ADIPOCYTE IRON REGULATES LEPTIN AND FOOD INTAKE

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Iron overload has been proposed to be a component of the insulin-resistance syndrome. On the other hand, dietary iron supplementation is associated with growth and increased appetite in children. We found the concentration of circulating ferritin is associated with low serum adiponectin, the insulin-sensitizing adipokine, and leptin, the adipokine that is responsible for regulating food intake. Similarly, mice fed a high iron diet and 3T3-L1 adipocytes treated with iron exhibited decreased adiponectin and leptin mRNA and protein.

Iron negatively regulates transcription of adiponectin promoter-driven luciferase activity in a FOXO1-dependent manner. However, iron decreases the inactivated form of FOXO1, acetyl-FOXO1, while leaving phosphorylated FOXO1 and total FOXO1 unaffected. The mechanism of iron's effect on adiponectin and promotion of metabolic syndrome is demonstrated via the binding affinity of multiple transcription factors in the adiponectin promoter using ChIP studies. The higher activation of FOXO1 in iron-treated cells contributes to more inactivation of PPARγ through direct interaction, leading to decreased adiponectin transcription and expression. These findings directly demonstrate a causal role for iron as a risk factor for metabolic syndrome and a role for adipocytes in modulating metabolism through adiponectin in response to iron stores.

We found iron negatively regulated leptin transcription via cAMP response element binding protein (CREB) activation. Two potential CREB-binding sites were identified in the mouse leptin promoter region. Mutation of both sites completely blocked the effect of iron on promoter activity. We also found enrichment of phospho-CREB

binding to those two sites by ChIP in 3T3-L1 adipocytes treated with iron. CREB is modified and destabilized by O-GlcNAc modification. Iron also negatively regulates O-GlcNAc modification both in 3T3-L1 and epididymal fat, and mice heterozygous for deletion of the gene encoding the enzyme that removes O-GlcNAc, O-GlcNAcase, showed significantly increased serum leptin compared to wild-type mice. Glucosamine treatment rescued high iron-induced decrease of leptin promoter activity. These results suggest that the effects of iron on leptin may be via crosstalk of O-GlcNAcylation and phosphorylation of CREB. These findings indicate that levels of dietary iron play an important role in regulation of appetite and fat metabolism through CREB-dependent modulation of leptin expression.

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LIST OF ABBREVIATIONS

AMPK: 5' AMP-activated protein kinase

cAMP: cyclic adenosine monophosphate or 3’-5’-adenosine monophosphate

BMI: body mass index

ChIP: chromatin immunoprecipitation

CRE: cAMP regulatory element

CREB: cAMP regulatory element binding protein

CVD: cardiovascular disease

DMT1: divalent metal transporter 1

EMSA: electrophoretic mobility shift assay

Fpn1: ferroportin 1

G6Pase: glucose 6-phosphatase gene

HBP: hexosamine biosynthesis pathway

IRE: iron regulatory element

IRP: iron regulatory protein

NHANES: National Health and Nutrition Examination Survey

SMAD: small 'mothers against' decapentaplegic

T2DM: Type 2 Diabetes Mellitus

TF: transferrin

TFR1: transferrin receptor 1

TFR2: transferrin receptor 2

O-GlcNAc: O-linked N-acetylglucosamine

OGTT: oral glucose tolerance test

OGT: O-linked N-acetylglucosamine (GlcNAc) transferase

PEPCK: phosphoenolpyruvate carboxykinase

WT: wild-type

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INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is major health problem in the United States and the world. Epidemiological studies have shown a positive correlation between iron levels in the body and insulin resistance (1). The mechanisms underlying these associations are largely unknown. Iron can contribute to hyperglycemia via reducing insulin secretion by islets or decreasing insulin sensitivity in muscle and adipose tissue. One hormone well recognized as a regulator of glucose homeostasis is adiponectin, a regulator of metabolism in many tissues that also sensitizes them to insulin. It is largely secreted by adipose tissue. It can activate 5' AMP-activated protein kinase (AMPK) that turns on fatty acid oxidation and increases glucose uptake in skeletal muscle. In liver, activation of AMPK by adiponectin can decrease glucose output through repressing gluconeogenesis via decreasing the genes encoding phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) (2). Opposite to iron, adiponectin levels are lower in patients with insulin resistance and T2DM, and decreased adiponectin levels have been causally linked to insulin resistance (3). We therefore hypothesized that iron and adiponectin are interrelated, and that adiponectin may be a mediator of iron's effect on insulin resistance. Supporting this, recent epidemiologic studies have found a negative correlation between serum ferritin and adiponectin levels (4-7).

Our lab therefore investigated the effect of iron on adiponectin and the mechanism of iron-induced decreases in adiponectin. We demonstrate in humans that the association between serum ferritin and adiponectin is independent of inflammation. Serum ferritin, even within its normal ranges, is among the best predictors of serum

adiponectin levels. Studies in cell culture, mouse models, and humans demonstrate that iron plays a direct and causal role in determining adiponectin levels. I have demonstrated the decrease of adiponectin transcription is via FOXO1-mediated repression of PPARγ occupancy on its promoter. The adipocyte responding to iron availability and adjusting expression of adipokines related to metabolism make it well suited to perform its broader nutrient-sensing function.

Obesity is also a national epidemic and currently affects about 1 out of 3 adults and 1 out of 6 children in the United States and accounts for 10% of the U.S. medical budget. Obesity and its related metabolic disorders are one of the leading causes of mortality in adult humans in the United States (8) and contributes to the risk for cardiovascular diseases (heart attack and stroke), T2DM, and some types of cancer. Obesity is characterized by increased adipose tissue mass. Unlike other tissues in the body, adipose tissue, typically known as a primary site for energy storage, constantly changes its size and distribution throughout adulthood because of changes in metabolic demand and caloric intake. Adipose tissue also functions as the largest endocrine organ, regulating metabolism through the secretion of many adipokines such as leptin, adiponection, tumor necrosis factor-α, and others. It is therefore not surprising that structural and functional alterations of the adipose tissue have been shown to have a severe impact on metabolism in the body. The developmental biology of adipose tissue is also important: Weight gain and obesity can be seen as cases of adipose over-development, wherein the need for maturation of adipocytes from preadipocytes is accelerated or even overwhelmed (9). Despite intense efforts to fully understand adipogenesis, however, an effective approach or drug to prevent and treat obesity is still lacking (10).

Much has been learned about obesity through studying rare and/or specific syndromes associated with obesity. For example, the leptin-deficient (ob/ob) mouse led

to the molecular definition of hypothalamic appetite regulation. Leptin is a hormone that is secreted primarily by adipose tissue and responsible for regulating feeding behavior. It is also a commonly used marker for total body fat and positively associated with body mass index (BMI). Elevated leptin levels play a central role in weight control through suppressing food intake and increasing energy expenditure (11). Scientific reports have shown iron plays a role in the regulation of appetite. Dietary iron supplementation is associated with growth and increased appetite in children (12, 13). The mechanism underlying this association is poorly understood. Low serum leptin levels have been linked to tissue iron overload (14, 15), and obesity is epidemiologically associated with iron deficiency (16). However, the possible mediators of the associations among iron and leptin levels are unknown.

On the other hand, iron deficiency is more prevalent in obese compared to normal weight individuals. If leptin is responsive to iron, decreased leptin in high iron consumption but lower adiposity may hide the association of iron and leptin. However, the adipocytes of β thalassemia major patients also fail to maintain adequate leptin production (17). The association between BMI and serum leptin levels is dampened or even abolished in transfusion-treated thalassemic patients (18), arguing iron may play a causal role in serum leptin independent of body weight. Dietary-induced obesity *in vivo* may be the outcome of increased leptin with decreased iron storage and leptin resistance (19). Furthermore, the clinically used biomarker for total body iron stores, soluble transferrin receptor (sTfR), is negatively associated with serum leptin (20). Serum ferritin, an iron storage protein, correlates significantly with appetite score ($r=0.680$, $P <0.001$) and food intake ($r =0.480$, $P < 0.01$) (21), suggesting iron may play a role in feeding behavior through reducing serum leptin.

In this introduction, I will review studies linking iron and adipocyte. I will describe iron homeostasis at the whole body level, and the relationship of iron, insulin resistance,

and obesity. Next, I will review the role of adipocytes in feeding behavior regulation, with particular emphasis on the satiety hormone, leptin. I will conclude by reviewing iron regulation in the adipocyte and the role that iron may play in the regulation of leptin.

Iron homeostasis

Iron is an essential constituent of many macromolecules involved in cell metabolism. Mammalian cells require iron for survival and proliferation. In general, an adult stores about 1 to 3 grams of iron in his or her body, and a balance between dietary uptake and loss is precisely regulated in healthy humans. About 1 to 3 mg of iron is lost daily through bleeding (e.g. menstruation in adult females) and sloughing of cells from skin and mucosal surfaces in the lining of the gastrointestinal tract (22, 23). However, there is no defined physiological means of active iron excretion. If intake of iron from the diet is not sufficiently compensated by loss of iron, a state of iron overload develops over time. Therefore, exquisite manipulation of iron absorption is crucial for the regulation of iron stores. Much of the iron in the lumen of the duodenum is in the ferric form. However, iron is mainly absorbed in mature villous enterocytes of the duodenum and upper jejunum via the brush border iron transporter divalent metal transporter 1 (DMT1) in the form of ferrous ion (24). Therefore, ferric ion must be reduced before it can enter enterocytes. The reduction of iron is probably carried out by the brush border enzyme, ferric reductase. Once iron enters enterocytes, it can be readily exported to the circulation through the iron transport protein ferroportin 1 (Fpn1), which is also the main iron export transporter in other cell types, including macrophages. When iron levels are high in the circulation, iron in enterocytes will not be transferred to the bloodstream because of reduction of ferroportin at the cell surface. The iron is then incorporated into the iron storage molecule, ferritin, and is lost when the cell is sloughed in the intestine. The circulating molecule that decreases ferroportin

expression in macrophages and enterocytes is hepcidin, which is mainly secreted from the liver. Hepcidin causes endocytosis and degradation of ferroportin, leading to sequestration of iron in enterocytes and protection from excess iron entering the circulation (25) (26). The so-called hepcidin-ferroportin axis is thus tightly regulated.

Adipokines and insulin sensitivity

Adipokines are a group of growth factors, hormones, and cytokines produced by mature adipocytes. Many of those factors are involved in diverse processes in the body, including glucose and lipid homeostasis, modulation of inflammatory responses, body weight control, blood pressure, and other diabetes-associated alterations such as inflammation (27).

People with metabolic syndrome—a collection of disorders including obesity, insulin resistance, hypertension, and abnormal glucose and lipid metabolism—display a characteristic imbalance of their adipokine profile. This altered adipokine profile functions through autocrine, paracrine, and endocrine mechanisms to lead to increased inflammation, decreased insulin sensitivity, and other metabolic disorders when the adipocytes are stressed by nutrient overload and obesity.

Two major adipocytokines, leptin and adiponectin, play a vital role in the regulation of metabolic homeostasis. Leptin acts directly on the leptin-receptor in the hypothalamus, thereby decreasing food intake and increasing energy expenditure (28). Plasma leptin concentrations are significantly elevated in obese people in proportion to BMI (29), and the chronically elevated results in leptin resistance may play a role in the pathogenesis of obesity-related complications (30). Obese subjects also preferentially secrete a low molecular weight isoform of adiponectin, which is not as effective as the larger isoforms, which also contributes to low rates of fat oxidation, failure to clear triglyceride and resulting lipid deposition in muscles and liver that contribute to

decreasing insulin sensitivity (31).

Metabolic syndrome is characterized by leptin resistance and low adiponectin, and thus decreased action of both hormones. Leptin and adiponectin activate AMPK and facilitate glucose uptake in muscle and repress gluconeogenesis in liver (32, 33). AMPK then phosphorylates and inhibits acetyl coenzyme-A carboxylase (ACC) in skeletal muscle and liver which results in lower rates of fatty acid synthesis. AMPK also activates peroxisome proliferator–activated receptor (PPAR) α and its targeted genes involved in peroxisomal and mitochondrial β-oxidation such as ACO, CPT1, and FABP3(34-37). Therefore, leptin and adiponectin levels serve as indices for metabolic homeostasis. The changes in each individual adipokine are the result of one or more changes of specific transcriptional programs that affect adipocyte gene control.

Iron overload and diabetes

It is well established that iron overload is associated with high risk of diabetes, including hyperglycemia, insulin resistance, chronic inflammation, and altered levels of adipokines (38). The first evidence showing increased iron deposition could contribute to hyperglycemia derived from the observation that the incidence of diabetes is higher in classic hereditary hemochromatosis (HH) (39, 40). Conversely, a causal role of iron overload in increasing insulin sensitivity was suggested in a cohort of subjects with frequent blood donation and decreased iron stores (41, 42). The role of iron in the pathogenesis of diabetes was further reinforced by the finding of an increased incidence of T2DM in increased iron deposition and improvement in glucose control with either phlebotomy or iron chelation therapy in a diverse set of medical conditions. For example, transfusional iron overload is a common cause of acquired iron overload, such as is seen in β-thalassemia patients. Impaired glucose tolerance (8.5%) and diabetes have high prevalences in transfusion-dependent β-thalassemic patients, 8.5%

and 19.5%, respectively (43). One risk factor for impaired glucose tolerance and T2DM was high serum ferritin.

Our laboratory has also found that iron overload results in elevated beta-cell oxidative stress and apoptosis, and desensitization of glucose-induced insulin secretion from mouse pancreatic islets, all of which result in decreased insulin secretory responses (44, 45). Conversely, impaired insulin secretory capacity may be attenuated by therapeutic iron chelation (46). However, the insulin secretion defect caused by iron overload is compensated by changes in insulin sensitivity such that these mice demonstrate normal glucose tolerance (45, 47, 48).

Iron deficiency and obesity

Obesity and iron deficiency are two common nutritional and health problems in the world and major cofactors in many other diseases. Obesity is a reflection of energy imbalance between expenditure and intake in the forms of increased fat accumulation. Excess body fat is correlated with increased all-cause mortality and increased risk for several medical morbidities (49). The foremost medical consequence of obesity is cardiovascular disease (CVD), which is also the leading cause of death in this country (50). It is urgent to develop new and effective strategies in controlling obesity and find potential targets for weight control.

Several studies have shown a greater prevalence of iron deficiency in overweight and obese people than normal-weight ones (51-55). Analysis of public-use data files indicated there is a negative relationship between levels of iron blood content and individual BMI after controlling for other individual characteristics (56).

The etiology of iron deficiency-associated obesity remains unknown. Several hypotheses have been put forth to explain the occurrence of obesity in iron deficiency. Some have suggested that decreased iron is a consequence, not a cause of obesity,

related to: (i) decreased iron intake associated with obesogenic diets or lifestyles; (ii) impairment of intestinal iron absorption and a reduced response to iron fortification in obesity (57); (iii) reduced iron release from iron stored cells and iron bioavailability (58, 59). Other studies indicate that iron may play a causal role in obesity. One report, for example, showed rats on an iron-deficient diet gained more fat mass than paired mice fed an iron-replete diet (60). We have investigated the mechanisms of iron's effects on lipid and carbohydrate metabolism in the liver and muscle. Muscle from mice on a low iron diet, for example, exhibits increased fatty acid synthesis but decreased lipolysis and fatty acid oxidation (61). On the other hand, iron overload mice change fuel usage from glucose to fatty acid oxidation in the muscle. Decreased glucose uptake and increased liver glucose production contribute to hyperglycermia in iron overloaded mice (62).

Hereditary hemochromatosis, type I

Hereditary hemochromatosis (HH), an autosomal recessive disorder of iron overload, is one of the most common genetic diseases in the Caucasian population. Symptoms are usually nonspecific or absent in the early development of HH, but progressive tissue iron overload may cause irreversible organ damage as patients age. A missense mutation in the HFE gene (C282Y) comprises up to 90% of HH cases (63). Mutation in HFE gene results in extremely low serum hepcidin levels, resulting in unregulated iron absorption from the gut and increased serum ferritin and transferrin saturation. Typical treatment for HH is phlebotomy or the use of iron chelators, with the frequency of treatment being guided by serial measurement of the above two indicators. The liver is the main organ that recycles iron from senescent erythrocytes and hepatocytes storage pools and releases iron according to the body's needs. It also secretes the hormone peptide hepcidin that controls iron absorption in enterocytes. The

regulation of hepcidin expression is the central point for maintaining the iron balance. Under low iron conditions, the HFE protein is associated with transferrin receptor 1 (TFR1). When iron level goes up, transferrin-Fe complex competes with HFE for the binding site in the TFR1 since they have the same binding site, while HFE then binds with transferrin receptor 2 (TFR2), which recruits the hemojuvelin (HJV)-SMAD signaling pathway (64, 65). Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and they signal through BMP receptors (BMPRs) to cytoplasmic SMAD protein transcription factors. Interestingly, HJV is a BMP co-receptor. Disruption or mutation of HJV also leads to hemochromatosis and reduction of hepcidin, indicating HJV's BMP signaling is critical for the regulation of hepcidin (66). Phosphorylation of serine/threonine kinase in BMPRs activates SMADs, including SMAD1, SMAD4, and SMAD8, causing translocation of SMADs to the nucleus and increase of the expression of target genes containing SMAD-binding elements. One of the most important genes is HAMP gene, which encodes hepcidin protein (Figure 1.1).

Obesity and leptin resistance

Obesity is an over-development of adipose tissue, with both hyperplasia (increased cell number) and hypertrophy (increased cell size) contributing to the excess fat (67, 68). Recently, efforts and progress have been made in understanding the process of adipocyte differentiation. These studies have laid the foundation for the understanding of the cellular and molecular basis of adipose tissue growth in physiological and/or patho-physiological states and provide a potential target to develop therapeutic strategies for the treatment and prevention of obesity. It has also been reported that transformation of adipocytes from cells that store triglycerides to cells that oxidize fatty acids results in profound loss of fat (69). Those transformed adipocytes, named BRITE (BRown-like to whITE), can be induced by hyperleptinemia and are

characterized by increased expression of enzymes of fatty acid oxidation and uncoupling proteins accompanied by loss of the adipocyte markers, tumor necrosis factor α and leptin. However, hyperleptinemia by itself does not eliminate excess fat in obese mice, indicating resistance of leptin action in obesity (70).

Leptin normally exerts negative feedback effects on food intake and positive feedback on energy expenditure (28, 71, 72). Leptin acts through the leptin receptor (ObR) (73). Animals with diet-induced obesity, however, are not leptin deficient. In contrast, they usually present high circulating leptin concentrations. Furthermore, leptin treatment of obesity fails to reduce energy intake and increase energy expenditure. This state is termed leptin resistance.

There are two dominant hypotheses that have been advanced to explain leptin resistance. One is that circulating leptin fails to transport to the brain to reach its target (74, 75), and the other is the failure of the leptin and leptin receptor signaling cascade (76). However, the detailed mechanism of leptin resistance is still not clearly investigated. Leptin resistance has been widely reported to be a consequence of obesity (77, 78), although emerging evidence also suggests that leptin resistance may not be purely a result of obesity, but also involved in its causation. For example, overexpression of leptin in specific tissues can result in induction of early leptin resistance (79), and inherent central leptin insensitivity contributes to dietary weight gain in certain obesity-prone rats (80). Therefore, more studies are required to explore the relationship between obesity and leptin.

Leptin and iron

Studies, both in healthy humans and patients with iron overload, have reported that high ferritin is associated with a decrease in plasma leptin levels (15). Another clinical marker for total body iron stores, soluble transferrin receptor (sTfR), is also

negatively associated with serum leptin (20). The adipocytes of patients with β thalassemia major, who are iron overloaded because of numerous blood transfusions, fail to maintain adequate leptin production (14, 15, 17, 20). In addition, we have also recently demonstrated that another adipokine, adiponectin, is regulated by iron, so there is precedent for possible regulation of leptin (81). As discussed in a previous section, epidemiologic studies suggest a relationship between iron deficiency and increased rates of obesity. We hypothesize that leptin deficiency and/or resistance may provide one link between dietary iron and obesity. If leptin is also responsive to iron, increased leptin in the population with low iron consumption but higher adiposity may hide the association of iron and leptin, since the high leptin could result from either. However, the association between BMI and serum leptin levels is dampened or even abolished in transfusion-treated β thalassemic patients (18), arguing iron may play a causal role in contributing to the levels of serum leptin. Dietary-induced obesity association *in vivo* thus may be the outcome of increased leptin with decreased iron storage and leptin resistance (19). Furthermore, serum ferritin, an iron storage protein, correlates significantly with appetite score ($r = 0.680$, $P < 0.001$) and food intake ($r = 0.480$, $P < 0.01$) (21), suggesting iron may play a role in feeding behavior through decreasing serum leptin.

Hexosamine biosynthesis pathway (HBP)

The HBP is a glucose metabolic pathway, usually accounting for only 2–5% of total glucose metabolism. The major product is UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is the unique donor for the modification of many cytoplasmic and nuclear proteins by O-linked GlcNAcylation, wherein a single N-acetylglucosamine molecule is transferred to serine or threonine (O-GlcNAc). Glucose entering the cytoplasm is first phosphorylated to glucose-6-phosphate (G6P) and then isomerized to

fructose-6-phosphate (F6P). The latter is then diverted to the HBP by the first and rate-limiting enzyme of the pathway, glutamine: fructose-6-phosphate amidotransferase (GFAT) that catalyzes the synthesis of glucosamine-6-phosphate. Subsequently, the addition of an acetyl moiety from acetyl-CoA yields N-acetyl glucosamine-6-phosphate, and the latter is rapidly modified to UDP-GlcNAc. Thus, substrate production in the HBP reflects cellular amino acid, fatty acid, and nucleotide metabolism, and there is evidence that it is rate limited at least by glucose and uridine. UDP-GlcNAc is then used as the substrate for the O-GlcNAcylation modification of proteins catalyzed by O-GlcNAc transferase (OGT). This modification is reversible and the removal of O-GlcNAc is via O-GlcNAcase (OGA). The HBP is precisely regulated via negative feedback circuits. The accumulated end product, UDP-GlcNAc, decreases GFAT activity and also modulates the affinity of OGT for individual substrates (82). Sites that are targets of O-GlcNAcylation are at or near sites of O-phosphorylation. Extensive crosstalk exists between O-GlcNAcylation and phosphorylation in organisms and contributes to signaling change in response to nutrients and a variety of stressors (83). While phosphorylation and dephosphorylation are catalyzed by a multitude of specific and distinct kinases and phosphatases, O-GlcNAcylation and its removal are catalyzed by products of single genes for OGA and OGT in mammals. O-GlcNAcylation cycles on and off nucleocytoplasmic proteins in response to different physiological stimuli, such as hormones, growth factors, and mitogens (84). About 600 targets have been identified, but little is known about the functional significance of their modification (85). O-GlcNAc directly regulates the activities of a variety of transcription factors, including Sp1 (86), CREB (87), members of the fork-head (FOXO) family (88), and others. Those changes may contribute to development of insulin resistance (89), glucose toxicity(90), heart failure (91), and memory loss (87), but a direct role and/or mechanisms remain to be established.

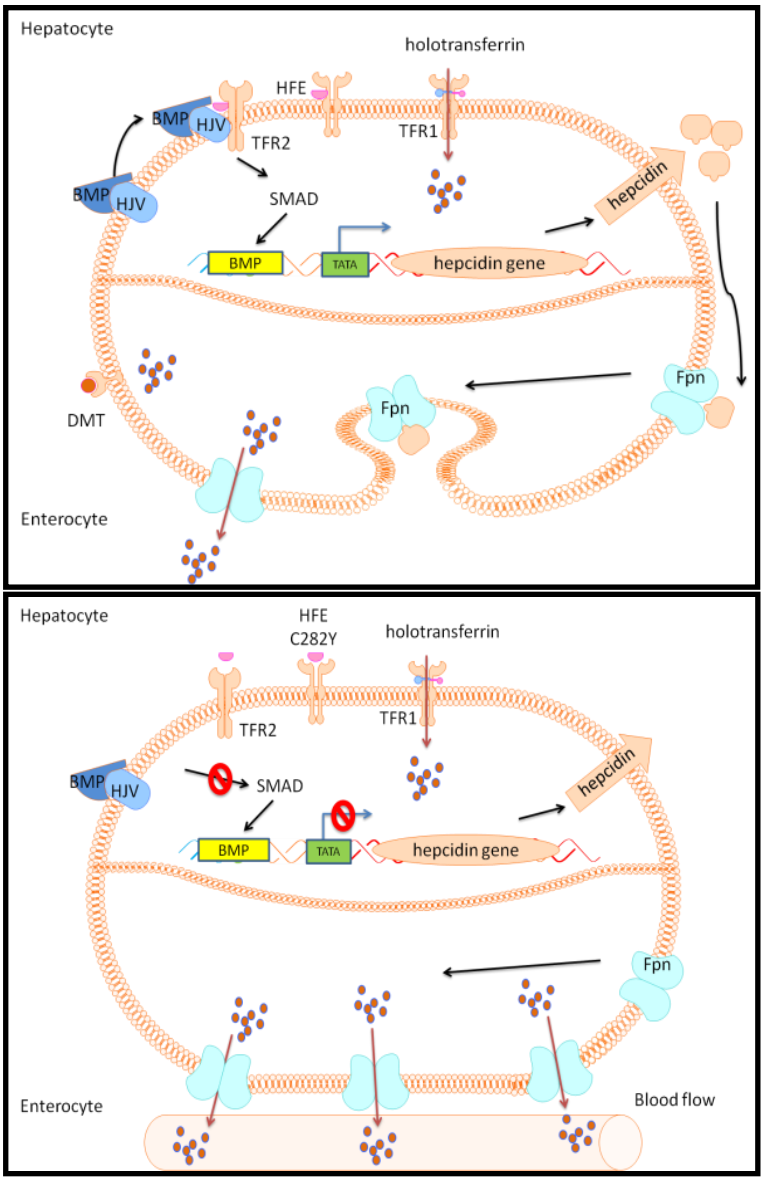

Figure 1.1 Schematic diagram shows the molecular pathogenesis for hereditary hemochromatosis.

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CHAPTER 1

ADIPOCYTE IRON REGULATES ADIPONECTIN THROUGH ACTIVATION OF FOXO*

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Research article

Adipocyte iron regulates adiponectin and insulin sensitivity

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Iron overload is associated with increased diabetes risk. We therefore investigated the effect of iron on adiponectin, an insulin-sensitizing adipokine that is decreased in diabetic patients. In humans, normal-range serum ferritin levels were inversely associated with adiponectin, independent of inflammation. Ferritin was increased and adiponectin was decreased in type 2 diabetic and in obese diabetic subjects compared with those in equally obese individuals without metabolic syndrome. Mice fed a high-iron diet and cultured adipocytes treated with iron exhibited decreased adiponectin mRNA and protein. We found that iron negatively regulated adiponectin transcription via FOXO1-mediated repression. Further, loss of the adipocyte iron export channel, ferroportin, in mice resulted in adipocyte iron loading, decreased adiponectin, and insulin resistance. Conversely, organismal iron overload and increased adipocyte ferroportin expression because of hemochromatosis are associated with decreased adipocyte iron, increased adiponectin, improved glucose tolerance, and increased insulin sensitivity. Phlebotomy of humans with impaired glucose tolerance and ferritin values in the highest quartile of normal increased adiponectin and improved glucose tolerance. These findings demonstrate a causal role for iron as a risk factor for metabolic syndrome and a role for adipocytes in modulating metabolism through adiponectin in response to iron stores.

Introduction

Increased iron stores are associated with increased risk of type 2 diabetes (1–4), gestational diabetes (5), prediabetes (6), metabolic syndrome (MetS) (7), central adiposity (8), and cardiovascular disease (9, 10). The mechanisms underlying these associations are poorly understood. The commonly used marker for total body iron stores, serum ferritin, is also responsive to inflammatory stress (11, 12), so increased ferritin in diabetes could simply reflect the inflammatory component of that disease (13). On the other hand, phlebotomy improves glycemia and MetS traits (14–17), arguing that iron may play a causal role in diabetes.

The possible mediators of the association between iron and diabetes risk are not known. Decreases in both insulin secretion and sensitivity have been linked to iron. Excess iron impairs pancreatic β cell function and causes β cell apoptosis (18–21). Recent studies have also found a negative correlation between serum ferritin and the insulin-sensitizing adipokine, adiponectin (3, 22–24). The hypothesis that adiponectin links iron and insulin resistance is appealing, as decreased adiponectin levels are associated with obesity and type 2 diabetes (25) and are causally linked with insulin resistance (26).

We therefore investigated the mechanisms underlying the relationships among serum ferritin, adiponectin, and MetS in mice and humans. We demonstrate in humans that the association between serum ferritin and adiponectin is independent of inflammation and that serum ferritin, even within its normal ranges, is among the best predictors of serum adiponectin. Studies in cell culture, mouse models, and humans demonstrate that iron plays a direct and causal role in determining adiponectin levels and diabetes risk. The adipocyte expresses specialized proteins related to iron metabolism that make it well suited to perform as an iron sensor, allowing it to integrate iron availability into its broader nutrient-sensing function.

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Results

Human ferritin levels are inversely associated with serum adiponectin independently of inflammation. We studied 110 individuals with (n = 49) and without (n = 61) diabetes recruited for an independent study of metabolic flexibility (27). Serum ferritin was negatively associated with serum adiponectin (r = –0.294, P = 0.0017). To mitigate the effects of inflammation and/or extreme iron overload and anemia, we next restricted the analysis to individuals with normal serum ferritin (men, >30 ng/ml and <300 ng/ml; women, <200 ng/ml) (28, 29). Excluding 28 individuals outside this range strengthened the association between ferritin and adiponectin (Figure 1A, r = –0.385, P = 0.0003). Serum levels of the inflammatory marker C-reactive protein (CRP) were elevated in diabetic subjects compared with those in nondiabetics (log[CRP] 3.40 ± 0.07 vs. 3.01 ± 0.08, P = 0.0005). Nonetheless, multivariate analysis, including CRP, BMI, and diabetes status, had little effect on the ferritin-adiponectin association (Table 1). There was no association of IL-6 or TNF-α levels with serum ferritin (r = –0.13, P = 0.29 and r = –0.010, P = 0.94, respectively) or adiponectin (r = 0.085, P = 0.51 and r = 0.13, P = 0.35, respectively).

Inclusion of gender in the analysis affected the serum ferritin and adiponectin association (Table 1). The relationship was weaker in women, likely because the range of ferritin values was significantly narrower and lower compared with that in men (average ferritin, 75.3 ± 10.5 ng/ml in women and 183.6 ± 20.9 ng/ml in men, P < 0.0001) (30). Overall, however, and consistent with previous reports (31), these lower ferritin levels were accompanied by higher adiponectin levels in women (3.42 ± 0.26 μg/ml vs. 2.39 ± 0.14 μg/ml

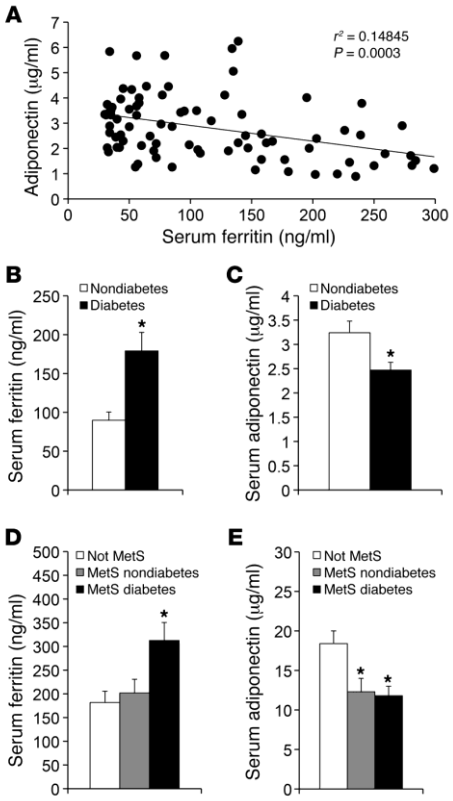


Figure 1
Serum ferritin levels are inversely associated with serum adiponectin ferritin levels and predict presence or absence of MetS. (**A**) Serum adiponectin and ferritin levels were measured and correlated in a cohort of subjects with normal ferritin. $P = 0.0003$. (**B**) Ferritin levels in the same subjects subdivided by diabetes status. *$P < 0.0004$. (**C**) Adiponectin levels in the same subjects subdivided by diabetes status. *$P < 0.0012$. (**D**) Ferritin levels in the obese subjects without MetS (white bars) or with MetS, without or with diabetes (gray and black bars, respectively). *$P < 0.03$ for the diabetic subgroup compared with either other group. (**E**) Adiponectin levels in the subset of the subjects described **D** in whom adiponectin levels were determined. *$P < 0.03$ for either MetS subgroup compared with the non-MetS group.

in men, $P = 0.0006$). In men, after adjusting for BMI and diabetes status, serum ferritin remained significantly associated with adiponectin (Table 1, $P = 0.04$) but was reduced to a trend after adjusting for CRP (Table 1, $P = 0.10$). Serum ferritin levels in men, however, remained more predictive of adiponectin levels than BMI, CRP, and diabetes status (Table 1, see *Relative contribution of variables*). In women, none of the variables were significantly associated with adiponectin in the multivariate analysis.

To further explore the physiological significance of the correlation between iron and adiponectin, we subdivided the cohort by diabetes status. In the subjects with diabetes, serum ferritin levels were 2-fold higher (Figure 1B, $P = 0.0004$) and adiponectin levels were 24% lower (Figure 1C, $P = 0.012$). We also determined that, in the entire cohort with normal ferritin levels, insulin sensitivity, as determined for the original study (27) by the glucose clamp technique, was inversely correlated with ferritin ($r = 0.365$, $P = 0.0003$, $n = 93$) and directly correlated with adiponectin levels ($r = 0.354$, $P = 0.0004$, $n = 95$).

Ferritin predicts adiponectin and the presence of MetS in obese men. To extend and validate these findings in a separate cohort, we measured serum adiponectin and ferritin levels of obese men, with or without MetS (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI44421DS1). BMI was similar between the groups (39.6 ± 0.7 kg/m^2 vs. 39.7 ± 0.6 kg/m^2). The MetS group had all 5 features of the syndrome: obesity, hypertension (>130/85 mmHg), HDL cholesterol <40 mg/dl, serum triglycerides >150 mg/dl, and either fasting glucose >100 mg/dl or diabetes (32). Those without MetS had no more than 1 factor in addition to obesity, and none had diabetes. Serum ferritin was measured in all subjects ($n = 125$), and adiponectin was measured in a randomly selected subset ($n = 38$). Overall, the MetS group had significantly higher ferritin (260 ± 23 ng/ml vs. 185 ± 21 ng/ml, $P < 0.01$) and lower adiponectin (11.5 ± 1.0 μg/ml vs. 18.9 ± 1.9 μg/ml, $P < 0.005$) than the non-MetS group. Higher ferritin values were seen in the MetS subset with diabetes compared with those in either the non-MetS group or the MetS subgroup without diabetes (Figure 1D, $P < 0.03$). Adiponectin was higher in the non-MetS group compared with that in either MetS subgroup (Figure 1E, $P < 0.03$). Consistent with the data in Figure 1A, ferritin was inversely correlated with adiponectin in this cohort, although in this smaller group the relationship did not quite reach the level of statistical significance ($r = 0.304$, $n = 38$, $P = 0.06$, data not shown). Insulin resistance estimated from the homeostasis model (HOMA-IR) was correlated positively with ferritin ($r = 0.264$, $n = 125$, $P < 0.01$) and negatively with adiponectin ($r = 0.48$, $n = 38$, $P < 0.005$).

Adipocyte iron increases and adiponectin mRNA and serum protein levels decrease in dietary iron overload. Several studies have identified effects of iron on adipocyte metabolism (e.g., refs. 33, 34). To explore the regulation of adipocyte iron levels, we first demonstrated that adipocyte iron levels respond to dietary iron content in WT C57BL6/J mice by measuring mRNA levels of the transferrin receptor (*Tfrc*). *Tfrc* mRNA contains iron response elements in its 3′ untranslated region that result in decreased *Tfrc* mRNA levels as cellular iron levels increase (35). We observed a 40% decrease in *Tfrc* mRNA in adipocytes from mice fed a high-iron diet (20 g/kg iron) compared with that in mice fed normal chow (330 mg/kg iron) (Figure 2A, $P < 0.05$). Technical difficulties precluded the direct assay of cytosolic iron in isolated adipocytes. To investigate a possible direct and causal role of iron in the regulation of adiponectin, we studied the effects of dietary iron overload on serum adiponectin levels in mice. We fed 129SvEvTac male mice high- (20,000 mg/kg carbonyl iron), normal (330 mg/kg), or low- (7 mg/kg) iron diets for 2 months. Body weights were significantly lower in mice fed high-iron diets and significantly higher in those fed low-iron diets compared with those of mice fed normal chow (low iron, 34.6 ± 1.1 g; normal chow, 29.0 ± 0.5 g; high iron, 26.7 ± 0.6 g, $P < 0.001$ between groups by ANOVA, $n = 8$–12/group). Despite decreased body weight, serum adiponectin levels were 29% lower in iron-overloaded mice (Figure 2B, $P = 0.0002$). Conversely, dietary iron restriction increased serum adiponectin levels by 31% despite increased body weight (Figure 2B, $P = 0.0036$). A similar 29% decrease in serum adiponectin was also seen in a different strain,

Table 1
Multiple regression models for the relationship of adiponectin to ferritin

Statistical model	Variables	Ferritin correlation coefficient	Ferritin *P* value	*t* ratio	*P* value
Multiple regression, men and women (n = 83)	Ferritin	0.006228	0.0004		
	Ferritin/log(CRP)	0.006102	0.0004	–	–
	Ferritin/log(CRP)/BMI	0.006124	0.0003	–	–
	Ferritin/log(CRP)/BMI/diabetes	0.005834	0.0006	–	–
	Ferritin/log(CRP)/BMI/diabetes/gender	0.002041	0.23	–	–
Multiple regression, men only (n = 47)	Ferritin	0.003887	0.021	–	–
	Ferritin/BMI	0.003567	0.033	–	–
	Ferritin/BMI/diabetes	0.003423	0.039	–	–
	Ferritin/BMI/diabetes/log(CRP)	0.002770	0.10	–	–
Relative contribution of variables, men only	Ferritin	–	–	1.681	0.10
	Log(CRP)	–	–	1.485	0.15
	Diabetes	–	–	1.012	0.32
	BMI	–	–	0.441	0.66

Multivariate analysis of the relationship between ferritin and adiponectin corrected for the variables of C-reactive protein (log CRP), body mass index (BMI), diabetes status, and gender. Results are presented for the entire cohort and for men only, with the relative contribution of variables also indicated for the men-only cohort.

namely male C57BL6/J mice fed normal chow and high-iron diets (6.46 ± 0.15 μg/ml vs. 4.55 ± 0.16 μg/ml, respectively, $P < 0.0001$). Adiponectin mRNA levels in isolated epididymal adipocytes were 30% lower in iron-overloaded mice (Figure 2C, $P = 0.07$), mirroring the changes in serum adiponectin levels. With a smaller cohort of high-iron diet– and normal chow–fed mice (n = 4/group), we determined body composition by magnetic resonance imaging, and the high-iron diet caused not only a decrease in weight but an even larger relative effect on fat mass because of a parallel increase in lean body mass (Figure 2D). Both food intake and oxygen consumption rates were higher in the lower-weight, high-iron group (Figure 2D). The differences in weight seen with the high-iron diet were not observed in mice with genetic deletion of adiponectin (Figure 2E). The expected inverse linear relationship between weight and adiponectin was observed in mice fed normal chow ($r = 0.48$, $n = 22$, $P = 0.02$, not shown), but this relationship was lost in mice fed the high-iron diet, in fact even trending toward a positive relationship ($r = 0.17$, $n = 17$, $P = 0.51$).

Finally, to demonstrate that the change in adiponectin was accompanied by changes in glucose metabolism, we performed hyperinsulinemic clamp studies on WT mice on normal chow and those that had been on the high-iron diet for 8 weeks. There was a trend toward lower glucose disposal, normalized to total body weight, in the mice fed high-iron diet (Figure 2F, $P = 0.22$). However, because most glucose uptake at hyperinsulinemia is into skeletal muscle, we also normalized to lean mass based on the magnetic resonance imaging findings, and the mice on high-iron diet had a significant decrease in their maximal glucose disposal rate per gram of lean tissue ($P < 0.001$).

Iron decreases adiponectin transcription. To demonstrate further that the decreased adiponectin mRNA levels are due to decreased transcription and that iron regulates adiponectin directly, we examined the effects of iron on adiponectin in a cell culture model. Treatment of 3T3-L1 adipocytes with iron sulfate decreased media adiponectin protein levels in a dose-dependent manner (Figure 3A, $P < 0.0001$). Adiponectin mRNA levels also decreased 30% with iron treatment (Figure 3B, $P = 0.02$). We measured luciferase activity driven by the proximal 1,460 bp of the murine adiponectin promoter, which contains most of the previously identified sites that regulate adiponectin transcription (36). Iron decreased promoter activity by 28% (Figure 3C, $P = 0.0025$). Iron did not decrease the half-life of the endogenous mRNA or the reporter construct measured after actinomycin C or cycloheximide D treatment of cells (data not shown). Most physiologic regulation of adiponectin gene transcription is attributable to the factors FOXO1 and PPARγ (36, 37). To explore the mechanism of regulation of adiponectin by iron, we first examined posttranslational modification of FOXO1. Iron caused decreased acetylation of FOXO1 without changing its level of phosphorylation or total protein (Figure 3, D and E). In agreement with the lack of change of FOXO1 phosphorylation, we also detected no differences in basal or insulin-stimulated phosphorylation of AKT in iron-treated cells (Figure 3F).

Contrary to the observed effects of iron on adiponectin transcription, deacetylation of FOXO1 is generally associated with increased adiponectin transcription (36, 38). We therefore measured FOXO1 occupancy at its 2 known sites of transcriptional activation using ChIP. As predicted by FOXO1 acetylation status, cells treated with iron exhibited a 3.1-fold increase in occupancy by FOXO1 at the sites reported to stimulate adiponectin transcription ($P < 0.01$, Figure 3G). FOXO1, however, has also been reported to transrepress adiponectin transcription when associated with the PPARγ response element (PPRE) of the adiponectin promoter (39). Iron treatment resulted in a 3.5-fold enhancement of FOXO1 binding to the PPRE ($P = 0.01$, Figure 3G). There was no significant increase in PPARγ binding to the PPRE (1.6 fold, $P = 0.07$, Figure 3G). Because the interaction of C/EBPα with FOXO1 has also been implicated in regulation of adiponectin transcription (36), we also measured the association of C/EBPα with FOXO1 by coimmunoprecipitation but saw no effect of iron on this association ($P = 0.93$, Figure 3H).

Adipocytes express the specialized iron channel ferroportin. Adipocytes express genes that are generally restricted to iron-sensing tissues (40, 41). We therefore sought to obtain evidence that adipocytes might have a specialized iron-sensing capacity by assessing adi-

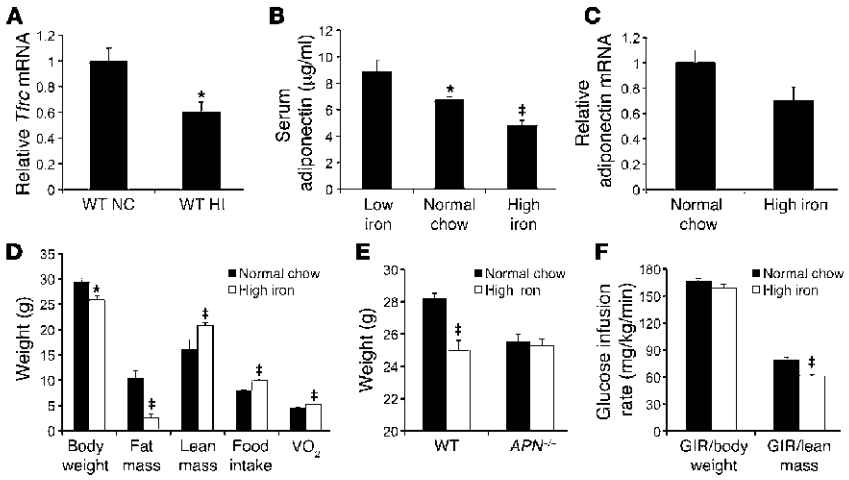


Figure 2

Serum adiponectin and adipocyte mRNA levels decrease with dietary iron overload and with iron treatment in 3T3-L1 cells. (**A**) *Tfrc* mRNA quantified by RT-PCR and normalized to cyclophilin in collagenased adipocytes from epididymal fat pads of mice fed normal chow (NC) or a high-iron diet (HI) for 8 weeks. $*P < 0.05$. (**B**) Serum adiponectin levels were measured in 7-month-old 129/SvEvTac background mice following 2 months of being fed low-iron (7 mg/kg carbonyl iron), normal chow (330 mg/kg), or high-iron (20 g/kg) diets. $*P = 0.004$, low iron vs. normal chow; $‡P = 0.0002$, high iron vs. normal chow. (**C**) Adiponectin mRNA levels in isolated epididymal adipocytes from mice fed high-iron diet or normal chow. $P = 0.07$. (**D**) Body weights were determined in C57BL6/J mice after 8 weeks on normal chow or high-iron diets, and body composition was determined by magnetic resonance imaging ($n = 6$/group or 8/group, $*P < 0.05$, $‡P < 0.01$). (**E**) Body weights were determined in mice with knockout of the adiponectin gene compared with those of controls after 8 weeks on normal chow or high-iron diets ($n = 5–12$/group, $‡P < 0.001$). (**F**) Euglycemic hyperinsulinemic clamps were performed on WT mice on normal or high-iron diets. Glucose infusion rates (GIRs) trended lower when normalized to total body weight ($P = 0.22$) but differed significantly when normalized to lean body mass ($‡P < 0.0005$).

pocyte expression of the iron export channel ferroportin, whose significant tissue expression has been reported to be limited to gut enterocytes, placenta, and reticuloendothelial cells, including macrophages (42, 43). Ferroportin mRNA and protein were detectable by quantitative RT-PCR and Western blot in differentiated 3T3-L1 adipocytes. Treatment with iron sulfate increased ferroportin mRNA in a dose-dependent manner (Figure 4A, $P < 0.0001$). Protein levels were responsive both to iron and to treatment by hepcidin, a ferroportin ligand that results in ferroportin downregulation (Figure 4B and ref. 44).

Deletion of adipocyte ferroportin results in increased adipocyte iron levels, decreased serum adiponectin, and increased insulin resistance. To demonstrate that ferroportin serves as a functional iron channel and exporter in adipocytes, we generated mice lacking the ferroportin gene in adipocytes (*Fpn1*$^{-/-}$ mice). An *Fpn1*$^{fl/fl}$ mouse (45), provided by Nancy C. Andrews (Duke University, Durham, North Carolina, USA), was crossed to a mouse expressing Cre recombinase under control of the 5.4-kB AP2 promoter. The *Ap2-Cre:Fpn1*$^{fl/fl}$ mice were subsequently backcrossed onto the 129 strain for at least 5 generations. Ferroportin mRNA was undetectable in adipocytes purified by collagenase digestion from *Fpn1*$^{-/-}$ mice (Figure 4C). Because macrophages can also express the *Ap2* gene, we examined ferroportin expression in splenocytes, wherein the only cell expressing significant ferroportin is the macrophage. There was no decrease in ferroportin mRNA in splenocytes from the *Ap2-Cre:Fpn1*$^{fl/fl}$ mice (Figure 4C).

To demonstrate a role for ferroportin in modulating adipocyte iron, we measured *Tfrc* mRNA in isolated adipocytes. Compared with WT *Fpn1*$^{fl/fl}$ adipocytes, *Ap2-Cre:Fpn1*$^{fl/fl}$ adipocytes exhibited a 44% decrease in *Tfrc* (Figure 4D, $P < 0.001$), consistent with increased cytosolic iron, and functionality of the ferroportin channel is in adipocytes.

The increased levels of adipocyte iron in the *Fpn1*$^{-/-}$ mice resulted in a 58% decrease in levels of adiponectin mRNA in adipocytes (Figure 4E, $P < 0.01$), and this was reflected in decreased serum adiponectin (Figure 4F). Because of the heterogeneity in the weights of the mice and the effect of weight on adiponectin, serum adiponectin was determined in a cohort of mice all weighing less than 30 g. In control (*Fpn1*$^{fl/fl}$) mice, the high-iron diet resulted in a 12% decrease in serum adiponectin (Figure 4F, $P < 0.05$). Serum adiponectin was also lower (13%, $P < 0.05$) in the *Ap2-Cre:Fpn1*$^{fl/fl}$ mice on normal chow compared with controls and did not decrease further in mice on the high-iron diet. No changes were noted in the distribution of adiponectin molecular weight isoforms, as analyzed by an adiponectin assay that detects both total and high molecular weight isoforms (Figure 4G) and as analyzed by native SDS-PAGE (data not shown).

To determine whether the change in adiponectin was physiologically significant, we performed glucose tolerance testing in WT and *Fpn1*$^{-/-}$ mice. *Fpn1*$^{-/-}$ mice had significantly higher glucose excursions at 30 and 60 minutes after challenge (Figure 4H, $P < 0.05$), and the areas under the glucose curve differed significantly between the groups (16,372 mg-min/dl in WT mice and 20,272 mg-min/dl in *Fpn1*$^{-/-}$ mice, 24% increase, $P < 0.001$, data not shown). Fasting glucose and insulin levels were also determined in WT and *Fpn1*$^{-/-}$ mice on different levels of dietary iron, and insulin resistance, as determined by homeostasis model assessment (HOMA-IR), was increased in the *Fpn1*$^{-/-}$ mice ($P < 0.01$,

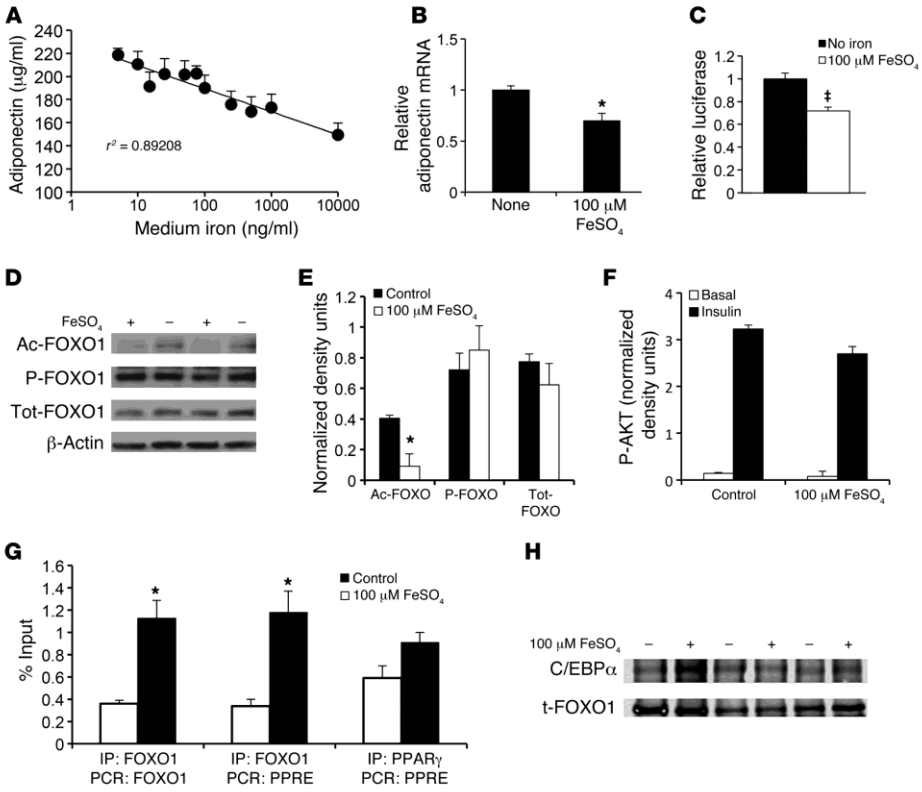


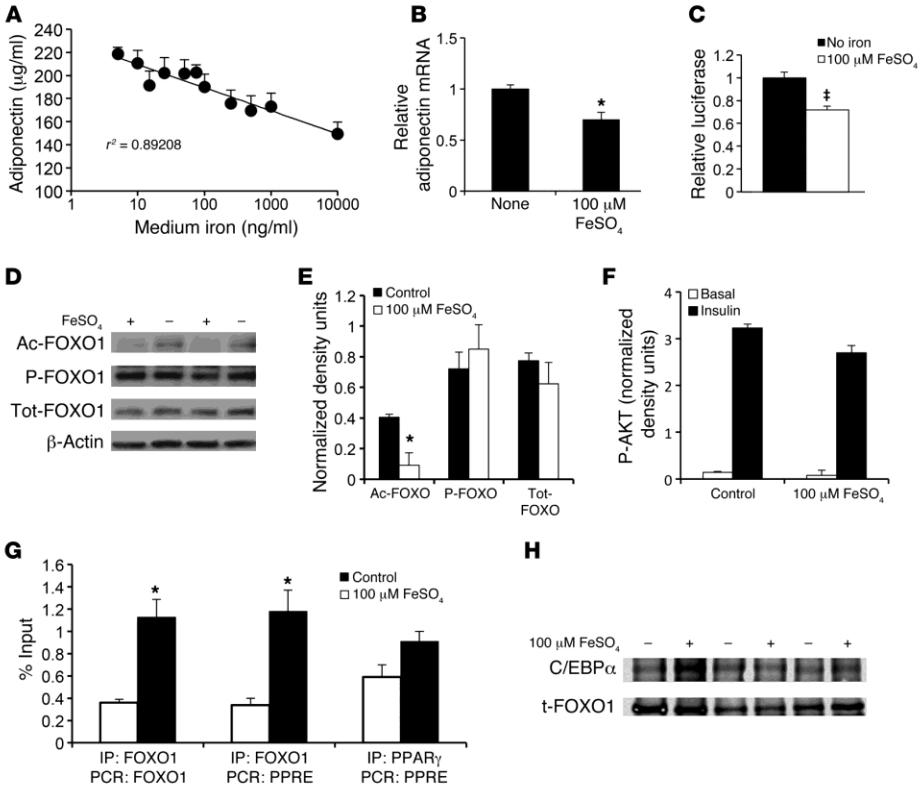
Figure 3

Transcriptional regulation of adiponectin by iron. (**A**) Media adiponectin levels in 3T3-L1 cells 12 hours following 12-hour pretreatment. $P < 0.0001$. (**B**) RT-PCR quantification of adiponectin mRNA levels in 3T3-L1 adipocytes treated with no iron or 100 μM $FeSO_4$ for 24 hours, normalized to cyclophilin A. *$P = 0.02$. (**C**) Adiponectin promoter-driven luciferase activity in the presence or absence of 100 μM $FeSO_4$. ‡$P = 0.0025$. (**D**) Western blot for acetylated FOXO1 (Ac-FOXO1), phosphorylated FOXO1 (P-FOXO1), total FOXO1 (Tot-FOXO1), and β-actin in 3T3-L1 adipocytes treated with no iron or 100 μM $FeSO_4$ for 8 hours. (**E**) Quantitation of Western blots (total $n = 6$ independent determinations) normalized to β-actin. *$P < 0.05$. (**F**) Quantitation of Western blots for phosphorylated AKT in 3T3-L1 adipocytes treated with no iron or 100 μM $FeSO_4$ for 8 hours and insulin (10 nM) for 1 hour. (**G**) ChIP showing FOXO1 occupancy of adiponectin promoter FOXO1 sites and PPRE and PPARγ occupancy of PPRE in 3T3-L1 adipocytes ($n = 3$ experiments each assayed in duplicate, *$P < 0.05$). (**H**) Immunoprecipitation of 3T3-L1 adipocyte extracts, treated overnight in the presence or absence of 100 μM $FeSO_4$, by antibodies to FOXO1, followed by immunoblotting for FOXO1 (t-FOXO1) and C/EBPα (0.58 ± 0.15 density units for control, 0.61 ± 0. 26 density units for iron-treated extracts, $P = 0.93$).

data not shown). The effects of high-iron diet on body weight and body composition were also lost in the *Fpn1*$^{-/-}$ mice. Body weights of the *Fpn1*$^{-/-}$ mice on normal chow compared with those of mice on high-iron diets did not differ (normal chow, 32.1 ± 1.5 g, high iron 31.5 ± 1.3 g, $P = 0.68$), and the changes in body composition induced by high-iron diet (Figure 2D) and replicated in a cohort of control *Fpn1*$^{fl/fl}$ mice (Figure 4I, $P < 0.05$ for both percentage of lean and fat mass) were also not seen in the *Fpn1*$^{-/-}$ mice (Figure 4I, $P = 0.37$ and $P = 0.56$ for percentage of lean and fat mass, respectively).

Lower adipocyte iron, higher serum adiponectin, and increased insulin sensitivity in hereditary hemochromatosis. The effects of iron on adiponectin levels present a paradox. Namely, adiponectin decreases with dietary iron overload, and yet serum adiponectin levels are increased in a mouse model of genetic iron overload, wherein the gene most commonly mutated in human hereditary hemochromatosis (HH) has been deleted (*Hfe*$^{-/-}$ mice) (46). It has been shown that the relative lack of hepcidin in HH results in failure to downregulate ferroportin, so that cells that express significant amounts of the iron channel are paradoxically less loaded with iron in HH (47, 48). We therefore sought to determine whether the same were true of adipocytes. *Tfrc* mRNA levels, which inversely reflect cytosolic iron, were increased by 67% in adipocytes from *Hfe*$^{-/-}$ mice compared with those from WT mice (Figure 5A, $P = 0.05$). Thus, the previously reported increased adiponectin in a mouse model of HH (46) is consistent with lower adipocyte iron levels.

Because humans with HH also trend toward increased insulin sensitivity prior to the onset of clinical diabetes (20), we hypothesized that serum adiponectin levels would likewise be increased in human HH. Serum adiponectin levels were increased by 89% in male subjects with HH, compared with non-HH, male sibling controls (Figure 5B, $P = 0.04$). In women, serum adiponectin lev-



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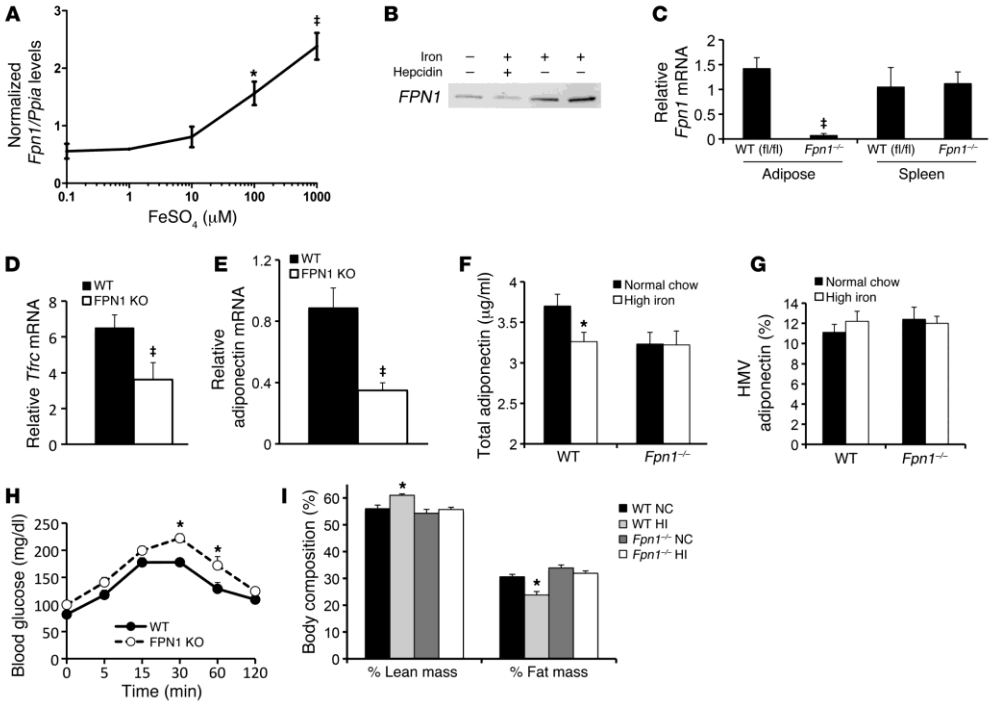

Figure 4

Functional expression of ferroportin in adipocytes. (**A**) *Fpn1* mRNA levels quantified by RT-PCR in 3T3-L1 adipocytes exposed to different concentrations of iron ($FeSO_4$) in the culture medium. **P* < 0.05 compared with 0.1 mM, ‡*P* < 0.01, *P* < 0.0001 for overall trend. (**B**) FPN1 protein levels in 3T3-L1 adipocytes, as detected by immunoblotting in adipocytes treated with no iron, 100 μM $FeSO_4$, or 1 μg/ml hepcidin for 8 hours. (**C**) *Fpn1* mRNA in adipose tissue and spleen from WT (*Fpn1*$^{fl/fl}$) and *Fpn1*$^{-/-}$ (AP2 *Cre* ferroportin knockout) mice. ‡*P* < 0.001. (**D**) *Tfrc* mRNA quantified by RT-PCR and normalized to cyclophilin in collagenased adipocytes from epididymal fat pads of WT and *Fpn1*$^{-/-}$ mice (*n* = 10–14/group, ‡*P* < 0.001). (**E**) Adiponectin mRNA in the adipocytes used in **E**. ‡*P* < 0.01. (**F**) Serum adiponectin in WT and *Fpn1*$^{-/-}$ mice (*n* = 9–12/group, **P* < 0.01). (**G**) High molecular weight (HMW) adiponectin determined as a percentage of total in the same group depicted in **F**. (**H**) Glucose tolerance testing of WT and *Fpn1*$^{-/-}$ mice (*n* = 5–6/group, **P* < 0.05 for individual glucose values). (**I**) Body composition by magnetic resonance imaging in WT and *Fpn1*$^{-/-}$ mice on normal chow or high-iron diets (*n* = 11–20/group, **P* < 0.05).

els also trended higher (136%) in patients with HH (Figure 5B, 3.3 μg/ml vs. 1.4 μg/ml, *P* = 0.06). In non-HH sibling controls of the subjects with HH, serum adiponectin and BMI were closely and inversely associated (Figure 5C, *r* = 0.77, *P* = 0.03), consistent with previous reports (31, 49). However, the association between BMI and adiponectin was lost in patients with HH (Figure 5C, *r* = 0.09, *P* = 0.87). Serum ferritin, which largely reflects hepatic iron stores, was not associated with serum adiponectin levels in patients with HH (data not shown).

To determine whether the increased adiponectin levels in HH mice were functionally significant, we generated mice with deletion of the adiponectin gene (*APN*$^{-/-}$, provided by Phillip Scherer, ref. 50) on the C57BL6/J-HH (*Hfe*$^{-/-}$) background. Because the effects of adiponectin deletion on glucose tolerance are more manifest in obese mice or mice exposed to a high-fat diet (26, 50), the mice were fed a high-fat diet for 8 weeks. The *Hfe*$^{-/-}$ mice exhibited a 19% decrease in fasting glucose compared with *APN*$^{-/-}$ mice, an improvement that was completely lost in the *APN*$^{-/-}$:*Hfe*$^{-/-}$ double-knockout mice (Figure 5D, *P* = 0.006 by ANOVA). A similar trend was seen in animals on normal chow but was not significant because of the relatively smaller effects of both the *Hfe*$^{-/-}$ and *APN*$^{-/-}$ genotypes on glucose in mice on normal chow (fasting glucose levels, 126 ± 7 mg/dl in WT, 115 ± 4 mg/dl in *Hfe*$^{-/-}$, and 129 ± 11 mg/dl in *APN*$^{-/-}$:*Hfe*$^{-/-}$, *P* = 0.16, data not shown).

Phlebotomy increases adiponectin and improves glucose tolerance in humans with high-normal serum ferritin. We next sought to determine whether iron plays a causal role in determining adiponectin levels and the risk of MetS in humans. We studied humans with impaired glucose tolerance (IGT) whose serum ferritin levels were in the highest quartile of normal (221 ± 42 ng/ml, see Supplemental Table 2). Individuals with chronic inflammatory states, such as hepatitis, arthritides, or infections, were excluded, as were individuals with HH. Subjects received oral and frequently sampled intravenous glucose tolerance tests (OGTTs and FSIVGTTs) before and approximately 6 months after phlebotomy, which was sufficient to result in a fall in serum ferritin to the lowest quartile of normal

also noted an association between serum ferritin and adiponectin, the adipocyte-specific, insulin-sensitizing hormone. We have verified that serum ferritin levels, reflecting tissue iron stores, are more tightly associated with adiponectin than its more common predictor, obesity. More importantly, the relationship is causal, reflecting regulation of adiponectin transcription by iron. We have demonstrated this in cultured cells, by manipulation of iron stores and adipocyte iron levels in rodents, and in humans. Adiponectin is causally linked to insulin sensitivity (26), and, consistent with this, the changes in adiponectin in response to iron are accompanied by changes in glucose tolerance and insulin sensitivity.

The fact that adipocytes use iron levels to regulate adiponectin suggests a role for adipocytes in coordinating organism-wide metabolic responses to iron availability, as they do for responses to overall macronutrient status. There is other evidence for cross-talk between iron and adipocyte metabolism. Insulin treatment, for example, increases iron uptake by increasing cell surface expression of transferrin receptor 1 in 3T3-L1 and rat adipocytes (52, 53). Iron induces lipolysis in cultured adipocytes and modulates the lipolytic response to norepinephrine (34, 54). Close coregulation of iron levels and metabolic parameters, such as fuel preference, is conserved from yeast (55, 56) to mammals (46). The need for this coregulation is consistent with the necessity of iron for electron transport and other redox reactions combined with its dangers as a potent oxidant.

Adipocytes are well suited for their iron-sensing role. They express not only common regulators of iron homeostasis, such as ferritin and iron regulatory proteins (57), but also iron-related proteins with restricted tissue expression, including transferrin receptor 2, HFE, hepcidin (40, 41), and, as shown herein, ferroportin. Ferroportin is the only known cellular iron export protein and is critical in the regulation of tissue iron stores (42, 43, 45). Hepcidin, a peptide secreted by the liver in response to serum iron, binds ferroportin, resulting in ferroportin internalization and degradation (44). Under conditions of high iron, hepcidin-induced downregulation of ferroportin in duodenal enterocytes prevents dietary iron from entering the circulation. Significant expression of ferroportin has been heretofore reported to be limited to duodenal enterocytes, hepatocytes, and macrophages (42, 43). Expression of ferroportin in adipocytes allows them to serve an iron-sensing function with greater dynamic range and sensitivity, as evidenced by the effect of tissue-specific ferroportin deletion.

Ferroportin expression dictates that adipocytes will respond to hepcidin by downregulating ferroportin and sequestering iron. Of added significance, hepcidin is not only regulated by iron but is also induced in inflammation (58). Thus, adipocytes will regulate adiponectin not only in response to the availability of iron but also to the inflammatory state of the organism. Such integration should be adaptive, given the dangers posed by the potentially additive dangers of oxidative stress, resulting from high rates of fuel oxidation, high tissue iron levels, and inflammation. We have previously shown, for example, that high iron itself activates fat oxidation (59), and we speculate that in situations of high-iron stores, dampening fatty acid oxidation by decreasing adiponectin (60) might be protective to tissues because of these additive oxidative stresses.

Most physiologic regulation of adiponectin levels is attributable to changes in transcription mediated by FOXO1 and PPARγ (36, 37). We demonstrate that iron causes decreased acetylation of FOXO1, without changing its level of phosphorylation. Deacetylation of FOXO1, however, is generally associated with increased adiponectin transcription (61). The resolution of this paradox appears to be that deacetylation is accompanied by increased binding of FOXO1, not only to activator sites in the adiponectin promoter, but also to a PPRE, which has been reported to repress transcription when co-occupied by FOXO1 and PPARγ (39). These results differ somewhat from those of Qiao et al. who reported that FOXO1 interaction with C/EBPα was enhanced by SIRT1, leading to increased adiponectin transcription in 3T3-L1 cells (36). Although we did see increased SIRT1 activity associated with increased binding of FOXO1 to the adiponectin promoter, we saw no enhancement of C/EBPα binding to FOXO1. Furthermore, the effect of SIRT1 activation was not sufficient to increase adiponectin transcription, so we can only speculate that the effect of occupancy of the PPARγ site that is stimulated by iron treatment is dominant, as has been reported for that site (39). Interestingly, fatty acid–induced reactive oxygen species have been shown to decrease adiponectin transcription by inducing FOXO1 phosphorylation and nuclear exclusion (62). We did not, however, observe any change in FOXO1 phosphorylation with iron, suggesting that inflammation is not the direct mediatory of adiponectin regulation by iron. FOXO1 also regulates adipocyte differentiation, size, and energy expenditure (63, 64), and the FOXO family of transcription factors is involved in nutrient sensing and adaptation of metabolism to available nutrients (65). Our results add iron to the list of factors that are integrated by FOXO1 in determining its regulation of adipocyte metabolism and adiponectin secretion.

The regulation of adiponectin by iron sheds light on glucose homeostasis in HH, a condition associated with a high prevalence of diabetes (20, 66). In HH, failure to induce hepcidin in the liver results in low hepcidin levels. This results in failure to downregulate ferroportin in enterocytes, leading to unregulated iron absorption from the gut. Iron overload of islets leads to diabetes, largely on the basis of decreased insulin secretion rather than insulin resistance (20, 67). In fact, a mouse model of HH and nondiabetic humans with HH exhibit increased insulin sensitivity (46), although they may also develop secondary insulin resistance from independent conditions such as obesity (20). This presents a paradox in that dietary iron overload is associated with typical type 2 diabetes, a condition of insulin resistance (1–4). The paradox is resolved by our finding of ferroportin in adipocytes. In the limited tissues expressing significant levels of ferroportin, such as macrophages, decreased hepcidin in HH results in increased ferroportin expression and therefore decreased iron levels despite total body iron overload (48, 68). As reported herein, adipocytes follow this pattern of decreased iron content in HH, explaining our previously reported increased levels of adiponectin in mouse HH (46) and the parallel findings in humans reported here. Adiponectin is the mediator of the effects of low adipocyte iron on insulin sensitivity, as demonstrated by the lack of improved glucose tolerance in *Hfe*$^{-/-}$ mice that also have deletion of the adiponectin gene. Decreased adipocyte iron may also explain our previous demonstration of increased size of *Hfe*$^{-/-}$ adipocytes, in that iron-deficient adipocytes exhibit decreased rates of lipolysis (34).

Within the range of iron used in the current studies the inverse relationship between iron and adiponectin is a continuum. It is possible, however, that adiponectin levels might also drop under conditions of severe iron deficiency. A modal relationship between iron and adiponectin would be adaptive, in that an organism might also seek to limit fat oxidation in situations of very low iron, wherein metallation of mitochondrial proteins might be limited.

Consistent with adiponectin levels falling with very low adipocyte iron, we have reported that insulin sensitivity decreases in HH subjects following phlebotomy (19), accompanied by decreased serum adiponectin levels (data not shown).

Proof of the effects of ferroportin in adipocytes, and of adiponectin as one of the mediators of the metabolic effects of iron, is provided by the phenotype of mice with adipocyte-specific deletion of ferroportin. These mice exhibit increased adipocyte iron, decreased adiponectin, insulin resistance, and decreased glucose tolerance. Thus, iron overload restricted to adipocytes is sufficient to affect glucose tolerance, although iron also exerts effects in other cells (e.g., muscle and β cells) that add to this diabetes risk (18, 19, 21, 59). Of note, in our tissue-specific deletion of ferroportin, we used a mouse expressing Cre recombinase under control of the AP2 promoter. Although certain AP2-driven constructs may be expressed in macrophages, we observed no change in macrophage ferroportin expression in the *Fpn1*$^{-/-}$ mice. Thus, the metabolic effects induced by changes in ferroportin expression in the knockout model can be confidently ascribed to the adipocyte.

The studies of the non-HH human populations also reveal close and causal relationships among serum ferritin, adiponectin, and glucose tolerance status. In men, ferritin predicted adiponectin approximately 4 times better than BMI. In the cohort of obese men, with or without MetS (Figure 1, D and E), ferritin was more strongly associated with diabetes than MetS. Although an association of MetS with ferritin has been reported (7), the stronger relationship with diabetes may be explained by the adverse effects of iron on both insulin secretion and adiponectin. The fact that MetS subjects without diabetes only trended toward higher ferritin but still had lower adiponectin demonstrates that other factors such as obesity modulate adiponectin independently of iron.

The relationship of ferritin to adiponectin was weaker in women, likely because the range of ferritin values was significantly narrower and lower in women than in men. On average, however, lower ferritin levels are accompanied by higher adiponectin levels in women, consistent with our hypothesis. The data suggest that ferritin may be a major determinant of the higher adiponectin levels that exist in women (31). The results may also speak to the differences between men and women in diabetes risk, with women having a decreased incidence of diabetes prior to menopause (69).

Insulin resistance, obesity, and diabetes are associated both with iron and with chronic inflammation (2, 3, 7, 8, 57), and ferritin is a known acute-phase reactant to inflammatory stimuli (11). We believe, however, that the association between serum ferritin and adiponectin reflects tissue iron rather than systemic inflammation for three reasons. First, while we found an association between serum ferritin and adiponectin in the human population as a whole, restricting the analysis to individuals with normal serum ferritin strengthened rather than weakened the association. In healthy adults, serum ferritin levels within this normal range are closely associated with mobilizable iron and reliably predict total body iron stores (70). Second, inclusion in multivariate analysis of CRP, another marker of the acute-phase response, had little effect on the correlation coefficient of serum ferritin with adiponectin, as was the case in other studies (3, 6, 7). In this study, the relationship between ferritin and adiponectin was also independent of IL-6 and TNF-α. Third, our in vitro and in vivo studies reveal that decreasing tissue iron stores is sufficient to decrease ferritin and increase adiponectin. Our studies do not eliminate the possibilities, however, that systemic inflammation is making an additional contribution to increased serum ferritin levels or that adiponectin transcription might also be regulated independently by signals that reflect oxidant stress and/or inflammation.

Because of the association of iron both with insulin resistance and insulin deficiency (18, 20, 21, 67, 71, 72), we reasoned that phlebotomy might improve both, leading to additive effects in decreasing diabetes risk. Phlebotomy in humans with IGT led to significant improvements in glucose tolerance and the glucose disposition index, a reflection of insulin secretion and sensitivity. The fact that phlebotomy decreased ferritin also confirms that the initially high-normal ferritin values were not due to inflammation. These results are consistent with our recent demonstration that iron restriction affords significant protection from diabetes to the *Lep*$^{-/-}$ (*ob/ob*) mouse, improving both insulin sensitivity and insulin secretion (21). The metabolic effects of iron, even within the "normal" range, therefore likely explain the many epidemiologic observations that have been made concerning iron and diabetes risk, such as the fact that periodic blood donation is associated with increased insulin sensitivity (73).

It should be emphasized that this small clinical study was designed as a proof of concept and does not serve as an adequate clinical trial to guide medical practice. To minimize variance, for example, we only studied men and, to maximize the effect size, we examined only those with IGT. The weight gain observed in 2 out of the 3 subjects is also of potential concern, although it also suggests that the improvements seen were not related to coincident changes in lifestyle. Iron deficiency is associated with obesity (74), although the phlebotomy was controlled such that none of these subjects became iron deficient. The mechanism underlying weight gain in iron deficiency is unknown, although we have demonstrated that decreased iron results in decreased capacity for fatty acid oxidation (59). The results suggest that larger clinical trials with more diverse populations are clearly indicated. In addition, because of the interrelationships between iron and oxygen sensing (75), and because this study was performed in subjects living at 4,000–5,000 feet altitude, any future trials should include subjects living at sea level to allow generalization of the results.

In sum, we have presented evidence that adipocyte iron levels regulate adiponectin transcription and serum protein levels. These data further highlight the role of the adipocyte as a key regulator of metabolism in all tissues, based on integrated sensing of nutritional stores and iron availability. Understanding the mechanisms by which adipocytes sense and respond to intracellular iron levels is fundamental to understanding this role and merits further investigation. In addition to regulating adiponectin, the fact that iron is also a factor in determining insulin secretory capacity (19, 21) demonstrates a rational basis for the observed associations among iron, glucose tolerance, and the MetS. These data also support the need for further clinical trials on the effects of iron reduction on diabetes and the MetS.

Methods

Experimental animals. Dietary iron manipulations were accomplished with diets containing 7 mg/kg, 330 mg/kg, 500 mg/kg, or 20 g/kg carbonyl iron (Harlan Teklad) for a period of 2 months before phenotyping. High-fat diet (Research diets D12451) contained 45% calories from fat. Targeted mutagenesis produced a knockout of the *Hfe* gene (76). This and the mutations listed below were bred onto either the 129/SvEvTac or C57BL6/J genetic backgrounds for at least 5 generations. Adipocyte-specific ferroportin knockout mice were generated by breeding mice with LoxP sites

flanking exon 6 and 7 of the ferroportin gene (45), provided by Nancy C. Andrews (Duke University), with mice containing an AP2-promoter–driven Cre recombinase (The Jackson Laboratory). Mice with deletion of the adiponectin gene (50) were provided by Phillip Scherer (University of Texas Southwestern, Dallas, Texas, USA). Age- and sex-matched WT littermates were used as controls. Body composition analysis was performed using magnetic resonance imaging.

Euglycemic clamp procedure. The jugular vein was catheterized under avertin anesthesia, using Micro-Renathane tubing (Braintree Scientific Inc., MRE 025). After a 48-hour recovery, mice were fasted overnight. A dual infusion pump (Harvard Apparatus, Pump 33) was used to infuse insulin at a constant flow rate and 50% dextrose at a variable rate to maintain glucose at 100–150 mg/dl. Glucose was measured at 10-minute intervals as described above for glucose tolerance testing. After steady state was reached for 3 successive glucometer readings, the glucose infusion rate was determined.

Human subjects. Subjects with HH have been described elsewhere (19). Studies with these subjects were approved by the University of Utah IRB. The subjects depicted in Figure 1, A–C, were part of a larger study and were described previously (27). Diabetes status was confirmed by OGTT. Diabetic subjects were on dietary therapy only, with fasting plasma glucose between 125 mg/dl and 175 mg/dl. The study protocol was approved by Pennington Biomedical Research Center's IRB. All sera were obtained after a 12-hour overnight fast. Insulin sensitivity was assessed by a 1-step, high-dose (120 mU × m^{-2} × min^{-1}) hyperinsulinemic-euglycemic clamp (27).

The obese men (BMI ≥35, with or without MetS) whose data are presented in Figure 1, D and E, were selected from subjects recruited from the general population as members of pedigrees for genetics studies or as subjects in a gastric bypass study (77), under a protocol approved by the University of Utah IRB.

For the phlebotomy study, informed consent was obtained, and OGTT, serum ferritin, and blood counts were measured to determine eligibility for phlebotomy. Subjects were excluded who had any significant chronic or inflammatory diseases, including cancer, hepatitis, renal failure (serum creatinine >1.4), arthritis, or autoimmune diseases. *HFE* genotyping using allele-specific PCR primers (20) was performed to rule out HH. OGTT and FSIVGTT (78) were performed in the CSC, each after a 12-hour fast. Glucose tolerance status was defined according to World Health Organization criteria (79). FSIVGTT results were analyzed by MINMOD software to determine the AIRg and Si (80). Subjects then donated blood at a Red Cross center (1 unit per month). After 2 to 3 units were donated by each subject, hematocrit, hemoglobin, and iron indices were determined, and phlebotomy continued until ferritin was in the lowest quartile of normal (goal = 50 μg/dl). In no case did blood counts decrease by more than 10%. Two to three months after reaching the ferritin goal, subjects were retested with OGTT and FSIVGTT.

Reagents and assays. Reagents were purchased from Sigma-Aldrich unless otherwise noted. Serum CRP and TNF-α were measured by ELISA (R&D Systems), IL-6 was measured by ELISA (Abnova), ferritin was measured by ARUP Laboratories, insulin was measured by radioimmunoassay (Diagnostic Products), and adiponectin was measured by ELISA (ALPCO).

3T3-L1 adipocyte culture and differentiation. 3T3-L1 adipocytes (ATCC) were maintained in high-glucose DMEM (HG-DMEM) supplemented with 10% bovine serum and penicillin/streptomycin (Invitrogen). For differentiation (81), cells were incubated in HG-DMEM with 10% FBS (Thermo Scientific) for 48 hours after confluence. Cells were then cultured in differentiation media I (HG-DMEM, 10% FBS, 1 μg/ml insulin, 0.25 μg/ml dexamethasone, 0.5 mM IBMX) for 4 days, followed by differentiation media II (HG-DMEM, 10% FBS, 1 μg/ml insulin) for 48 hours. Prior to experiments, cells were cultured overnight in low-glucose DMEM (LG-DMEM, Invitrogen) with 10% FBS. All experiments were performed in LG-DMEM.

Plasmids, nucleofection, and luciferase assay. The proximal 1,460 bp of the murine adiponectin promoter was amplified from genomic epididymal fat pad DNA and inserted into the pGL4.10 vector (Promega). Plasmids were nucleofected into differentiated 3T3-L1 adipocytes using an Amaxa Nucleofector with Kit L (Lonza). Luciferase and renilla activity were quantified using the Dual Luciferase Reporter Assay System (Promega) and a 96-well plate luminometer.

Isolation of primary adipocytes. Epididymal and retroperitoneal fat pads were removed from male and female mice, respectively, and incubated in HBSS with 1% BSA and 20 mg collagenase, type I, for 30 minutes at 37°C, rotating at 180 rpm. Adipocytes were filtered through 100-μm nylon mesh and rinsed with HBSS–1% BSA. Following centrifugation at 500 *g*, the fat cake was transferred to a clean tube, rinsed, and centrifuged. Isolated adipocytes were either used immediately or flash frozen in liquid nitrogen.

Quantification of transcript. Quantitative RT-PCR was performed as described previously (46). Briefly, mRNA was extracted from primary and 3T3-L1 adipocytes using TRIzol (Invitrogen), purified using an RNeasy column (QIAgen), and synthesized into cDNA using a First-Strand cDNA Synthesis Kit (Invitrogen). Real-time PCR was performed with a LightCycler (Roche Diagnostics). cDNA products were quantified using the LightCycler software ($\Delta\Delta C_T$ method). mRNA levels of specific genes were normalized to cyclophilin A.

Western blotting. Antibodies used included pFOXO1 (Ser 256), acFOXO1, total FOXO1, and pAKT from Santa Cruz Biotechnologies Inc. Rabbit α-mouse ferroportin antibody was provided by Jerry Kaplan (University of Utah).

ChIP. ChIP studies were performed as described previously (82). Chromatin was extracted from 3T3-L1 adipocytes on day 9 after differentiation using the SimpleChIP Kit (Cell Signaling Technology). Cells were treated with $FeSO_4$ or 0.1 N HCl control for 24 hours prior to cross-linking for 10 minutes with 1% formaldehyde. Cells were then lysed and sonicated 3 times for 20 seconds using a sonic dismembrator (Fisher Scientific). Lysates were precleared with protein A agarose beads (Millipore). FKHR or PPARγ antibody (Santa Cruz Biotechnology Inc.) was applied. DNA was released from protein-DNA complexes by proteinase K digestion and then subjected to quantitative real-time PCR for the APN response elements for PPARγ (PPRE) (83) and FOXO1 using the Power SYBR Green Kit (Applied Biosystem). ChIP-qPCR data were normalized to input samples for the amount of chromatin and for immunoprecipitation efficiency by normal IgG controls.

Statistics. Descriptive statistics in the text and figures are represented as average ± SEM. The Pearson's correlation coefficient was calculated to test for correlation between 2 parameters. Difference between 2 slopes was calculated by analysis of covariance. Multivariate logistic regression analysis was sequentially performed to investigate the role of CRP, BMI, diabetes, and gender on the ferritin-adiponectin association. An unpaired 2-tailed Student's *t* test was used to determine significance between controls and individual experimental groups, whereas a paired *t* test was used to compare values in the same subjects before and after phlebotomy. One-way ANOVA was used to compare series of data. $P < 0.05$ was considered significant for all tests. All statistical analyses were performed with GraphPad PRISM 5.0d for Macintosh. In the studies depicted in Figure 1, adiponectin values ($n = 2$) that were more than 3 SD from the mean were excluded from the analysis.

Study approval. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Utah, under IACUC approval A3031-01. The human studies were approved by the IRB of the University of Utah, protocols 0880 and 20094. Studies were also reviewed and approved by the Advisory Committee to the Clinical Services Core (CSC) of the Center for Clinical and Translational Science. Additional pre-

viously unpublished data was used from human studies completed at the Pennington Biomedical Research Center and at the University of Utah. Both studies were approved by the IRBs of the respective institutions, as previously published (27, 77).

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CHAPTER 2

ADIPOCYTE IRON REGULATES LEPTIN AND FOOD INTAKE VIA ACTIVATION OF CREB

Abstract

Dietary iron supplementation is associated with increased appetite. We therefore investigated the effect of iron on leptin, a hormone regulating food intake and energy homeostasis. Serum ferritin is negatively associated with serum leptin in a cohort of humans with metabolic syndrome. This correlation is recapitulated in mice fed a high iron diet. Loss of the iron exporter, ferroportin, in adipocyte resulted in iron loading and decreased leptin. Conversely, decreased levels of hepcidin in hereditary hemochromatosis result in increased adipocyte ferroportin expression, decreased adipocyte iron, and increased leptin. Treatment of 3T3-L1 adipocytes with iron decreased leptin mRNA in a dose-dependent manner. We found iron negatively regulated leptin transcription via cAMP-responsive element (CRE)-binding protein (CREB) activation. Two potential CREB-binding sites were identified in the mouse leptin promoter region. Mutation of both sites completely blocked the effect of iron on promoter activity. We also found enrichment of phosphor-CREB binding to those two sites by ChIP in 3T3-L1 adipocytes treated with iron. Consistent with the changes in leptin, dietary iron content was also directly related to food intake, independently of weight. These findings indicate that levels of dietary iron play an important role in regulation of appetite and metabolism through CREB-dependent modulation of leptin expression.

Introduction

Iron deficiency is the most common nutrient deficiency in the United States (1). Individuals with iron deficiency have appetite loss, while dietary iron supplementation is associated with growth and increased appetite in children (2, 3). The mechanism underlying these associations is poorly understood. Leptin, the protein product of the *ob* gene, is a hormone that is secreted primarily by adipose tissue and responsible for

regulating feeding behavior. It is also strongly and positively associated with BMI and total body fat. Individuals with iron deficiency also have a higher prevalence of obesity (4-8). Any direct association between leptin and iron would be difficult to discern given the findings above, because either high iron consumption or lower adiposity would result in decreased leptin. There are, however, suggestions for a direct relationship between iron and leptin. Patients with β thalassemia major, a condition usually accompanied by transfusional iron overload, fail to maintain adequate leptin production (9-11). Furthermore, the association between BMI and serum leptin levels is dampened or even abolished in these patients (12). Soluble transferrin receptor (sTfR), a clinical marker for total body iron stores, is negatively associated with serum leptin (13). Finallly, serum ferritin, an iron storage protein and marker of both tissue iron stores and inflammation, is significantly correlated with appetite score and food intake (14). They do not, however, demonstrate causality nor mechanism for any of the relationships.

We therefore investigated the effect of iron on leptin. We demonstrate in humans that the association between serum ferritin and leptin is independent of inflammation and body fat mass, and that serum ferritin, even within its normal range, is among the best predictors of serum leptin. Studies in cell culture, mouse models, and humans demonstrate that iron plays a direct and causal role in determining leptin levels. Thus, the adipocyte responds to iron availability as well as macronutrient status to adjust expression of leptin, a major regulator of appetite, energy homeostasis, and metabolism.

Results

Human ferritin levels are inversely associated with serum leptin independently of inflammation and BMI. We examine the relationship of iron and leptin in a cohort of 76 individuals with (n = 36) and without (n = 40) diabetes recruited for an independent

study of metabolic flexibility (15). Ferritin was measured as an indicator of tissue iron stores. Ferritin is also an acute phase response protein whose concentrations increase during inflammation and no longer reflect the level of iron stores. To mitigate the effects of inflammation and/or extreme iron overload and anemia, we restricted the analysis to individuals with normal serum ferritin (men, >30 ng/ml and <300 ng/ml; women, >15 ng/ml and <200 ng/ml) (16, 17). Excluding 12 individuals outside this range, we found a significant negative association between ferritin and leptin (Figure 2.1A, r = -0.474, P = 0.0002). Serum ferritin is also negatively associated with serum leptin when individuals with abnormal ferritin are included, although the association is somewhat weaker (r = -0.409, P = 0.002, Supplementary data Figure 2.S1A). Correcting for potential covariates including an independent marker of inflammation (C-reactive protein, CRP), BMI, and diabetes status, had little effect on the ferritin-leptin association (Table 2.1). Serum iron was also significantly negatively correlated with leptin (r = 0.407, p=0.003, Figure 2.1B).

Inclusion of gender in the analysis has little effect on the serum ferritin and leptin association (Table 2.1), and serum ferritin level is second only to BMI in predicting leptin (Table 2.1). Average ferritin values are significantly lower compared with that in men (average ferritin, 77.07 ± 12.38 ng/ml in women and 162.64 ± 17.89 ng/ml in men, *P* = 1.26 X 10^-4) (18), and these lower ferritin levels are accompanied by higher leptin levels in women (22.98 ± 1.85 µg/ml vs. 9.41 ± 1.27 µg/ml in men, *P* = 7.84 X 10^-7), despite their BMI values being equivalent (31.56 ± 1.21 in women and 30.62 ± 1.29 in men).

Dietary iron overload increases adipocyte iron and decreases leptin mRNA and serum protein levels in mice. Our previous studies have identified effects of iron on adiponectin and adipocyte metabolism (19, 20). To explore the regulation of adipocyte iron levels, we first measured the cytosolic iron in isolated adipocytes by inductively coupled plasma atomic emission spectroscopy (ICP-AES). We observed a 215%

increase of iron in adipocytes from mice fed a high iron diet (2000 mg/kg iron) compared with that in mice fed normal chow (low normal iron diet, 35 mg/kg iron) (Figure 2.2A, P<0.05). We also demonstrated that adipocyte iron levels respond to dietary iron content in wild-type C57BL6/J mice by measuring mRNA levels of the transferrin receptor (*Tfrc*). *Tfrc* mRNA contains iron response elements in its 3' untranslated region (UTR) that result in decreased *Tfrc* mRNA levels as cellular iron levels increase (21). We observed a 50% decrease in *Tfrc* mRNA in adipose tissue from mice fed a high iron diet (2000 mg/kg iron) compared with that in mice fed the low normal iron chow (35 mg/kg iron) (Figure 2.2B, $P < 0.05$). Serum leptin decreased with dietary iron (linear regression, p=0.003, n = 36/group, Supplementary data Figure 2.S3). Serum leptin levels were 42% lower in mice on the high iron diet (2000 mg/kg iron) compared to those on the low normal iron diet (35 mg/kg iron) (Figure 2.2C, P = 0.0009). Intracellular leptin levels assayed by western blotting were 35% lower (Figure 2.2D and 2.2E) and leptin mRNA levels in epididymal pads were 41% lower in iron overloaded mice (Figure 2.2F, P = 0.009), mirroring the changes in serum leptin levels. To further confirm the effect of iron on leptin, transgenic mice expressing the luciferase reporter gene under the control of leptin regulatory sequences were fed the low normal or high iron diets (35 mg/kg vs. 2000 mg/kg iron). Fluorescence after intraperitonal injection with D-luciferin was 15% lower in the mice on high iron, both after fasting (48 h) or 6 h after refeeding (Figure 2.2G and 2.2H).

Deletion of adipocyte ferroportin results in increased adipocyte iron levels, decreased serum leptin. Previously, we have generated mice lacking the gene encoding the iron export channel ferroportin (*Fpn*) in adipocytes. An *Fpn1fl/fl* mouse, provided by Nancy C. Andrews (Duke University, Durham, North Carolina, USA) (22), was crossed to a mouse expressing Cre recombinase under control of the ap2 promoter to obtain the adipocyte-specific ferroportin knockout mice (aFpnKO) (23).

Ferroportin mRNA was undetectable in adipocytes purified by collagenase digestion from aFpnKO mice. *Tfrc* mRNA exhibited a decrease in isolated adipocytes of aFpnKO mice, indicating relative iron overloading compared to wide-type mice (19). The increased levels of adipocyte iron in the aFpnKO mice resulted in a 49% decrease in levels of serum leptin (Figure 2.3A, $P < 0.01$). There was no change in body weight or body composition as analyzed by a Magnetic Resonance Imaging (MiniSpec, Bruker) (Figure 2.3B).

Lower adipocyte iron is associated with higher serum leptin in hereditary hemochromatosis. If iron decreases leptin secretion, we would expect high leptin levels in models of decreased adipocyte iron. *Hfe*$^{-/-}$ mice are a model of human hereditary hemochromatosis (HH), a disease of genetic iron overload wherein the gene most commonly mutated in HH has been deleted. HFE protein is required for normal regulation of a master iron regulatory hormone, hepcidin, which reduces the iron export channel ferroportin (24, 25). HH or HFE deletion results in low hepcidin, increased ferroportin expression, and increased iron egress from cells that express ferroportin, such as duodenal enterocytes. Adipocytes also express ferroportin as demonstrated previously by us, and are therefore paradoxically low iron level in this disease of overall iron overload (19). HFE-/- mice therefore have less iron in adipocytes, and we determined that this results in higher serum leptin compared to littermate WT mice (Figure 2.3C).

Iron decreases leptin transcription and activates CREB. To demonstrate that the decreased leptin mRNA levels are due to decreased transcription and that iron regulates leptin directly, we examined the effects of iron on leptin in a cell culture model. We observed a decrease of leptin after treatment with ferric ammonium citrate (FAC) in primary mouse adipocytes isolated from inguinal white adipose tissue (iWAT) and progenitor cells isolated by fluorescence-activated cell sorting (FACS) and

differentiated *in vitro* (Figure 2.4A and Supplementary data Figure 2.S4). 3T3-L1 adipocytes differentiated with isobutylmethylxanthine/dexamethasone/insulin express very little leptin transcript, but when differentiated in the presence of a PPARγ agonist, ciglitazone, the adipocytes have been shown to exhibit a 5-fold increase in leptin mRNA and maintain insulin sensitivity and their differentiated phenotype (26). Using this cell culture model, treatment of 3T3-L1 adipocytes with FAC decreased intracellular leptin protein levels measured by western blotting (Figure 2.4B and 2.4C). Secreted leptin in the medium decreased 36% with iron treatment (Figure 2.4D, P <0.05), which was reversed by an iron chelator, deferoxamine (DFO). Similarly, leptin mRNA levels also decreased 48% with iron treatment (P <0.05) and DFO treatment abolished the iron-induced decrease of leptin mRNA (Figure 2.4E). The decrease in leptin by iron treatment was dose-dependent (Figure 2.4F, r = 0.668, P < 0.01). The levels of iron used in treating the cultured adipocytes have been shown to cause a parallel dose-dependent decrease in the expression of *Tfrc* and iron level measured by ICP-AES (19), indicating that the resulting intracellular iron levels are in a physiologically sensed range. Iron can regulate mRNA levels posttranscriptionally by iron-response elements (IRE) and iron-responsive proteins (IRP). Binding of IRP to IRE in the 3'-UTR of mRNA typically increases mRNA stability, while binding of IRP to the 5'-UTR typically decreases translation. Binding of free iron to IRPs causes their dissociation from the mRNA and either enhanced mRNA degradation (3'-IRE) or enhanced translation (5'-IRE). Leptin mRNA possesses a 59bp 5'-UTR and 2.7kb 3'-UTR. We transfected 3T3-L1 adipocytes with constructs containing luciferase with each or both UTRs and found no effect of iron on luciferase activity, indicating leptin is not an IRE-IRP targeted gene (Supplementary data Figure 2.S5). Iron also did not decrease the half-life of the endogenous mRNA measured after actinomycin D treatment of cells (data not shown).

We next constructed two luciferase reporter plasmids with the proximal 5,986bp

and 764bp of the murine leptin promoter. We first measured luciferase activity driven by the proximal 764 bp of the murine leptin promoter, a region that contains sites known to regulate leptin transcription, including CCAAT/enhancer binding protein (C/EBP), specificity protein 1 (SP1), and the hypoxia response element (HRE). Iron did not decrease the activity of this proximal promoter (Supplementary data Figure 2.S6). Luciferase activity driven by 5,986bp leptin promoter, however, decreased 33% after FAC treatment of the cells (Figure 2.5A). We found this region, as well as the human leptin promoter (27), contains potential cAMP response elements (CRE) as predicted from the transcriptional database of GenBank. We therefore investigated the effect of iron on CRE binding protein (CREB) activation. FAC treatment resulted in increased phosphorylation of CREB and slightly decreased total protein levels (Figure 2.5B and 2.5C). Consistent with the increased phosphorylation of CREB, we also observed increased expression of a downstream target of CREB activation, PGC1 (Supplemental Figure 2.S7A). Increased CREB phosphorylation in iron-treated cells occurred independently of two of its known activators, cAMP and calcium, with FAC treatment actually decreasing both of them (Supplementary data Figure 2.S8). A membrane-permeable cAMP analogue (8-Br-cAMP) activates a known CREB kinase, cyclic AMP-dependent protein kinase. Treatment of 3T3-L1 adipocytes with 8Br-cAMP or the adenylyl cyclase activator forskolin decreased luciferase activity and abolished iron's effect on expression of the 5,986bp luciferase construct (Figure 2.5D and 2.5E). 8Br-cAMP treatment also decreased leptin transcript levels (Supplementary data Figure 2.S7B), but did not affect iron metabolism in terms of *Tfrc* expression (Supplementary data Figure 2.S7C). We generated 3T3-L1 cells stably expressing a dominant negative CREB inhibitor, ACREB, a synthetic polypeptide that heterodimerizes with and disrupts binding of CREB1, CREM, and ATF1, but not unrelated bZIP proteins to DNA (28). In cells expressing ACREB, iron lost its effect on decreasing the 5,986bp promoter

luciferase activity, confirming that functional CREB sites confer iron-responsiveness to the promoter (Figure 2.5E).

Iron increases CREB occupancy of the leptin promoter. We mutated three potential CREB sites in each of two 5,986bp plasmids (plasmids 425 and 533, Supplementary data Figure 2.S9A). Iron lost its ability to decrease luciferase activity in 3T3-L1 adipocytes stably expressing plasmid 533 but not in cells expressing wild type or 425 plasmids (Figure 2.6A). Plasmid 533 contains two potential CRE sites and one typical AP-1: CRE-like site, from -2590 bp to -2583 bp (CRE-1, CTACTTCA), from -2080 bp to -2073bp (CRE-2, CTATGTCA) and from -877 to -883 (AP-1: CRE-like, TTAGTCA) (Supplementary data Figure 2.S9B). AP-1, and CRE proteins recognize a similar DNA sequence and there is evidence that the fos/jun (components of AP-1) complex and CREB families are able to dimerize and bind to AP-1/CRE-like sequences, allowing crosstalk between the two signaling systems (29-31), and such degenerate CRE sites can be functional, as shown in other systems (32-34). We performed EMSA on the CRE-1, CRE-2, and AP-1/CRE-like sites. Three oligonucleotide probes containing the CRE-1, CRE-2, and AP-1/CRE-like sequences were synthesized and biotinylated. Both biotin-labeled CRE-1 and CRE-2 formed a complex with nuclear extracts (Figure 2.6B). Coincubation with a 200-fold excess of a nonbiotin-labeled CRE-1 or CRE-2 oligonucleotide effectively abolished complex formation. A mutated oligonucleotide in which the potential CRE-1 binding sequence (CTACTTCA) was mutated to mCRE-1 (CTAAATCA) failed to compete for binding with the biotin-labeled CRE-1 probe (Figure 2.6B). Similarly, mutation of the potential CRE-2 site (TATGTCA) to mCRE-2 (TATGTCA) resulted in failure to compete for the binding of biotin-CRE-2 to nuclear protein (Figure 2.6C). We did not observe any band shifts of biotin-labeled AP1 oligonucleotides after incubation with 3T3-L1 nuclear extracts (Supplementary data Figure 2.S10). These findings indicate that the CRE-1 and -2 sequences in the leptin

promoter are functional binding sites. We next measured CREB occupancy at those two potential CRE sites using ChIP. As predicted by CREB phosphorylation status, cells treated with iron exhibited a 3- to 4-fold increase in occupancy by pCREB at the CRE1 and CRE2 sites ($P < 0.01$, Figure 2.6D).

Iron-induced decrease of leptin is associated with increased food intake. To demonstrate that iron-induced changes in leptin are physiologically significant, we studied the effects of chows with different dietary iron content on serum leptin and food intake in mice. We fed C57BL6/J male mice very high (20 g/kg carbonyl iron), high (2000 mg/kg), high normal (500 mg/kg), low normal (35 mg/kg) or low (4 mg/kg) iron diets for 2 months. The 35 mg/kg diets are derived from TestDiet® AIN-93G recommended by the American Institute of Nutrition. The 500 mg/kg iron diet is representative of standardized diets in animal facilities which range from 200 to 500 mg/kg iron. The 2000 mg/kg iron diet results in a modest increase of liver iron (2-fold) which is within the 4-fold range seen in normal humans (35). The highest iron diet is 2% carbonyl iron (20 g/kg iron diet), which increases plasma iron by two-fold after 24 weeks on diet (36). Mice were studied in metabolic chambers with monitoring of food intake after 9 to 12 weeks on the diets. We observed a positive correlation of dietary iron levels and food consumption (Figure 2.7A, $r = 0.5914$, $P < 0.001$). Mice on the high iron diet (2000 mg/kg) tend to eat more during each 15 min interval in the chamber during 3 days (Figure 2.7B, upper panel, $P = 0.0084$) and accumulated food intake is higher in high iron diet (2000 mg/kg) compared to low iron diet (4 mg/kg iron) (Figure 2.7B, lower panel, $P = 0.0002$). Body weights did not change with different dietary iron after 9 weeks on diet (low iron, 28.88 ± 0.31 g; low normal chow, 29.55 ± 0.33 g; high normal iron, 28.26 ± 0.37 g; high iron, 28.76 ± 0.30 g, linear regression, $P = 0.104$, $n = 36$/group, supplementary data 2.S11). To demonstrate that the change of feeding behavior under different levels of dietary iron is mediated through changes in leptin

levels, we fed the same diets to ob/ob mice, where the leptin gene is mutated and nonfunctional. In ob/ob mice, the association of dietary iron level with food intake is abolished (Figure 2.7C). Similarly, no difference in food intake was apparent comparing mice on the high iron diet (2000 mg/kg) to those on the low iron diet (4 mg/kg iron) in lively food intake and accumulated food intake (Figure 2.7D), and in bar-graphed data (Figure 2.7E) in ob/ob mice.

Discussion

Iron is an essential constituent of many macromolecules involved in cell metabolism, and is required for survival and proliferation. At the same time, iron can act as a potent and potentially dangerous oxidant. Iron levels are therefore highly regulated at the levels of uptake, storage, and recycling in the whole organism as well as individual tissues and cells. A typical adult has total body iron stores of ~1-3 g. A balance between dietary uptake and tissue loss is usually maintained in healthy humans wherein 1 to 3 mg daily losses through bleeding and sloughing of cells from skin and mucosal surfaces is balanced by gastrointestinal uptake (37, 38). However, there is no defined physiological means of active iron excretion, so the levels of tissue iron can increase over time with dietary excess, such that normal levels of serum ferritin, a marker of tissue iron stores in otherwise healthy individuals, vary 15- to 20-fold. We have previously hypothesized that the central role of iron in metabolism dictates that its availability should be reflected in the metabolic state of the organism, and that cells that regulate metabolism such as adipocytes therefore need to sense not only the macronutrient status of the organism but also the availability of iron to support fuel oxidation. This hypothesis is supported by the regulation of adiponectin by iron (19). Recent studies have also noted an association between serum ferritin, soluble transferrin receptor, and leptin, the adipocyte-specific satiety hormone (13, 14). In this

paper, we have verified that serum ferritin levels, reflecting tissue iron stores, are among the best predictors of serum leptin under physiological conditions. We have demonstrated this in humans, in cultured cells, and by manipulation of iron stores and adipocyte iron levels in rodents. More importantly, the relationship is causal, reflecting regulation of leptin transcription by iron. Leptin is causally linked to feeding behavior (39), and, consistent with this, the changes in leptin in response to iron are accompanied by changes in food intake.

The regulation of leptin by iron is operative across physiologic and pathophysiologic iron levels. The current report demonstrates regulation across the range of normal human serum ferritin values. BMI is well known to be positively correlated with serum leptin (40), and consistent with this, we found serum leptin in the obese subjects (upper 25th percentile BMI) to be 3.7-fold higher than that in the lean subjects (lower 25th percentile BMI) (Supplemental data Figure 2.S2A). At the same time, we found serum leptin in the low iron subjects (lower 25th percentile ferritin) is 3.2-fold higher than that in the high iron subjects (higher 25th percentile ferritin) (Supplemental data Figure 2.S2B), indicating ferritin is also a good clinical predictor of leptin. Our results suggest that this may be a direct result of adipocyte iron loading even within the physiologic range. In the mouse studies, as we have previously reported, the low normal (35 mg/kg) and high normal (500 mg/kg) iron diets represent the range of iron content seen in normal chows, and the high iron diet (2000 mg/kg) does not cause overt iron pathology and results in increases in hepatic iron within the ranges seen in normal humans (41). Likewise, in the cell culture models, addition of iron to cells results in modulation of the transferrin receptor in a manner that mirrors normal iron physiology. Thus, we think the ranges of iron represented in these model systems reflect the variation seen in normal humans, and leptin is regulated throughout these ranges of iron.

Although we did not study humans with overt iron deficiency or overload, previous reports are consistent with the current results and demonstrate that the association of leptin and iron is also maintained in pathophysiologic states. Leptin levels are lower (10, 11, 13) and the normal positive relationship between BMI and leptin is lost in males suffering from thalassemia major, a condition in which transfusional iron overload develops (12). Leptin levels remain low in those thalassemic patients even at high BMI. At the other end of the spectrum, mice on the low iron diet (4 mg/kg) have low leptin. Although these mice maintain normal blood hemoglobin concentrations and are not overtly iron deficient, their hemoglobin concentrations are lower than animals on the higher iron diets. Consistent with this observation, dietary iron supplementation in iron-deficient children results in increased growth and appetite (2). The normal association between leptin and body fat is also lost in the elderly, who are more likely to be effectively iron-deficient (16, 42). Overall, these mouse and culture models accurately reflect not only the broad range of “normal” physiology but pathologic human tissue iron levels as well.

Obesity and iron deficiency are two common nutritional and health problems in the world and major cofactors in many other diseases (43, 44). Given this wide range of health implications, it is urgent to develop new and effective strategies in controlling obesity and find potential targets for weight control. Several studies have shown a greater prevalence of iron deficiency in overweight and obese people than normal-weight ones (4, 5, 7, 45, 46). Analysis of public-use data files from the continuous National Health and Nutrition Examination Survey (NHANES) (1999-2006) indicate there is a negative relationship between levels of blood iron content and individual BMI after controlling for other individual characteristics (47). Consistent with this, rats on an iron-deficient diet gain more fat mass than paired mice fed an iron-replete diet (48). However, the etiology of iron deficiency-associated obesity remains unknown. Several

hypotheses have been put forth to explain the occurrence of obesity in iron deficiency. Some have suggested that decreased iron is a consequence, not a cause of obesity, related to: (i) decreased iron intake associated with obesogenic diets or lifestyles; (ii) impairment of intestinal iron absorption and a reduced response to iron fortification in obesity (49); (iii) reduced iron release from iron stored cells and iron bioavailability because of obesity-associated inflammation that results in high levels of hepcidin, a protein that decreases ferroportin and iron release into the systemic circulation (50, 51). However, our current data suggest that another factor in the obesity associated with iron deficiency may be increased leptin and the resultant leptin resistance (45, 52). Additional mechanisms include changes in metabolism induced by iron. Previous results from our laboratory, for example, demonstrate that muscle from mice on a low iron diet exhibits increased fatty acid synthesis but decreased lipolysis and fatty acid oxidation (53). Conversely, iron overloaded mice change fuel usage from glucose to fatty acid oxidation in the muscle (54). All of these data demonstrate the importance of iron stores in determining the metabolic state of the organism, and suggest that iron sufficiency may aid in the prevention or treatment of obesity. At the same time, however, higher levels of iron, even within the “normal” range, may predispose to other metabolic abnormalities such as diabetes (41), metabolic syndrome, and nonalcoholic steatohepatitis (55). Thus, optimal tissue iron levels may represent a significantly narrower range than that seen in the population as a whole.

The molecular mechanism for the regulation of leptin by iron includes phosphorylation of CREB. Besides iron, CREB is another well-known factor that can change fuel utilizaiton from glucose to fat burning in mammals under fasting. Leptin is responsive to nutritional status, and fasting leads to a gradual decline in leptin (56, 57). CREB activation under fasting may play a role in diminishing leptin expression, and the cellular mechanism that causes leptin to fall with iron may be similar to that seen with

fasting. Fasting evokes a number of responses, including an increase of glucagon, a known activator of CREB (58). Thus, CREB may play a role in connecting not only iron but also macronutrient status to leptin. Prior to the current study, there was limited evidence for the regulation of leptin by CREB. It has been reported that activation of CREB during the differentiation process suppresses leptin secretion and expression in mesenchymal stem cell (59), and bioinformatics analysis indicates there are two potential CRE sites in the human ob promoter, but no study of their function has been reported (27). Interestingly, we have found that another nutrient-sensing pathway, the hexosamine biosynthesis pathway (HBP), which is also known to play a crucial role in regulation of CREB (60) and has been reported to regulate leptin expression (61), also contributes to iron-induced changes in leptin expression. Namely, increased phosphorylation of CREB is associated with a decrease of O-GlcNAcylation of CREB, and activation of the HBP by glucosamine overcomes the iron-induced inhibition of leptin (data not shown, manuscript in preparation). Thus, reciprocal interplay of “starvation” (CREB) and “overfeeding” (HBP) pathways modulate leptin expression in an iron-responsive manner. These observations further underline the close relationship of iron levels with overall nutritional status and metabolic regulation.

In sum, the current results demonstrate that adipocytes serve to integrate not only the macronutrient status, but also the iron status of the organism to modulate metabolism, appetite, and energy homeostasis. Presumably, this would help maintain food-seeking behavior in an iron-rich environment that would support high rates of oxidative metabolism, energy production, growth, and reproduction.

Methods

Experimental animals. Dietary iron manipulations were accomplished with diets containing 60% carbohydrate, 17.7% protein, and 7.2% fat by weight, and either 4

mg/kg (TD. 10210), 35 mg/kg (TD. 10211), 500 mg/kg (TD. 10212), 2000 mg/kg (TD. 10214), or 20 g/kg carbonyl iron (TD. 10213) (Harlan Teklad) for a period of 2 months before phenotyping. Wild-type mice were of the C57BL6/J strain. In addition, mice with targeted deletion of the Hfe gene were studied after being bred onto either the 129/SvEvTac or C57BL6/J genetic backgrounds for at least 5 generations (62). Mice with adipocyte-specific deletion of the ferroportin gene were generated by breeding mice with LoxP sites flanking exon 6 and 7 of the ferroportin gene (22) (generously provided by Nancy C. Andrews, Duke University), with C57BL6/J mice containing an ap2-promoter-driven Cre recombinase (The Jackson Laboratory). Age- and sex-matched WT littermates were used as controls. Body composition analysis was performed using a nuclear magnetic resonance system (Minispec LF-50 BCA Analyzer, Bruker). Transgenic mice expressing the luciferase reporter gene under the control of leptin regulatory sequences were generously provided by Dr. Jeffry M. Friedman (63).

Human subjects. The subjects were part of a larger study and were described previously (15), and their samples were deidentified for this study. Diabetes status was confirmed by oral glucose tolerance testing (OGTT). Diabetic subjects were on diet therapy only, with fasting plasma glucose between 125 mg/dl and 175 mg/dl. All sera were obtained after a 12-hour overnight fast.

Reagents and assays. Reagents were purchased from Sigma-Aldrich unless otherwise noted. Serum leptin levels were measured by ELISA kit (Millipore). Serum ferritin and iron were measured by ARUP laboratories (Salt Lake City, UT).

In vivo imaging. In vivo imaging of leptin/luciferase transgenic animals was performed using the Xenogen IVIS Lumina II imaging system (Caliper). Because of the heterogeneity in the weights of the transgenic mice and the effect of weight on leptin, firefly levels were determined only in weight-matched mice. Isoflurane-anesthetized animals were injected intraperitonally with luciferin (300 µl of 15 mg/ml in PBS). The

animals were imaged in an imaging chamber 15 min after injection and the photon image was analyzed by Living Image 3.0 software (Xenogen) (63).

3T3-L1 adipocyte culture and differentiation. 3T3-L1 adipocytes (ATCC) were maintained in high-glucose DMEM (HG-DMEM) supplemented with 10% bovine calf serum (BCS, Hyclone) and penicillin/streptomycin (Invitrogen). For differentiation (64), cells were incubated in HG-DMEM with 10% BCS for 48 h after confluence. Cells were then cultured in differentiation medium I (HG-DMEM, 10% FBS, 1 µg/ml insulin, 0.25 µg/ml dexamethasone, 0.5 mM IBMX, 4µM ciglitazone) for 4 days, followed by differentiation medium II (HG-DMEM, 10% FBS, 1 µg/ml insulin) for 48 h. Prior to experiments, cells were cultured overnight in DMEM (Invitrogen) with 0.5% BSA. All experiments were performed in MEM-α medium, which is iron-free, supplemented with 10% FBS and FAC as indicated.

Plasmids, nucleofection, and luciferase assay. The ~6kb (5,986bp) and 764bp 5'-flanking region of the murine leptin promoters were amplified with BglII-overhung or HindIII-overhung primers from genomic epididymal fat pad DNA and inserted into the pGL4.14 vector (Promega). Site-specific mutagenesis studies were carried out using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). Plasmids were nucleofected into undifferentiated 3T3-L1 preadipocytes using an Amaxa Nucleofector with Kit V (Lonza) and an electroperator (Nucleofector II AAD-1001N, device no. 400414; Amaxa Biosystems, Cologne, Germany). After overnight incubation, positively transfected cells were selected by hygromycin. After two weeks' selection, pools of cells were differentiated and also used to select clonal cell lines. Luciferase activity was quantified using the Luciferase Reporter Assay System (Promega) and a 96-well plate luminometer. Luciferase levels of specific genes were normalized to protein levels.

Isolation of primary adipocytes. Subcutaneous and epididymal fat pads were removed from male mice, and incubated in HBSS with 1% BSA and 1.5 mg/ml collagenase, type I, for 45 min at 37°C, rotating at 180 rpm. Adipocytes were filtered through 100-µm nylon mesh and rinsed with HBSS-1% BSA. Following centrifugation at 880rpm, the fat cake was transferred to a clean tube, rinsed, and centrifuged. Isolated adipocytes were either used immediately or flash frozen in liquid nitrogen.

Labeling, analysis, and sorting of adipose progenitor cells. Subcutaneous and epididymal adipose tissue was excised and the stromal vascular fraction (SVF) isolated as described (65). The SVF was resuspended in ice-cold HBSS with 10% FBS for labeling. Antibody incubations were performed on ice for 20 min. Cells were washed and resuspended in HBSS for sorting. Samples were sorted on a BD FACSAria cell sorter and analyzed on a BD LSRII (BD Biosciences) flow cytometer, each equipped with BD FACSDiva Software. For sorting, the cells were separated on the basis of the cell-surface markers indicated in the literature (66).

Quantification of transcripts. Quantitative RT-PCR was performed as described previously (53). Briefly, mRNA was extracted from primary or 3T3-L1 adipocytes or fat pad using TRIzol (Invitrogen), purified using an RNeasy column (QIAgen), and synthesized into cDNA using a First-Strand cDNA Synthesis Kit (Invitrogen). The fat cake in TRIzol was cleared by transferring the infranatant to a clean tube using a glass syringe before extraction. Real-time PCR was performed with a QuantStudio real-time PCR systems (Applied Biosystems). cDNA products were quantified using the relative standard curve method. mRNA levels of specific genes were normalized to cyclophilin A or RPL13A.

Antibodies. Antibodies used included pCREB (Ser 133) (87G3) and CREB (86B10) from Cell signaling Inc., ob (A20, sc842) from Santa Cruz Inc., and α-tubulin (T9026) from Sigma-Aldrich.

EMSA. Oligonucleotides were end labeled with biotin. Differentiated 3T3-L1 adipocyte nuclear extracts were prepared using a nuclear extraction kit (Abcam ab113474). The binding reaction was done according to the LightShift Chemiluminescent kit manual with some modification (Thermo Scientific, Cat#20148). Briefly, nuclear extract was added to binding buffer containing 5 nM MgCl2, 0.05% NP-40, 50 ng/uL poly(dI-dC), and 2.5% glycerol and incubated at room temperature for 10 min add biotinylated oligonucleotide probe and incubated 25 min at room temperature. Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels and visualized by chemiluminescent nucleic acid detection module (Thermo Scientific, Cat# 89880). For DNA competition EMSA, nonbiotinylated wild-type or mutant oligonucleotides were added first.

ChIP. ChIP studies were performed as described with minor modification (67). Chromatin was extracted from 3T3-L1 adipocytes on day 10 to 13 after differentiation using the SimpleChIP kit (Cell signaling #9002) according to the manufacturer's instructions. Briefly, cells were treated with 100 µg/mL of FAC for 24 h prior to crosslinking for 10 min with 1% formaldehyde. Cells were then lysed by micrococcal nuclease and sonicated three times for 20 s using a sonic dismembrator (Fisher Scientific). pCREB was then immunoprecipitated from precleared lysates with Protein A/Salmon sperm DNA agarose beads (Millipore). DNA was released from protein-DNA complexes by proteinase K digestion, and then subjected to quantitative real-time PCR using a power SYBR green kit (Applied Biosystem). The following primers, Fwd-5'-GCA CGA TGT AAC CAC GAA TG -3' and Rev-5'- ACG TCC ATT CAG CAA AAA CC -3', were used to amplify the CRE1 site. Fwd-5'- GGC GAA AGG CAA ACA TAA GA-3' and Rev-5'- TTC CCG CTC TGA CAT TCT TT-3' primers were used to amplify the CRE2 site. ChIP-qPCR data were normalized for amount of chromatin by input samples and normal IgG samples.

Food intake measurement. Food intake was measured over a 3-day period with the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) using a four-chamber open-circuit system. Animals were acclimatized to the chambers overnight prior to data collection and maintained at 24°C under a 12:12-h light-dark cycle. Mice were housed individually with food and water freely available. Four mice were measured simultaneously. Food consumption was monitored by electronic scale for each mouse, and each cage was sampled for 1.5 min at 15 min intervals. Accumulated food intake was the sum of all acquired points.

Statistics. Descriptive statistics in the text and figures are represented as average ± SEM. The Pearson's correlation coefficient was calculated to test for correlation between 2 parameters. Multivariate logistic regression analysis using STATA was sequentially performed to investigate the role of CRP, BMI, diabetes, and gender on the ferritin-leptin association. An unpaired 2-tailed Student's t test was used to determine significance between controls and individual experimental groups. A paired 2-tailed Student's t test was used to determine significance between controls and individual experimental groups in leptin-luciferase mice. One-way ANOVA was used to compare series of data. Wilcoxon signed-rank test was applied to compare real-time feeding behavior and accumulated food intake in different mouse groups in the metabolic chamber. $P < 0.05$ was considered significant for all tests.

Study approval. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Utah, under IACUC approval A3031-01. The human studies were approved by the IRB of the University of Utah, protocols 0880 and 20094. Studies were also reviewed and approved by the Advisory Committee to the Clinical Services Core (CSC) of the Center for Clinical and Translational Science. Additional previously unpublished data were from human studies completed at the Pennington Biomedical Research Center and at the University of Utah. Both studies

were approved by the IRBs of the respective institutions, as previously published (15, 68).

Acknowledgments

Yan Gao conducted all experiments and wrote the manuscript, except the portions indicated below. Zhonggang Li aided with statistical analysis of human study results by STATA and western blots. J. Scott Gabrielsen performed human ELISA assay and constructed the 764bp leptin promoter luciferase plasmid. Judith A. Simcox aided with tissue harvest and provided tissues from mice fed with different dietary iron. Soh-hyun Lee helped with tissue harvest and luciferase mice imaging. Robert Cooksey aided with tissue harvest, food intake measurement, and analysis. Deborah Jones aided with tissue harvest and took care of mice. William T. Cefalu provided human samples for leptin and ferritin assay. Donald A. McClain oversaw experimental design, experiments, data analysis, and interpretation, in addition to writing of the manuscript.

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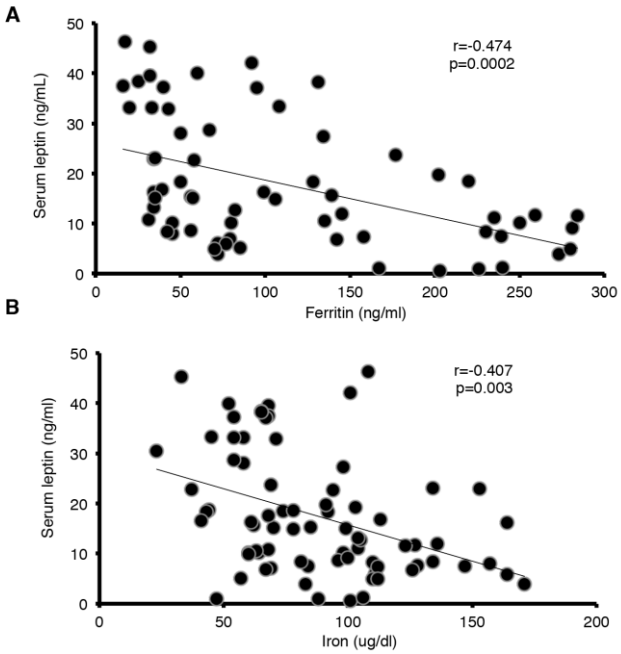

Figure 2.1 Serum ferritin and iron levels are inversely associated with serum leptin levels. (A) Serum leptin and ferritin levels were measured and correlated in a cohort of subjects with normal ferritin (n=65). (B) Serum iron levels are inversely associated with serum leptin levels in a cohort of patients with type 2 diabetes as well as in obese subjects with metabolic syndrome (n=76).

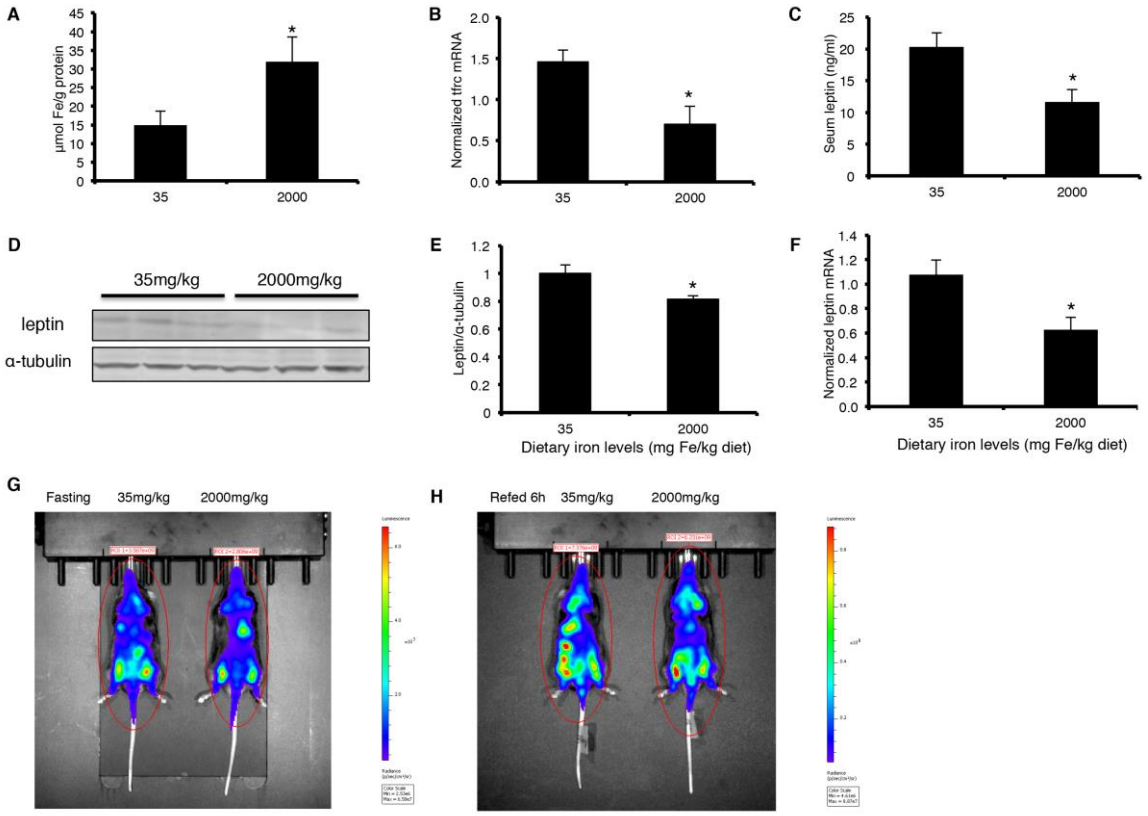

Figure 2.2 Serum leptin and adipocyte leptin mRNA levels decrease with dietary iron overload. (A) Iron levels measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) in mice fed low normal (35mg/kg) and high (2000 mg/kg) iron diet (n=4 or 5 per group). (B) *Tfrc* mRNA levels quantified by RT-PCR and normalized to cyclophilin A in adipose tissue from mice fed different levels of dietary iron (n=36 per group). (C) Serum leptin levels from the mice (n=36 per group). (D) Intracellular leptin levels by western blotting (n=4 per group). (E) Quantification of leptin protein levels from panel D normalized to tubulin (n=4 per group). (F) Leptin mRNA levels quantified by RT-PCR and normalized to cyclophilin A in adipose tissue from mice fed different dietary iron (n=36 per group). (G, H) Leptin expression is significantly decreased in high iron-fed transgenic mice expressing the luciferase reporter gene under the control of leptin regulatory sequences both in fasting (G) and refed (H) state (n=5 per group). *P<0.05 compared to 35 mg/kg dietary iron-fed mice.

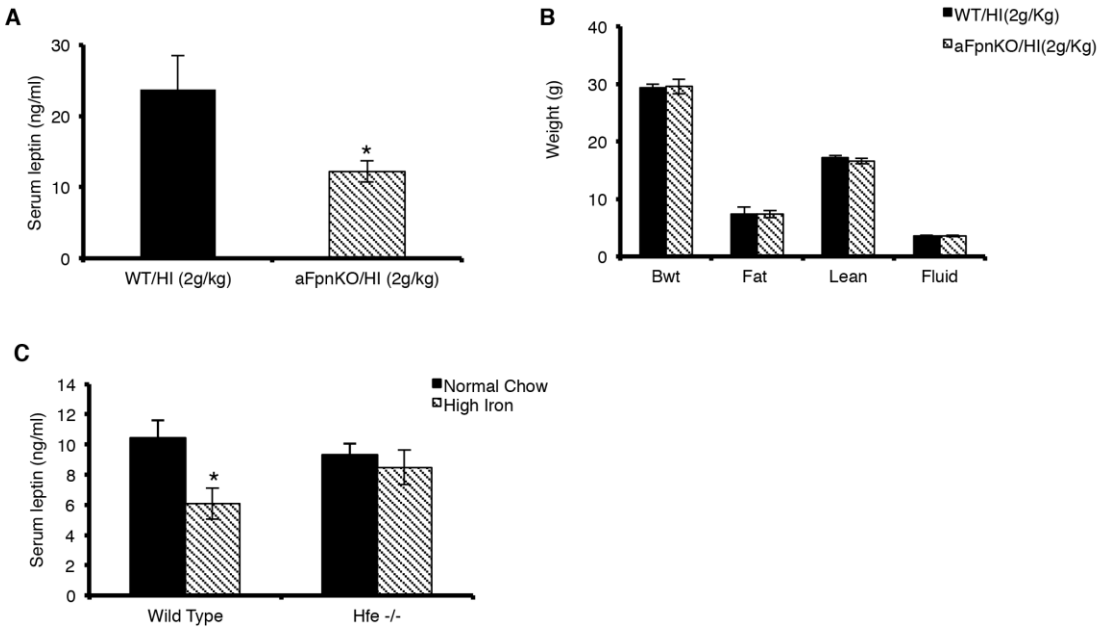

Figure 2.3 Serum leptin levels in mouse models with increased and decreased adipocyte iron. (A) Decreased serum leptin levels in adipocyte-specific ferroportin knockout mice (aFpnKO) (n=4 or 5). (B) Body composition in aFpnKO compared to WT mice (n=4 or 5). (C) Serum leptin in WT and HFE-/- mice on the 129/SvEvTac background fed normal chow (330 mg/kg) and high-iron (20 g/kg) diets (n=10 to 20 per group). *P <0.05 compared to wild-type group.

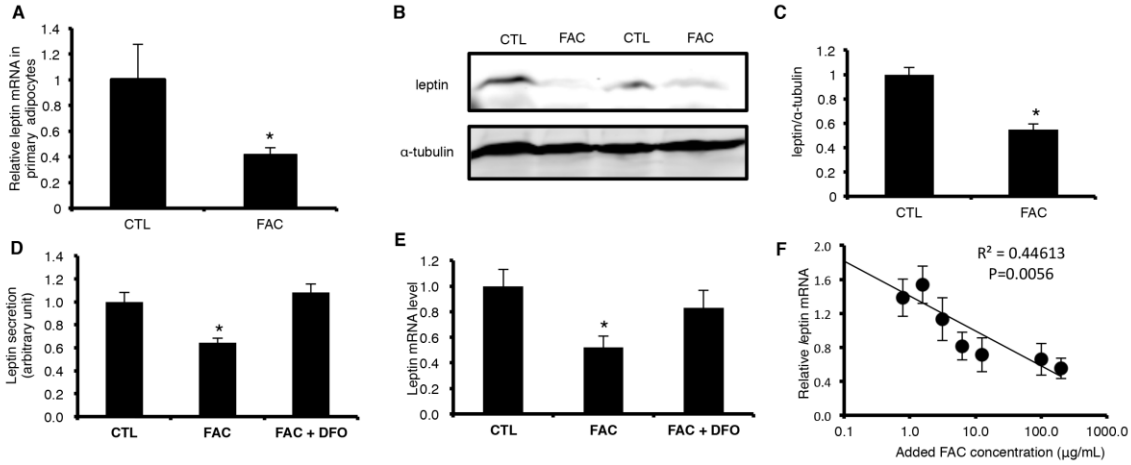

Figure 2.4 Leptin mRNA and protein levels decrease with iron treatment in primary adipocytes or 3T3-L1 adipocytes. (A) Leptin mRNA levels decrease in collagenased floating primary adipocytes treated with FAC (n= 4 per group). (B) Intracellular leptin protein decreases with FAC treatment. (C) Quantificaton of western blots (total n = 6 independent determinations) normalized to α-tubulin. (D, E) Leptin-secreted protein (D) and mRNA (E) levels in 3T3-L1 cells following 6 h treatment with FAC and FAC plus the iron chelator desferoxamine (DFO) (n=3). (F) Dose response for suppression of leptin mRNA by iron (n=3, p=0.0056). *P <0.05 compared to control.

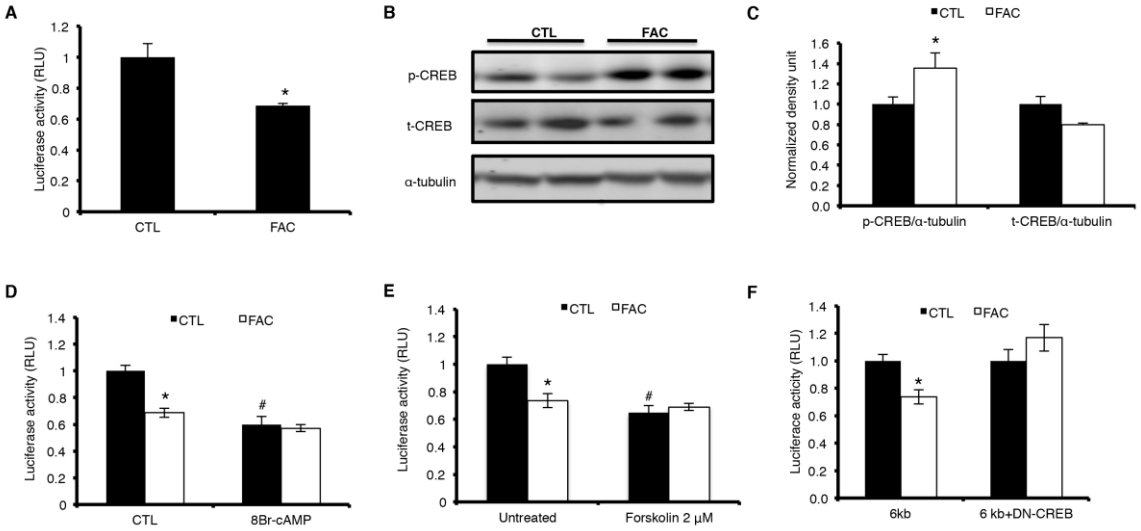

Figure 2.5 Iron decreases leptin promoter activity and activates cAMP-response element binding protein (CREB). (A) Distal leptin promoter (-5986 bp) -driven luciferase activity in the presence or absence of 100 μg/ml FAC (n = 6). (B) Western blot for phosphorylation of CREB (p-CREB), total CREB (t-CREB) and α-tubulin in 3T3-L1 adipocytes treated with 100 μg/ml FAC for 24 h. (C) Quantitation of western blots (total n = 3 independent determinations) normalized to α-tubulin (n = 3). (D) Distal leptin promoter luciferase activity in 3T3-L1 adipocytes treated with cAMP agonist, 8Br-cAMP (1 mM) and/or 100μg/ml FAC for 24 h (n = 6). (E) Distal leptin promoter luciferase activity in 3T3-L1 adipocytes treated with cAMP agonist, Forskolin (2 μM) and/or 100 μg/ml FAC for 24 h (n = 6). (F) Dominant-negative CREB (ACREB) abolished the iron-mediated decrease of luciferase activity driven by the distal leptin promoter construct (n = 6). *P <0.05 compared to control. #P <0.05 compared to untreated group.

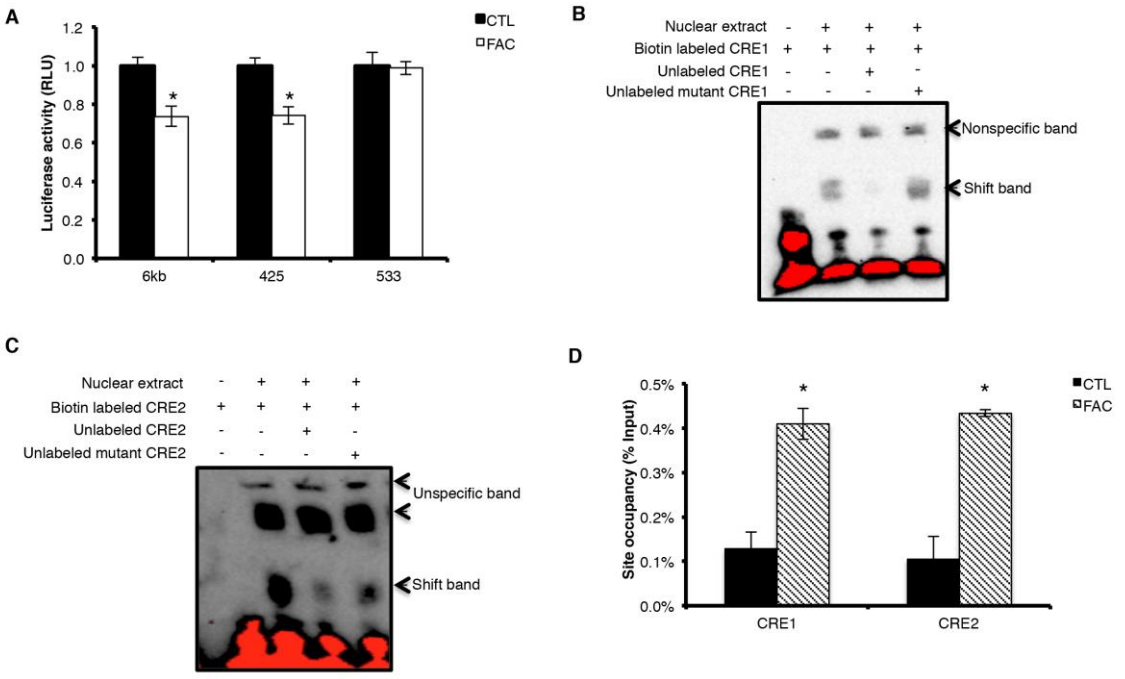

Figure 2.6 Iron decreases leptin promoter activity via potential CRE binding sites. (A) Iron decreases promoter activity in control and the 425 mutant construct but not the 533 mutant construct (n = 6). (B) EMSA with the CRE1 oligos as indicated. Unlabeled CRE1 abolished protein-probe complex formation and mutation of the CRE1 failed to compete the protein-probe interaction. (C) EMSA with the CRE2 oligos as indicated. Unlabeled CRE2 abolished protein-probe complex formation and mutation of the CRE2 failed to compete the protein-probe interaction. Addition of phosphor-CREB (S133) antibody markedly decreased CRE2-protein binding. (D) Phosphor-CREB (S133) occupancy at leptin promoter sites were induced by iron. Quantitative real-time PCR amplification of DNA sequences flanking CRE1 and CRE2 sites from ChIPs performed with pCREB (S133) antibody (n=3). *p<0.05 compared to control group.

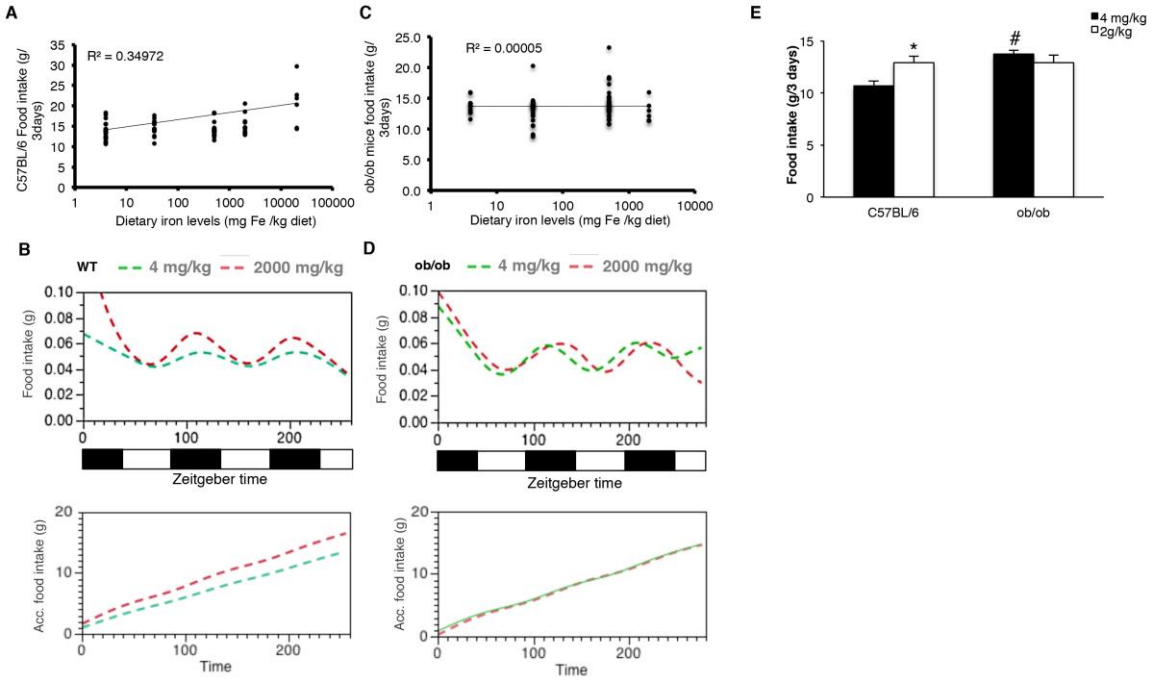

Figure 2.7 Decreased leptin in mice fed different levels of dietary iron is associated with increased food intake. (A) Food intake is positively associated dietary iron levels in mice (****P* <0.001, n = 69). (B) Real-time recording (upper panel) and accumulation of food intake (lower panel) in mice on low (4 mg Fe/kg) and high iron (2000 mg Fe/kg) diets (Welcoxon signed-rank test, *P* = 0.0084 for food intake and *P* = 0.0002 for accumulated food intake, respectively). Black bar in zeitgeber time is night time (12h/daily) and white bar is day time (12h/daily). (C, D) Dietary iron association with food intake is abolished in ob/ob mice. (E) Significant increase of food intake was observed in 2000 mg/kg group compared to 4 mg/kg and iron-fed mice.

Supplementary experimental procedures

The leptin UTRs constructs were generously provided by Susan Fried, Boston University. The leptin UTRs were cloned into the pGL3 control vector containing SV40 promoter and enhancer sequences (Promega) to drive a high and stable expression of luciferase. UTR plasmids and Renilla as an internal control were nucleofected into well-differentiated 3T3-L1 preadipocytes using an Amaxa Nucleofector with Kit L (Lonza) and an electroperator (Nucleofector II AAD-1001N, device no. 400414; Amaxa Biosystems, Cologne, Germany). The proximal 764bp of the murine leptin promoter were amplified with BamHI-overhung primers from genomic epididymal fat pad DNA and inserted into the pGL4.14 vector (Promega). Plasmids were nucleofected into undifferentiated 3T3-L1 preadipocytes using an Amaxa Nucleofector with Kit V (Lonza). After overnight incubation, positively transfected cells were selected by hygromycin. After 2 weeks' selection, pools of cells were differentiated and also used to select clonal cell lines. Luciferase activity was quantified using the Luciferase Reporter Assay System (Promega) and a 96-well plate luminometer. Luciferase levels in different constructs were normalized to Renilla or protein levels. 3T3-L1 adipocytes differentiated for 10-14 days were starved by DMEM containing 0.5% BSA overnight, following by iron treatment. Cyclic AMP or calcium levels were measured by cyclic AMP EIA kit or calcium assay kit (Cayman Chemicals Ann Arbor, MI).

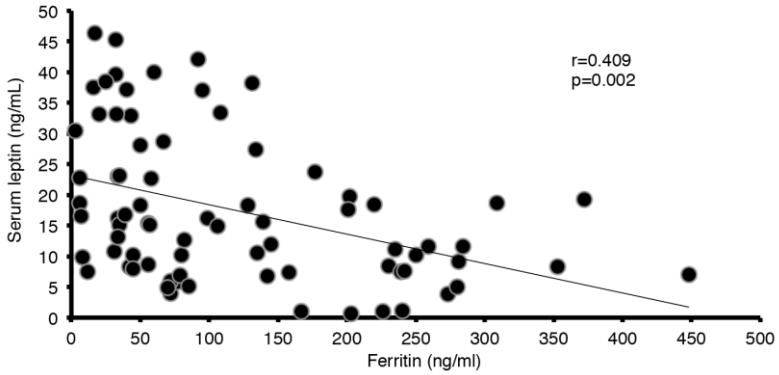

Figure 2.S1 Serum ferritin levels are inversely associated with serum leptin levels. Serum leptin and ferritin levels were measured and correlated in a cohort of patients with type 2 diabetes as well as in obese subjects with metabolic syndrome (n=77). *P = 0.002

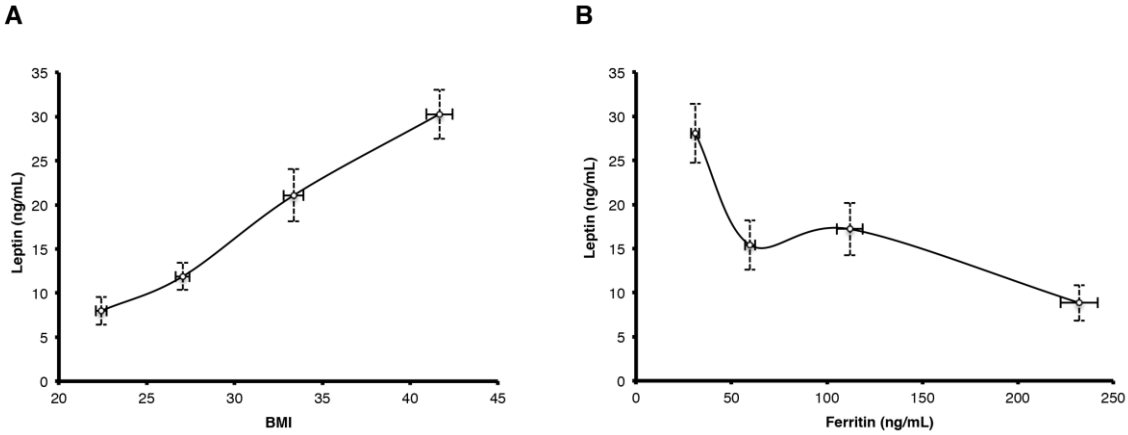

Figure 2.S2 The association between leptin and BMI or ferritin by quartiles in human. (A) Leptin in the high BMI group (upper 25% percentile BMI) is significantly higher than that in the other three low BMI groups. (B) The leptin in the high ferritin group (upper 25% percentile ferritin) is significantly lower than that in the other three low ferritin groups (n=65). ***P < 0.001(one way ANOVA for leptin by BMI category); * P < 0.05(one way ANOVA for leptin by ferritin category).

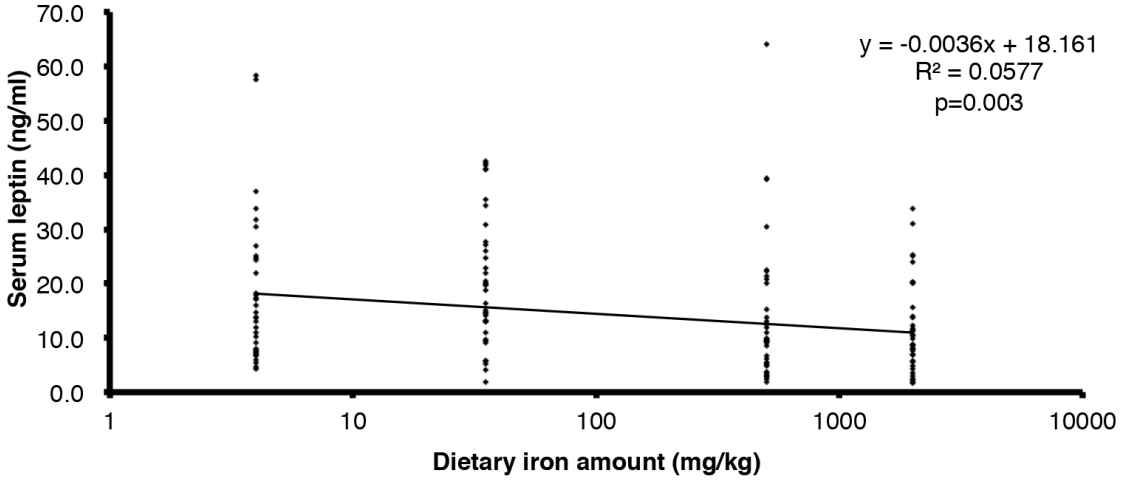

Figure 2.S3 Serum leptin levels were determined in 5-month-old C57BL/6J mice following 9 weeks of being fed low iron (4 mg/kg carbonyl iron), normal chow (35 mg/kg and 500 mg/kg), or high iron (2000 mg/kg) diets. Serum leptin decreased with dietary iron amounts (linear regression, P = 0.003) (n =36).

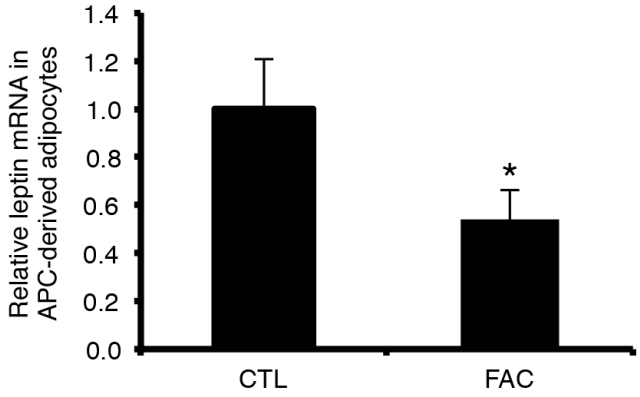

Figure 2.S4 Leptin mRNA levels decrease in adipocyte progenitor cells (APC)-derived primary adipocytes by fluorescence-activated cell sorting (FACS) (n=3). * $P < 0.05$ compared to control (n=6).

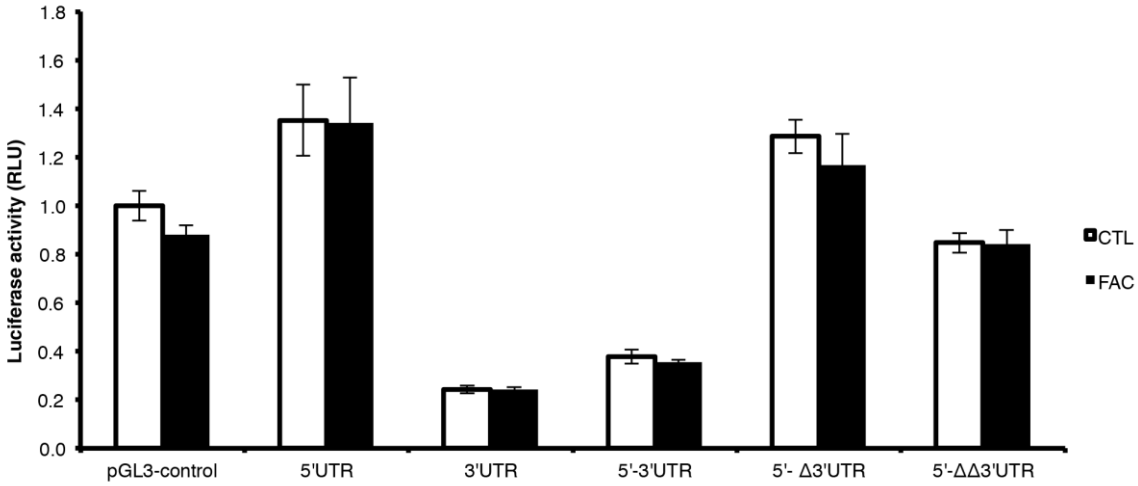

Figure 2.S5 Iron had no effect on translational regulation of leptin by its 5'- or 3'-UTRs. Differentiated 3T3-L1 cells were transiently co-transfected with the chimeric leptin UTR-LUC constructs and the pRL-TK (*Renilla* luciferase vector) as described under "Supplementary Experimental Procedures" (n=6). Chimeric UTR-LUC constructs carrying the 5'-UTR and/or various lengths of 3'-UTR of leptin mRNA (56). The pGL3 control contained no leptin UTR sequences and was used as control.

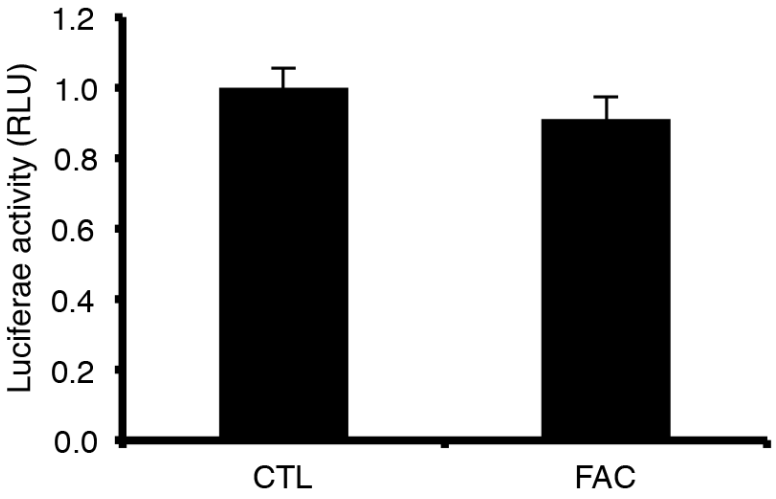

Figure 2.S6. Proximal leptin promoter (-764bp)-driven luciferase activity is unchanged in the presence or absence of 100 µg/ml FAC (n=6).

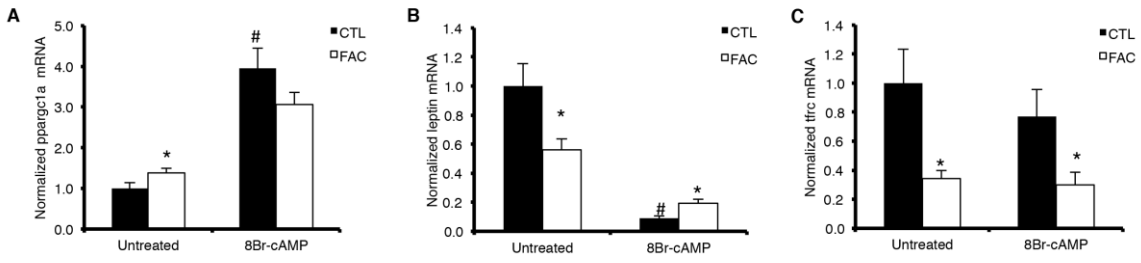

Figure 2.S7 Gene expression with iron and/or 8Br-cAMP treatment. (A) 8Br-cAMP abolished increment of ppargac1a expression seen after 6 h iron treatment (n = 3). (B) Leptin expression in 3T3-L1 adipocytes treated with cAMP agonist, 8Br-cAMP (1mM), and/or 100 µg/ml FAC for 6 h (n = 3). (C) Tfrc mRNA in 3T3-L1 adipocytes treated with or without cAMP agonist, 8Br-cAMP (1 mM) for 6 h and no iron or 100 µg/ml FAC for 6 h (n = 3).

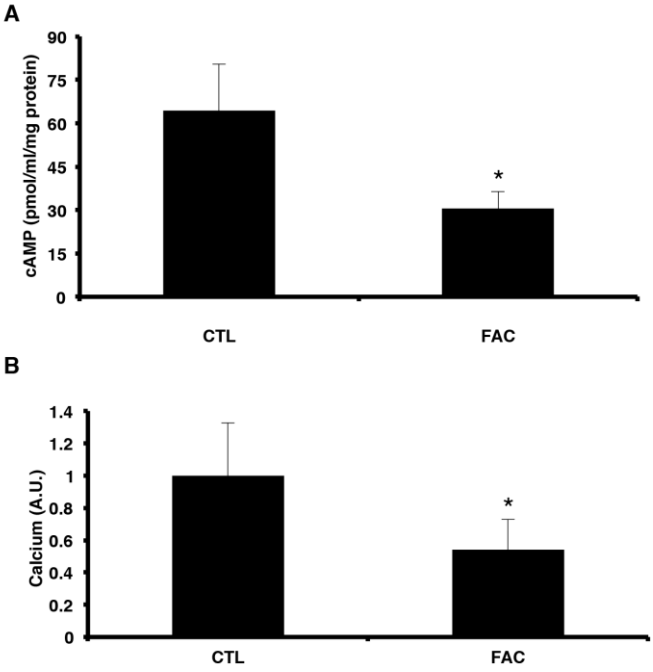

Figure 2.S8 cAMP and calcium levels were decreased in FAC treated 3T3-L1 adipocytes (n = 3).

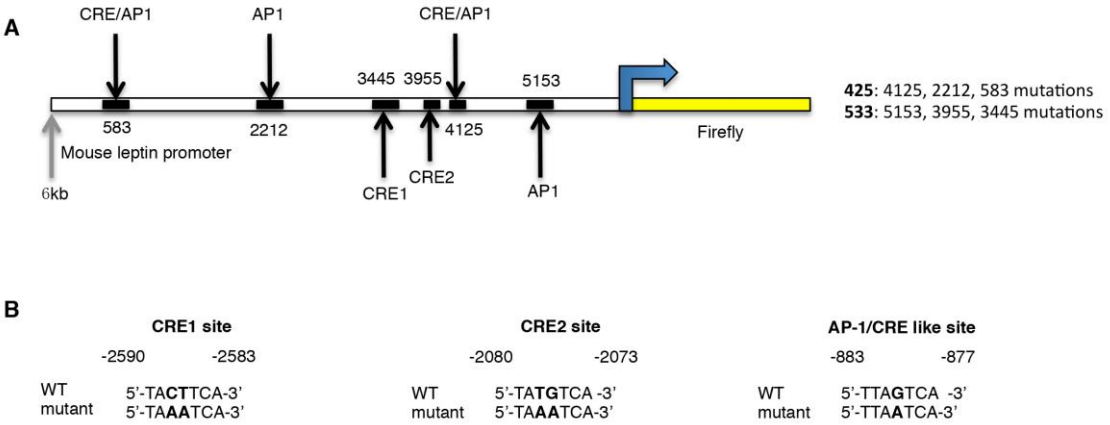

Figure 2.S9 Model of leptin promoter. (A) Mouse leptin promoter and mutagenized constructs showing with potential CRE. (B) Three potential CRE sites and mutagenesis used in plasmids or EMSA assay.

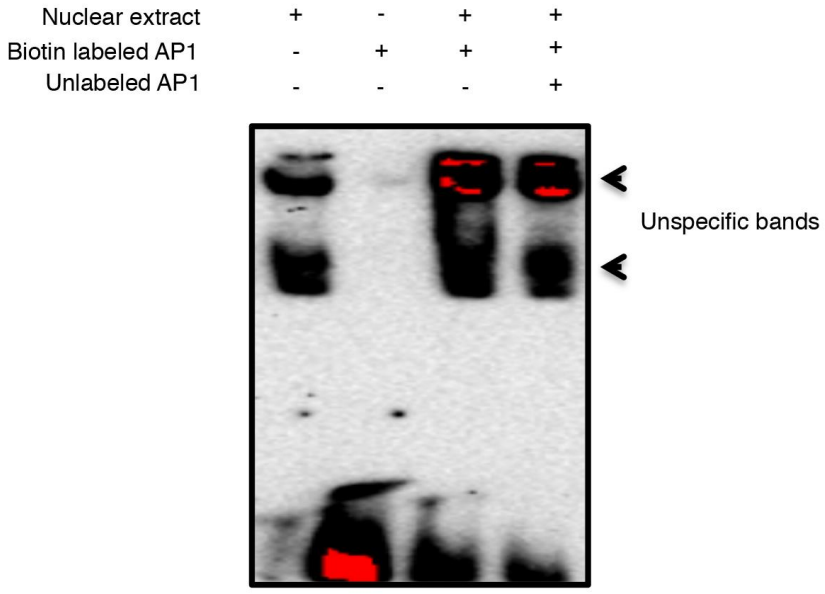

Figure 2.S10 EMSA with the AP1 oligos as indicated.

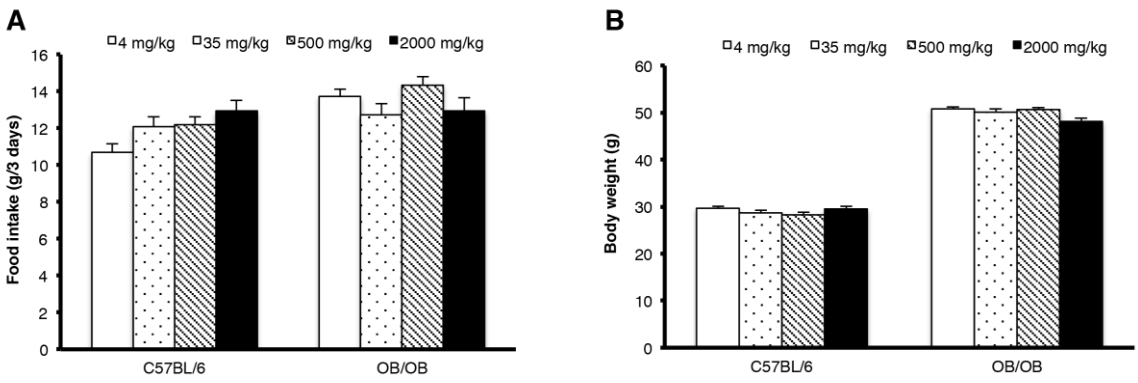

Figure 2.S11 Accumulated food intake and body weight in C57/BL6 and ob/ob mice on different iron diet. (A) Mice fed with high iron diet (2000 mg/kg) had more food intake compared to low iron diet. (B) Body weights did not change with different dietary iron after 9 weeks on diet. *$P < 0.05$ (1-way ANOVA for food intake in C57BL/6 mice under different dietary iron); $P = 0.123$ (1-way ANOVA for food intake in ob/ob mice under different dietary iron), $P = 0.524$ (1-way ANOVA for body weight in C57BL/6 mice under different dietary iron); $P = 0.856$ (1-way ANOVA for body weight in ob/ob mice under different dietary iron).

CHAPTER 3

IRON-ACTIVATED CREB VIA DECREASED O-GLCNACYLATION

Introduction

Prior to the current study, there was little evidence that CREB regulates leptin in mature adipocytes. It has been reported that activation of CREB during the differentiation process suppresses leptin secretion and expression in mesenchymal stem cell (1), and bioinformatics analysis indicates there are two potential CRE sites in the human ob promoter, but no study of their function has been reported (2). In the previous chapter, we have demonstrated that leptin expression can be mediated by modulation of CREB. The molecular mechanism for the regulation of leptin by iron includes phosphorylation of CREB is demonstrated. However, how iron regulates phosphorylation of CREB is totally unknown. Besides iron, another well-know factor that can change fuel consumption from glucose to fat burning in mammals is fasting. Leptin is responsive to nutritional status and fasting leads to a gradual decline in leptin (3, 4). The cellular mechanism that causes leptin to fall with iron may be similar to that seen with fasting. Fasting evokes a number of responses, including an increase of glucagon, a known activator of CREB (5). Thus, CREB may play a role in connecting not only iron but also nutritional status to leptin. Interestingly, in this study, we have found that nutrient-sensing pathway, the hexosamine biosynthesis pathway (HBP), which is also known to play a crucial role in regulation of CREB (6) and has been reported to regulate leptin expression (7), also contributes to iron-induced changes in leptin expression.

In this chapter, I will explore the relationship of iron, O-GlcNAcylation, and leptin, trying to establish the role of micronutrition status in metabolic regulation.

Methods

Experimental animals. Dietary iron manipulations were accomplished with diets containing 60% carbohydrate, 17.7% protein, and 7.2% fat by weight, and either 35

mg/kg (TD. 10211) or 2 g/kg carbonyl iron (TD. 10214) (Harlan Teklad) for a period of 2 months before phenotyping. Wild-type (WT) mice were of the C57BL6/J strain.

Reagents and assays. Reagents were purchased from Sigma-Aldrich unless otherwise noted. Serum leptin levels were measured by ELISA kit (Millipore).

3T3-L1 adipocyte culture and differentiation. 3T3-L1 adipocytes (ATCC) were maintained in high-glucose DMEM (HG-DMEM) supplemented with 10% bovine calf serum (BCS, Hyclone) and penicillin/streptomycin (Invitrogen). For differentiation (8), cells were incubated in HG-DMEM with 10% BCS for 48 h after confluence. Cells were then cultured in differentiation medium I (HG-DMEM, 10% FBS, 1 μg/ml insulin, 0.25 μg/ml dexamethasone, 0.5 mM IBMX, 4 μM ciglitazone) for 4 days, followed by differentiation medium II (HG-DMEM, 10% FBS, 1 μg/ml insulin) for 48 h. Prior to experiments, cells were cultured overnight in DMEM (Invitrogen) with 0.5% BSA. All experiments were performed in MEM-α medium, which is iron-free, supplemented with 10% FBS.

Plasmids, nucleofection, and luciferase assay. The proximal 5,986bp of the murine leptin promoter were amplified with BglII-overhung or HindIII-overhung primers from genomic epididymal fat pad DNA and inserted into the pGL4.14 vector (Promega). Site-specific mutagenesis studies were carried out using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). Plasmids were nucleofected into undifferentiated 3T3-L1 preadipocytes using an Amaxa Nucleofector with Kit V (Lonza) and an electroperator (Nucleofector II AAD-1001N, device no. 400414; Amaxa Biosystems, Cologne, Germany). After overnight incubation, positively transfected cells were selected by hygromycin. After 2 weeks' selection, pools of cells were differentiated and also used to select clonal cell lines. Luciferase activity was quantified using the Luciferase Reporter Assay System (Promega) and a 96-well plate luminometer. Luciferase levels of specific genes were normalized to protein levels.

Quantification of transcripts. Quantitative RT-PCR was performed as described previously (9). Briefly, mRNA was extracted from primary or 3T3-L1 adipocytes or fat pad using TRIzol (Invitrogen), purified using an RNeasy column (QIAgen), and synthesized into cDNA using a First-Strand cDNA Synthesis Kit (Invitrogen). The fat cake in TRIzol was cleared by transferring the infranatant to a clean tube using a glass syringe before extraction. Real-time PCR was performed with a QuantStudio™ Real-Time PCR Systems (Applied Biosystems). cDNA products were quantified using the relative standard curve method. mRNA levels of specific genes were normalized to cyclophilin A or RPL13A.

Antibodies. Antibodies used included pCREB (Ser 133) (87G3) and CREB (86B10) from Cell signaling Inc., ob (A20, sc842) from Santa Cruz Inc., anti-O-Linked N-Acetylglucosamine antibody (10) (ab2739) from Abcam Inc., CTD 110.6 from Covance, and α-tubulin (T9026) from Sigma-Aldrich.

Immunoprecipitations (IP) assay. Well-differentiated adipocytes were washed with PBS once and collected in lysis buffer with protease inhibitor (Roche Complete Tablet 11836153001) and phosphatase inhibitor (sigma P2850). After centrifugation at 15000 rpm for 10 min, the supernatant was transferred to a new tube and protein concentration was measured. The lysates (600ug) were immunoprecipitated with 4ug of CREB antibody (Cell signaling) at 4°C for overnight and added with 100 µl of (50% slurry) Protein A agarose beads (Millipore) overnight after precleared with 20 µl of free protein A agarose beads (Millipore) for 2 h. The supernatant was discard and the immune complex on beads was washed with lysis buffer with protease inhibitor and phosphatase inhibitor 3 times and boiled in sample buffer. The immunoprecipitated products were subjected to 7.5% SDS-PAGE, transferred onto the membrane, and immunoblotted with the antibodies, RL-2 and phosphor-CREB.

Statistics. Descriptive statistics in the text and figures are represented as average ± SEM. An unpaired 2-tailed Student's t test was used to determine significance between controls and individual experimental groups. One-way ANOVA was used to compare series of data. $p < 0.05$ was considered significant for all tests. All statistical analyses were performed with STATA.

Study approval. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Utah, under IACUC approval A3031-01.

Results and discussion

Dietary iron overload decreases O-GlcNAcylated protein level. To explore the regulation of dietary iron's effect on O-GlcNAcylation, we first measured the O-GlcNAcylated protein in epydidymal white adipose tissue by western blots. We observed a decrease of O-GlcNAcylated protein in fat from mice fed a high iron diet (2000 mg/kg iron) compared with that in mice fed low normal chow (35 mg/kg iron) (Figure 3.1A). We also demonstrated that adipocyte iron levels respond to dietary iron content in WT C57BL6/J mice by measuring mRNA levels of the transferrin receptor (*Tfrc*). *Tfrc* mRNA contains iron response elements in its 3' untranslated region (UTR) that result in decreased *Tfrc* mRNA levels as cellular iron levels increase (11). W observed a decrease of Tfrc mRNA in fat pad from high iron diet-fed mice compared with that in mice fed the normal iron chow (35 mg/kg or 500mg/kg iron) (Chapter 2, Figure 2.2B, $P < 0.05$). Serum leptin decreased with dietary iron (Chapter 2, Figure 2.S3). We also supplemented FAC to the medium and measured the total O-GlcNAcylation levels. We found a decrease of O-GlcNAcylated protein in well-differentiated adipocytes (Figure 3.2A and 3.2B).

$OGA^{+/-}$ mice relieved decreased serum leptin in high iron diet mice. To investigate the effect of iron and O-GlcNAcylation on leptin, OGA heterozygous mice

were fed the low normal or high iron diets (35 mg/kg vs 2000 mg/kg iron). We then measured the leptin in the serum from those mice. High iron diet decreased serum leptin in wild-type mice (Figure 3.3A, $P < 0.05$). We found leptin level was higher in $OGA^{+/-}$ mice in high iron diet (Figure 3.3A). This finding is consistant with our previous in vitro data in human adipocytes, suggesting increased O-GlcNAcylation plays a role in increase of leptin (12). We also found the decreased serum leptin in high iron diet-fed wild-type mice was relieved in $OGA^{+/-}$ mice independent of epididymal fat pad mass (Figure 3.3A and 3.3B). In 3T3-L1 adipocytes, glucosamine treatment relieved decreased leptin promoter activity by iron (Figure 3.4). Iron's effect on leptin promoter activity was abolished when co-treated with GFAT inhibitor, 6-diazo-5-oxo-L-norleucine (DON) (Figure 3.5).

Decreased O-GlcNAcylation of CREB is associated with increased phosphp-CREB in iron-treated 3T3-L1 adipocytes. We have preciously demonstrated activation of CREB by agonist blunted iron's effect on leptin reduction; similarly dominate-negative CREB, A-CREB, is also sufficient to block iron's effect on leptin, suggesting iron-mediated phosphorylation of CREB may be responsible for leptin regulation. Previously, it has been shown that crosstalk between phosphorylation and O-GlcNAcylation of CREB can modulate CREB activity to CRE-mediated promoter activity. We then measure iron's effect on CREB post-modification.

When we pulled down the CREB, and found iron supplementation to 3T3-L1 adipocytes decreased O-GlcNAcylation of CREB and total CREB protein while increased phospho-CREB levels (Figure 3.6). Decreased total CREB may be due to phosphorylation of CREB increase its degradation by protease (13, 14). Turnover of other transcriptional regulators including β-catenin and I-κB (15, 16) are also regulated through phosphorylation-targeted proteasomal degradation. Namely, increased phosphorylation of CREB is associated with a decrease of O-GlcNAcylation of CREB,

and activation of the HBP by glucosamine overcomes the iron-induced inhibition of leptin. These observations further underline the close relationship of iron levels with overall nutritional status and metabolic regulation.

In sum, the current results demonstrate that adipocytes serve to integrate not only the nutrient status, but also the iron status of the organism to modulate metabolism, appetite, and energy homeostasis. Presumably, this would help maintain food-seeking behavior in an iron-rich environment that would support high rates of oxidative metabolism, energy production, growth, and reproduction. The adipocyte responding to iron availability and adjusting expression of adipokines related to metabolism make it well suited to perform its broader nutrient-sensing function.

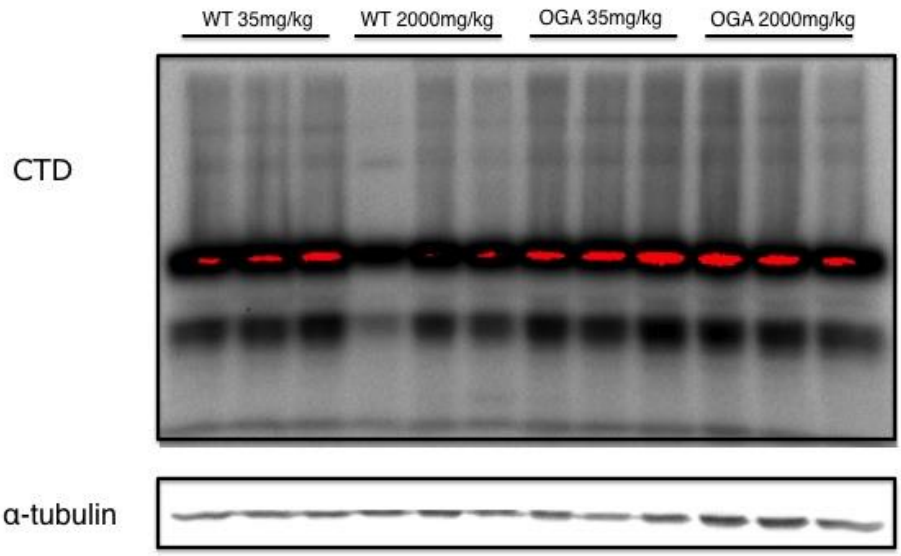

Figure 3.1 Dietary iron supplementation decreased O-GlcNacylation in adipose tissue.

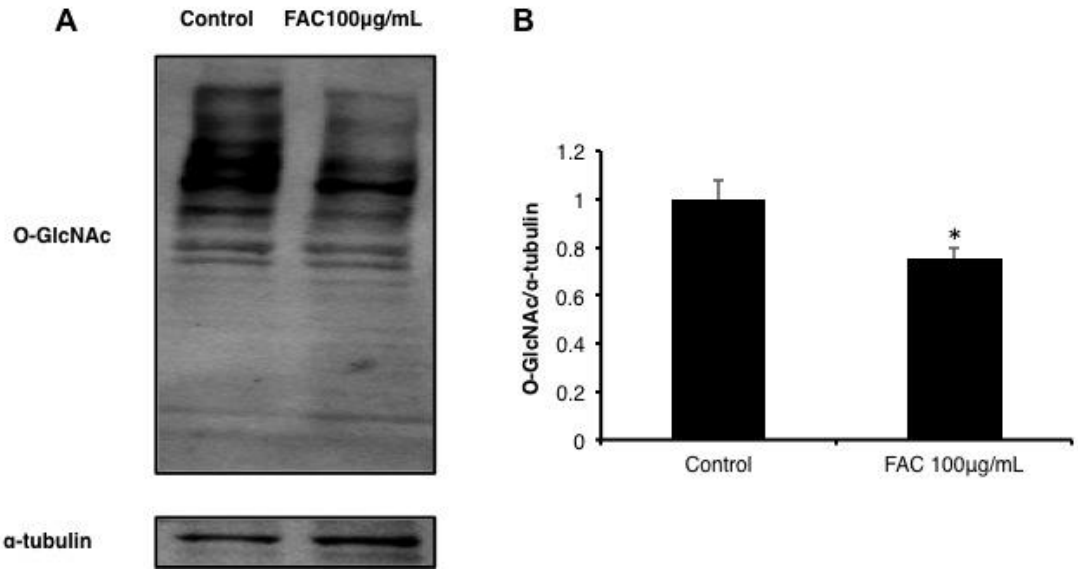

Figure 3.2 Iron treatment decreased total O-GlcNAcylation *in 3T3-L1 adipocytes. (*A) Whole O-GlcNAcylated protein decreases with 100µg/ml FAC treatment. (B) Quantificaton of western blots (total n = 3 independent determinations) normalized to α-tubulin. Significance denoted *P < 0.05 using Two-tailed Student's t tests (A). Data represent mean ± SEM.

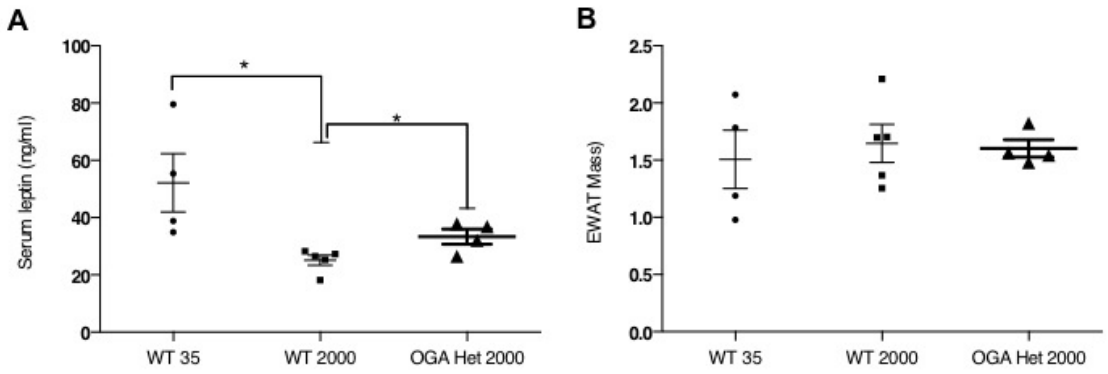

Figure 3.3 Decreased serum leptin under high iron diet is relieved in $OGA^{+/-}$ mice relieved. (A) High iron diet decreased serum leptin in wild-type mice, while the decrease of leptin is relieved in $OGA^{+/-}$ mice. (B) No difference in epididymal fat mass was observed in both wild-type and $OGA^{+/-}$ mice under 35 mg/kg and 2000 mg/kg iron diet. Significance denoted *$P < 0.05$ using Two-tailed Student's t tests (A). Data represent mean ± SEM.

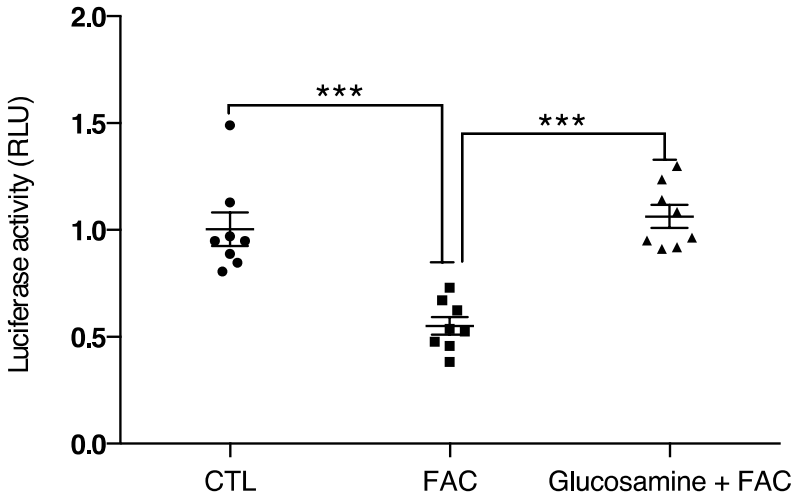

Figure 3.4 Glucosamine relieved decreased serum leptin promoter activity in 3T3-L1 adipocytes by iron. Significance denoted *** $P < 0.001$ using Two-tailed Student's t tests. Data represent mean ± SEM.

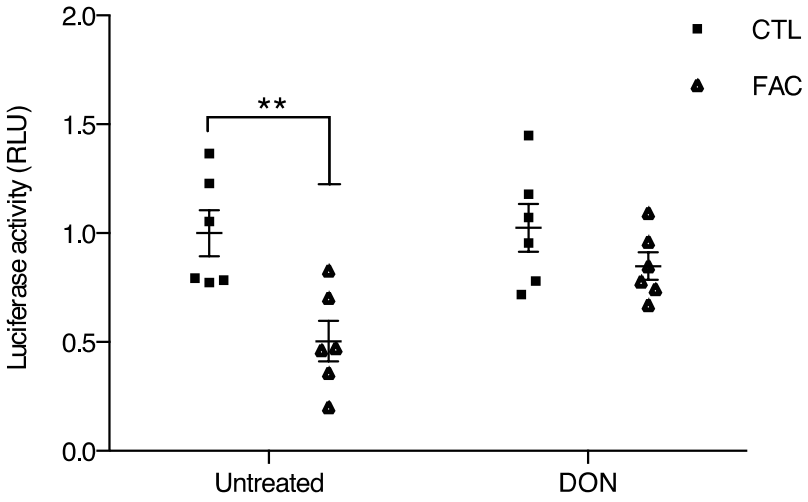

Figure 3.5 GFAT inhibitor, 6-diazo-5-oxo-L-norleucine (DON), abolished FAC-mediated decreased serum leptin promoter activity in 3T3-L1 adipocytes.

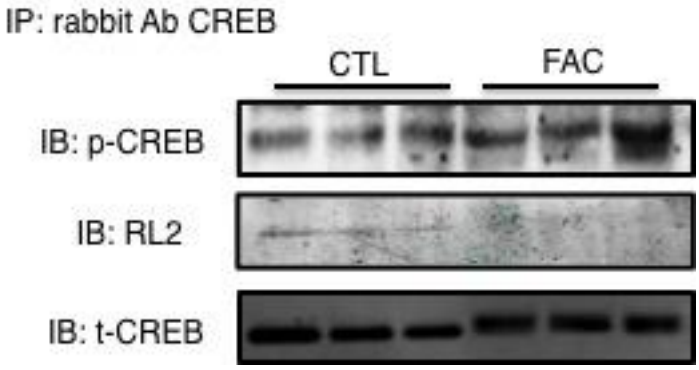

Figure 3.6 Decreased O-GlcNAc-CREB is associated with increased phospho-CREB with iron treatment in 3T3-L1 adipocytes. Total-CREB antibody was used in immunoprecipitation, and blot for phosphor-CREB and O-GlcNAcylated-CREB protein by RL2 antibody.

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CONCLUSION

It is well-established that iron overload is associated with insulin resistance and development of diabetes (1). Because adiponectin is an insulin-sensitizing adipokine, it is reasonable to speculate that iron stores might result in decreased levels of adiponectin. Scientific reports have also investigated the role of iron in the regulation of appetite. Dietary iron supplementation, for example, is associated with growth and increased appetite in children (12, 13).

In this dissertation, we have therefore investigated the effect of iron on adiponectin and leptin, and the mechanism of iron-induced decreases in the levels of these adipokines. We first verified that the concentration of circulating ferritin, a marker of iron stores, is associated with low serum adiponectin and leptin in humans. We demonstrate in humans that the association between serum ferritin and adiponectin or leptin is independent of inflammation. And serum ferritin, even within its normal ranges, is among the best predictors of serum adiponectin or leptin. Similarly, mice fed a high iron diet and 3T3-L1 adipocytes treated with iron exhibited decreased adiponectin and leptin mRNA and protein.

We found that iron negatively regulates transcription of adiponectin promoter-driven luciferase activity in a FOXO1-dependent manner. However, iron decreases the inactivation form of FOXO1, acetyl-FOXO1, while leaving phosphorylated FOXO1 and total FOXO1 unaffected. The mechanism of iron decreased adiponectin and promotion of metabolic syndrome is demonstrated via the binding affinity of multiple transcription factors to the adiponectin promoter by ChIP studies. The higher activation of FOXO1 in iron-treated cells contributes to more inactivation of PPARγ through directly interaction, leading to decreased adiponectin transcription and expression. These findings directly

demonstrate a causal role for iron as a risk factor for metabolic syndrome and a role for adipocytes in modulating metabolism through adiponectin in response to iron stores.

We also demonstrate that transcription of leptin also responds to iron. Consistent with the changes in leptin, dietary iron content was also directly related to food intake, independently of weight. Loss of the adipocyte iron export channel, ferroportin, in mice resulted in adipocyte iron loading and decreased leptin. Conversely, decreased levels of hepcidin in hereditary hemochromatosis result in increased adipocyte ferroportin expression, decreased adipocyte iron, and increased leptin. Treatment of 3T3-L1 adipocytes with iron decreased leptin mRNA levels in a dose-dependent manner. We found iron negatively regulated leptin transcription via cAMP response element binding protein (CREB) activation. Two potential CREB-binding sites were identified in the mouse leptin promoter region. Mutation of both sites completely blocked the effect of iron on promoter activity.

Iron also negatively regulates O-GlcNAc modification both in 3T3-L1 and epididymal fat, and mice heterozygous for deletion of the gene encoding the enzyme that removes O-GlcNAc, O-GlcNAcase, showed significantly increased serum leptin compared to wild-type mice. Glucosamine treatment rescued high iron-induced decrease of leptin promoter activity. These results suggest that the effects of iron on leptin may be via crosstalk of O-GlcNAcylation and phosphorylation of CREB. These findings indicate that levels of dietary iron play an important role in regulation of appetite and fat metabolism through CREB-dependent modulation of leptin expression.