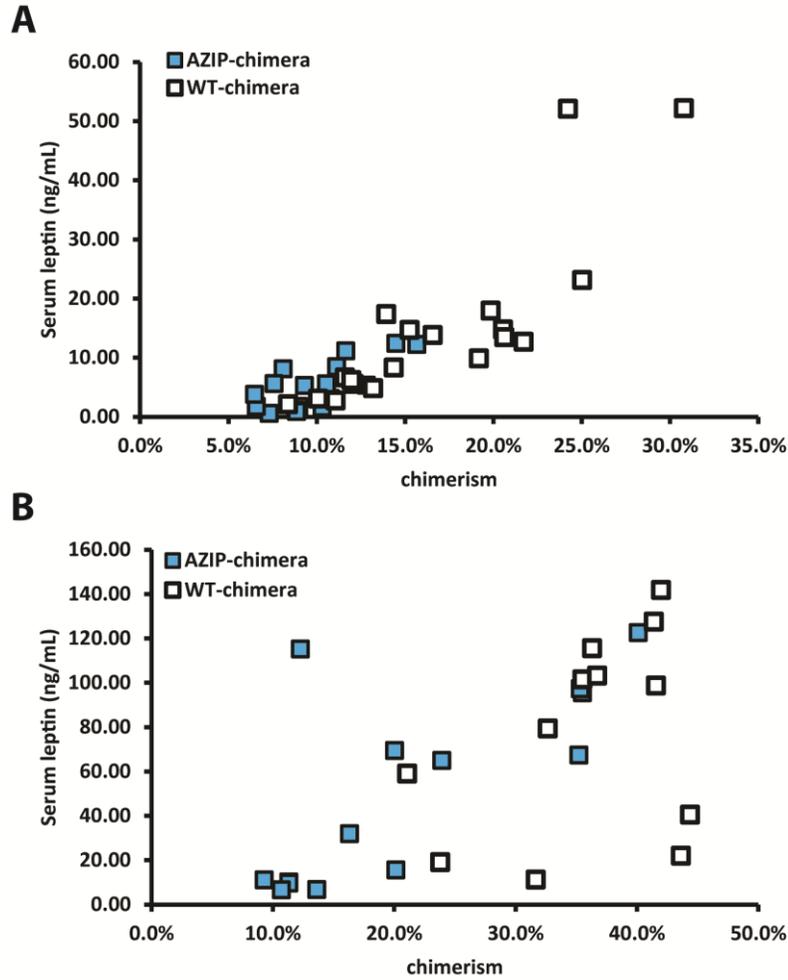
FIGURE 5.10 Serum leptin versus body adiposity of AZIP(B6)-chimeras measured by MRI. (A). Animals are fed on chow diet. Both AZIP-and WT-chimeras have serum leptin level positively correlated with adiposity. n=17 for AZIP-chimera and n=19 for WT-chimeras. (B). Animals are fed on HFD. n=14 for AZIP-chimeras and n=11 for WT-chimeras.

resistance compared with WT-chimeras under HFD, consistent with previous

observation that limited expandability of adipose tissue is causative to

metabolic complication (Huang-Doran et al., 2010; Virtue and Vidal-Puig,

2008). However, low adiposity phenotype compared to WT-chimeras was

observed under both chow and high-fat diet cross all range of chimerism,

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suggesting that low contribution of ES-cell to the adipocyte per se cannot fully account of the observed phenotype. Additional factors such as the presence of AZIP-cells in the SVF can limit adipogenesis, including accumulation of lipid during terminal differentiation, must be considered.

It has been a long debate whether hyperplasia contributes to obesity. Hyperplasia is strongly correlated with severity of obesity, especially in morbidly obese individuals (Hirsch and Batchelor, 1976). However, the data was collected cross-sectionally, and therefore gives no direct information on the longitudinal relationship between adiposity and adipocyte cellularity. Therefore it is impossible to conclude whether the average increase in adipocyte number seen in obese individuals is the result of adult adipocyte recruitment or rather a reflection of a population of people predisposed (by their pre-adulthood fat cell number) to obesity. At least in short-term studies, adult humans do not show any increase in adipocyte number following significant weight gain (Salans et al., 1971). Consistently, our data demonstrated that resistance to obesity as a result of predisposed limited adipocyte numbers, although complication from other tissues including the SVF cannot be ruled out.

TABLE 5.1 AZIP(FVB)-chimeric animals.

Animal ID	DOB	Sex	AZIP?	Blood Chimerism	DEXA	Leptin	Insulin	Glucose (8w)	Glucose (12w)
0419-3	5/6/2012	M	+	97%	18.1	19.2	5.5	166	160
0419-7	5/6/2012	M	+	49%	15.0	11.3	2.2	146	181
0419-8	5/6/2012	M	+	32%	11.7	5.3	1.0	144	169
0419-26	5/6/2012	M	+	32%	14.9	6.4	1.9	157	186
0508-2	5/25/2012	M	+	56%	26.2	11.9	2.2	152	174
0614-1	7/1/2012	M	+	20%	16.2	4.6	8.7	200	225
0614-2	7/1/2012	M	+	1%	10.7	3.0	10.4	142	199
0626-2	7/12/2012	M	+	77%	21.4	10.3	1.1	186	147
0626-5	7/12/2012	M	+	40%	24.1	13.1	1.8	205	166
0628-3	7/14/2012	M	+	60%	21.5	6.3	2.9	156	159
0628-7	7/14/2012	M	+	7%	12.6	1.9	0.9	160	190
0419-1	5/6/2012	M		32%	23.5	24.2	0.5	166	190
0419-10	5/6/2012	M		1%	17.1	9.1	0.7	152	167
0419-14	5/6/2012	M		0%	16.4	3.7	0.5	149	188
0419-2	5/6/2012	M		28%	25.6	28.5	1.2	156	200
0419-21	5/6/2012	M		8%	15.5	4.6	0.6	155	205
0419-23	5/6/2012	M		69%	24.7	28	4.2	251	600
0419-5	5/6/2012	M		0%	19.5	5.1	1.5	156	180
0419-6	5/6/2012	M		1%	11.5	1.2	0.7	131	180
0628-5	4110400%	M		44%	20.3	4.6		180	168
0501-6	4104700%	M	+	2% (uncomp.)	9.1	ND	>9.6	551	600

5.3 Application

The presented blastocyst complementation assay provides a novel platform to establish the role of specific molecules to regulate adipose tissue mass *in vivo*. This is potentially a better alternative to the common employed *loxP-CRE* system for the blastocyst complementation requires fewer breeding and provides higher tissue specificity.

The most widely used adipocyte-specific *CRE*-mouse model has been the *aP2* promoter, which drives expression of fatty acid binding protein 4 (FABP4). However, in vitro studies have shown that *aP2* is induced late in adipogenesis (Bernlohr et al., 1985) and in vivo results have suggested that *aP2* is not expressed in adipocyte precursors (Rodeheffer et al., 2008). In addition, the aP2-promoter expresses in other tissues during developments such as macrophages and brain (Lee et al., 2013). Alternatively, *Adiponectin*-CRE provides higher adipocyte-specificity, but it is also only expressed in mature adipocytes (Jeffery et al., 2014). Lastly, PPARg-driven mouse model has been shown to label adipogenic stromal cells *in vivo* (Tang et al., 2008), yet its application is also limited stages in late adipogenesis. Most recently, a novel preadipocyte factor, *Zfp423*, was identified as adipogenic marker both *in vitro* and *in vivo* (Gupta et al., 2010; Gupta et al., 2012). Although *Zfp423* is expressed prior to aforementioned adipocyte factors in adipogenesis, its specificity *in vivo* remained to be accessed. Indeed, as adipogenesis *in vivo* and accompanying cell markers are poorly known, no suitable genetic mouse model for studying preadipocytes have been constructed. With the AZIP-
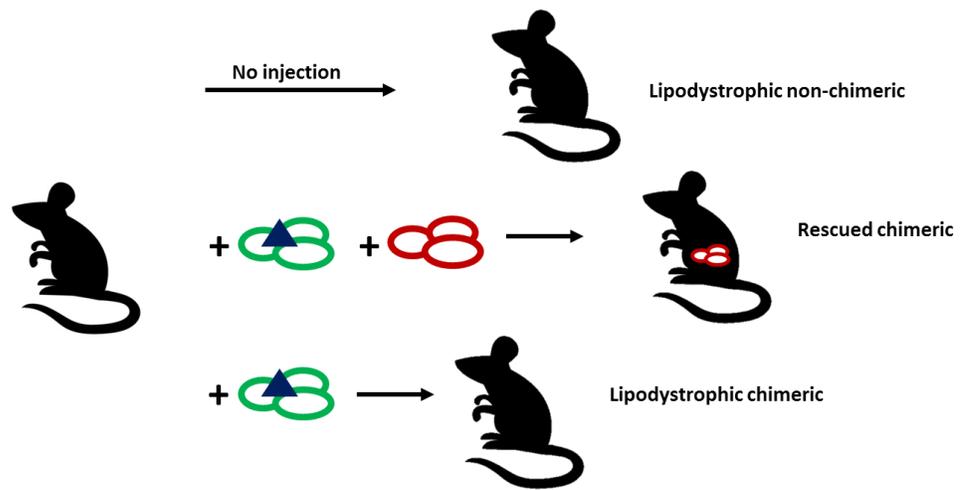
FIGURE 5.11 AZIP-blastocyst complementation as a method to generate adipocyte specific knockout in one generation. Green and red color depicts ES-cells that are labeled with different marker. Blue triangle represents a homozygous knockout of a gene of interest, presumably generated by CRISPR mediated genome modification.

chimera model, adipocyte specific gene modifications can be achieved in one step by introducing gene targeted mutations into the donor ES cells (FIGURE 5.11). As such, gene modification is achieved in early progenitor cells, which is very useful to study the genetics of adipose tissue development *in vivo*.

However, the current AZIP-chimera model is limited in several regards. First, since AZIP-transgene is driven by *aP2*-promoter, tissue specificity remains concerning. Additionally, since any complementing effect, in theory, cannot be assayed before the expression of AZIP-transgene, defects in early adipocyte stages remain masked. Secondly, the moderate lipodystrophy observed in AZIP-chimeras complemented with wild-type ES cells is indicative of inherent developmental defects of cultured ES-cells. It

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has been shown that cultured ES-cells accumulate epigenetic modifications that affect *in vivo* development (Eckardt et al., 2011). Even if a specific ES-cell clone has been proven pluripotent (defined as capable of producing live born through tetraploid complementation), it could be a result of developmental compensation and that it does not provide direct information on its developmental capacity under normal conditions. In addition, AZIP-blastocyst is likely developmentally dominant over ES-cells. Although the mechanistic details are unclear, it has been shown that, through inter-species blastocyst complementation, the blastocyst host is instructive in determine overall animal size and organ morphology in interspecies chimeras (Solter, 2010). As such, it is likely that the developmental potential of wild-type ES-cells in terms of its adipogenic capacity is limited by AZIP-blastocyst. Two possible strategies can circumvent this limitation. First, aggregation chimeras using two embryos will address the concern of developmental discrepancy between live embryo and cultured cells. Second, reverse the combination of blastocyst and ES-cell by injecting AZIP-transgenic ES-cells into wild-type blastocysts. This provides additional advantage of lightening up the breeding load on AZIP-animals, which has compromised fertility.

Despite having limitation in studying adipocyte development, we plan to apply blastocyst complementation method to characterize the *in vivo* functions of novel factors involved in leptin regulation. As described in Chapter 3, we mapped the *cis*-elements that regulate leptin expression *in vivo* using leptin-luciferase reporter mice. Combining biochemical assays and

genome-wide bioinformatics, we have identified several candidate trans-

factors that interact with these sequences. In Chapter 5, we have performed

RNA-sequencing on leptin specific gene expression pattern, upon which we

have identified different genes co-regulated with leptin. These data provided

gene candidate that can be subject to generating ES-cell knockout using

CRISPR technique (Wang et al., 2013a). The presented blastocyst

complementation assay will be useful in generating adipocyte-specific

knockout of these candidates in one generation and reveal their *in vivo*

function in adipose tissue.

CHAPTER 6: CONCLUSION

Since the discovery of leptin in 1994, a staggering amount of effort has advanced our understanding of leptin's critical role in the regulation of energy homeostasis and glucose metabolism. Yet the molecular and cellular mechanisms regulating leptin production in adipose tissue have remained as a gap in knowledge. In the face of the growing prevalence of obesity, the molecular basis of adipose tissue expansion in response to diet-induced obesity is a pressing question for disease management. While leptin serves as the afferent signal of a feedback system for regulating energy homeostasis, common obesity are characterized by leptin resistance developed from chronic hyperleptinemia. Understanding the mechanism of increase leptin production in response to increase adiposity is therefore fundamental to study of pathogenesis of obesity. Since serum leptin correlates with body adiposity and correlates with mRNA levels in each adipocyte, it was hypothesized that *leptin* expression is regulated by an adipocyte cell-autonomous mechanism that senses lipid content within adipocytes.

Lack of a suitable cell culture model to study *leptin* regulation compounded with a lack of understanding of adipose tissue development *in vivo* had made the study of leptin regulation *in vivo* particularly challenging. We address this by developing a luciferase BAC-transgenic reporter mouse model that allows us to assay qualitative and quantitative leptin expression *in vivo*. In Chapter 3, using a series of reporter mice, we first narrowed down

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a 40kb region from -22kb to +18kb that is sufficient for quantitative and qualitative *leptin* expression *in vivo.* We further demonstrated that the 5'-end of this construct alone is sufficient for *leptin* transcriptional regulation. Combination of sequence analysis and gel-shift assays identified a novel distal *cis*-element of the *leptin* gene at -16.5kb that is essential for *leptin* transcription. Since this *cis*-element contains a CCAAT-box sequence, a candidate approach was taken in a super-shift assay and identified an adipogenic factor, NF-Y, as the *trans*-element for this enhancer site.

To study the function of NF-Y in adipose tissue *in vivo*, we generated lentiviral mediated knockdown of *NF-Y* in 3T3-L1 cells and generated adipocyte specific *NF-Y* knockout mice via crossing *adiponectin-CRE* to *NF-YA fl/fl* mice. These experiments, described in Chapter 4, consistently showed that NF-Y is required for adipogenesis, since *NF-Y* knockdown 3T3-L1 cells do not differentiate and NFY-KO mice showed progressive lipodystrophy. NFY-KO mice were insulin resistant and dyslipidemic, both of which can be ameliorated by leptin treatment. These results show that the metabolic complications in NFY-KO mice are secondary to adipose tissue loss cause by the deletion. Unfortunately, *leptin* expression is reduced in NFY-KO mice but likely as a result of lipodystrophy. To study the direct function of NF-Y in *leptin* transcriptional regulation *in vivo*, an inducible model such as the *adiponectin-ER-CRE* system can be considered. Alternatively, deletion or point mutation of the -16.5kb *cis*-element directly in the genome, which is

made accessible with CRISPR technique, may provide insight to the endogenous function of this regulatory sequence.

While the leptin-luciferase transgenic model proved to be an excellent tool in identifying *cis*-elements within the leptin gene, there is no good unbiased method in identifying the corresponding *trans*-factors. We address this by performing RNA-sequencing experiments in adipose tissue with animals under varying ambient leptin. Leptin treatment, in both wild-type and *ob/ob* mice, produces unique metabolic effects that result in fat loss. As such, it is postulated that gene expression profiles vary with *leptin* expression levels and that by comparing among samples, one can identify molecular and cellular profiles that modulate leptin expression. Sequencing experiments were performed with untreated wild-type, *ob/ob*, wild-type leptin withdrawal (model of acute leptin deficiency), and *ob/ob* leptin replacement (model of leptin induced weight loss). Coupled with other genomic methods such as the DNAse hypersensitivity assay and ChIP-seq assay, we aim to understand the transcriptional network modulated by leptin levels in white adipose tissue. This part of study is under progress.

Lastly, we took the developmental approach to study leptin regulation *in vivo*. Adipose tissue is the most plastic organ in the body, of which the size is determined by coordinated processes between hypertrophy and hyperplasia. Regarding adipose tissue as an endocrine organ for leptin and other adipokines, it is intriguing how organ size control is achieved. We

performed an AZIP-blastocyst complementation experiment to study

developmental control of adipose tissue and leptin regulation. The unique

property of blastocyst complementation allows the distinction between

adipocyte autonomous versus non-autonomous factors that contribute to

determine body adiposity and regulate metabolic functions. Specifically,

because the AZIP-blastocyst cannot develop into mature adipocytes, all

mature adipocytes were progenies of wild-type ES cells in the chimeric

animals. As such, we first showed that adipocyte progenitor number as

represented by degree of chimerism does not affect adiposity in adult

chimeras in chow diet. Nonetheless, at least in the case of B6 background,

AZIP-chimeras are partially lipodystrophic with moderate insulin resistance.

Moreover, AZIP-chimeras with low chimerism were unable to expand

adipose tissue upon HFD challenge, suggesting that hyperplastic expansion is

required to accommodate excess dietary fat. Adipose tissue expansion is

critical to metabolic health, as excess lipid that cannot be stored in adipose

tissue results in lipotoxicity and insulin resistance in other peripheral tissues.

Overall, an incomplete rescue of AZIP-phenotype by ES-cell complementation

showed that the broad spectrum of metabolic health is controlled by intricate

mechanisms involving other cell types and tissues. Nonetheless, under both

chow and HFD conditions, serum leptin is correlated with body adiposity,

suggesting adipocyte-autonomous regulation of *leptin* transcription.

Altogether, this thesis presents the molecular basis as well as the

cellular basis of leptin regulation by using different mouse models. Leptin-

luciferase BAC transgenic mice can be applied to the study of acute leptin regulation by live luciferase imaging following pharmaceutical or genetic manipulations. The AZIP-blastocyst complementation model is useful in generating adipocyte specific knockout within one generation, avoiding time consuming crossing with the traditional genetic approach. Both models will be instrumental in further elucidating the molecular and cellular mechanisms regulating leptin gene expression in adipose tissue *in vivo*.

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