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Functional analysis of Nrf1 and Nrf2 transcription factors in adipose tissue

DISSERTATION

Submitted in satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Biomedical Sciences

by

Kevin Schneider

Dissertation Committee
Professor Jefferson Y. Chan, Chair
Professor Bogi Andersen
Professor Robert Edwards

2016

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DEDICATION

To

My parents, my grandparents and my brother for their unwavering support, boundless love, and for believing in me all these years.

My friends, for putting up with my strange schedules and odd hours during these many years.

My girlfriend Janice, for without you none of this would have been possible. You are the most wonderful person in the world and have brightened my life from the moment I met you. I love you with all of my heart.

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LIST OF ABBREVIATIONS

Prefixes

m	milli
μ	micro
n	nano

Units

°C	degree Celcius
bp	base pair
g	grams
h	hours
m	meters
min	minutes
s	seconds
M	molar
L	liter

Genes

Nrf1	Nuclear factor (erythroid-derived 2)-like 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
Nrf3	Nuclear factor (erythroid-derived 2)-like 3
Keap1	Kelch-like ECH-associated protein 1
Ucp1	Uncoupling Protein 1
Ucp2	Uncoupling Protein 2
Ucp3	Uncoupling Protein 3
Metrn1	Meteorin-like 1
Nqo1	NAD(P)H quinone oxidoreductase 1
Ho-1	Heme Oxygenase 1
Gclm	Glutamate-cysteine ligase regulatory subunit
Gclc	Glutamate-cysteine ligase catalytic subunit
Gpx1	Glutathione Peroxidase 1
Prdm16	PR Domain Containing 16
HSL	Hormone Sensitive Lipase
Pparγ	Peroxisome proliferator-activated receptor gamma
Pgc1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pgc1β	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
Tfam	Transcription Factor A
Cox7a1	Cytochrome c oxidase polypeptide 7A1
Cox8b	Cytochrome c oxidase polypeptide 8B

Sirt3	NAD-dependent deacetylase sirtuin-3
CideA	Cell death-inducing DFFA-like effector A
Txnrd1	Thioredoxin reductase 1
Txnip	Thioredoxin interacting protein
Lxr	Liver x Receptor
Rip140	Receptor-interacting protein 140
Fgf21	Fibroblast growth factor 21
Vegf	Vascular endothelial growth factor
Rplpo	Ribosomal Protein, Large, P0

Compounds

ATP	Adenosine triphosphate
DCFHDA	Chloromethyl-2,7-dichlorodihydrofluorescein diacetate
MNTBAP	Mn(III)tetrakis(4-benzoic acid)porphyrin chloride
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
NAC	N-Acetyl Cysteine
GO	Glucose Oxidase
BHA	Butylated hydroxyanisole
FCCP	Carbonyl cyanide- p-trifluoromethoxyphenylhydrazine

Others

ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription polymerase chain reaction
WAT	White adipose tissue
BAT	Brown Adipose Tissue
ShRNA	Short hairpin RNA
CLAMS	Comprehensive Lab Animal Monitoring System
OCR	Oxygen Consumption Rate

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ABSTRACT OF DISSERTATION

Functional analysis of Nrf1 and Nrf2 transcription factors in adipose tissue

By

Kevin Scott Schneider

Doctor of Philosophy in Experimental Pathology

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Professor Jefferson Y. Chan, Chair

The Nrf1 and Nrf2 (also known as NFE2L1 and NFE2L2) transcription factors are critical regulators of genes involved in defense against oxidative stress. Previous studies have suggested that Nrf2 plays a role in adipogenesis in vitro, and deletion of the Nrf2 gene protects against diet-induced obesity in mice. Here, we demonstrate that loss of either Nrf1 or Nrf2 results in a lean phenotype. Resistance to diet-induced obesity in Nrf2^{-/-} mice is associated with a 20-30% increase in energy expenditure. This increase in whole animal energy expenditure is mirrored in the white adipose tissue of Nrf2^{-/-} animals, which exhibit greater oxygen consumption in isolated tissue explants. Nrf2^{-/-} WAT showed a greater than two-fold increase in Ucp1 gene expression along with increased expression of a variety of brown adipose tissue markers. Cell data mirrored that of animals with oxygen consumption increased nearly 2.5 fold in Nrf2 deficient fibroblasts. Oxidative stress induced by glucose oxidase resulted in increased Ucp1 expression and oxygen consumption. Conversely, antioxidant chemicals, such as N-acetylcysteine and MnTBAP, and SB203580, a known

suppressor of Ucp1 expression, decreased Ucp1 and oxygen consumption in Nrf2 deficient fibroblasts. Examination of animals deficient in Nrf1 specifically in adipose tissue showed similar findings with increased expression of UCP1 and brown adipose tissue genes in the white adipose tissue. Additionally, Nrf1 deficient mice expressed higher levels of Metrn1, a factor associated with increased Ucp1 levels. These findings suggest that modulating Nrf1 and Nrf2 levels in adipose tissue may be a novel means to regulate energy balance as a treatment of obesity and related clinical disorders.

CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

OXIDATIVE STRESS

Oxidative stress is referred to as a buildup of reactive oxygen species (ROS) within a biological system, and excessive oxidative stress is associated with a wide variety of pathologic conditions such as cancer ⁽¹⁾. Reactive oxygen species come in a variety of types and are generated in a variety of ways due to normal cellular functions. Superoxide is released as a byproduct of the mitochondrial electron transport chain when 1-3% of electrons escape from Complex I or III and react with oxygen ⁽²⁾. Excessive buildup of superoxide is associated with a wide number of pathologic conditions ⁽³⁾. Additional reactive radicals derived from oxygen such as the hydroxyl radical and the peroxy radical as well as hydrogen peroxide also result from normal cellular processes and can cause damage within the cell including DNA damage, as well as lipid peroxidation and protein oxidation ⁽⁴⁻⁶⁾.

In order to deal with the harmful effects of excessive buildup of these reactive oxygen species, cells have developed a wide variety of defense mechanisms. Cells employ antioxidant enzymes such as superoxide dismutase, thioredoxins, as well as glutathione to scavenge and capture free radicals and reduce peroxides ⁽⁷⁾. While excessive buildup of oxidative stress is deleterious to the cell, reactive oxygen species at low and moderate levels also serve as

important signaling molecules within the cell, and serve as second messengers in a wide number of pathways regulating everything from cell proliferation to programmed cell death ⁽⁸⁾. Due to the importance of reactive oxygen species within the cell both as a deleterious molecule and as a beneficial signaling molecule, it is of great importance to understand those transcription factors and enzymes that are important to the regulation of reactive oxygen species. One such family of transcription factors that is critical to the regulation of reactive oxygen species are the CNC-bZIP transcription factors Nrf1 and Nrf2.

NRF2 AND ARE-MEDIATED TARGET GENES

Nrf1 and Nrf2 are members of the Cap-N-Collar subfamily of bZIP transcription factors that also includes p45NFE2 and Nrf3 ⁽⁹⁻¹¹⁾. Under basal, non-stressed conditions, Nrf2 remains sequestered in the cytoplasm, bound to Kelch-like ECH-associated protein 1 (Keap1). This association results in the ubiquitination and subsequent proteasomal mediated degradation of Nrf2, which allows for the cell to maintain low intracellular concentrations until such time as they are needed ⁽¹¹⁻¹²⁾. However in response to oxidative stress due to buildup of reactive oxygen species, key cysteine residues of Keap1 are modified, leading to the release and subsequent nuclear translocation of Nrf2 ⁽¹³⁾. Upon entering the nucleus, Nrf2 is able to form heterodimers with other transcription factors including small Maf proteins (sMaf), JunD, and activation transcription factor 4 (ATF4) which then bind and activate gene transcription via cis-acting antioxidant response elements (ARE) in the promoters of key cellular defense genes ⁽¹⁴⁻¹⁷⁾.

There are a wide variety of cytoprotective genes that are regulated by CNC-bZIP transcription factors such as Nrf2. Antioxidant response elements are present in the regulatory regions of many genes, and Nrf2 has been shown to regulate enzymes involved in detoxification such as NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) ⁽¹⁸⁻¹⁹⁾. Nrf2 is also involved in driving the expression of key antioxidant enzymes such as the catalytic and regulatory subunits of γ -glutamate cysteine ligase (GCLC and GCLM) ⁽²⁰⁾. It is important to note that mice lacking the Nrf2 gene demonstrate no obvious outward defects, however they have an increased susceptibility to toxicant and oxidative stress-induced diseases such as heavy metal toxicity and are also more prone to developing autoimmune disorders as they age ⁽²¹⁻²⁵⁾. In another example, the livers of Nrf2 knockout mice are more prone to acetaminophen-induced hepatocellular necrosis ⁽²⁶⁾. This trend continues throughout a variety of tissues within the animal with Nrf2 being responsible for protecting the lungs from hypoxic or chemical injury by upregulating the pathways involved in cellular detoxification ⁽²⁷⁾. Finally, Nrf2 mutant mice have also been shown more sensitivity to carcinogens in the GI tract, suggesting an important role of Nrf2 and antioxidant response in the protection against cancer ⁽²⁸⁾.

NRF1

In contrast to Nrf2, the biological function of Nrf1, its targets, and its regulation are less well understood. Compared with Nrf2, Nrf1 germ line mutant mice are mid to late gestational embryonic lethal, suffering from anemia brought

on by abnormal fetal liver erythropoiesis ⁽²⁹⁾. This suggests that Nrf1 plays a critical role during normal embryonic development. Previous experiments have indicated that Nrf1-Maf heterodimers bind preferentially to ARE sequences identical to that of Nrf2 ⁽³⁰⁾. Displaying similar binding characteristics, it comes as no surprise that Nrf1 is also involved in the regulation of key antioxidant response genes that are also regulated by Nrf2. Previous experiments have indicated that Nrf1 plays a role in regulation of genes involved in glutathione synthesis including GCLC and GCLM ⁽³¹⁾. Additionally, fibroblasts from Nrf1 mutant and Nrf1/Nrf2 compound mutant embryos express lower levels of glutathione, a critical antioxidant compound, and thus are under an increased level of oxidative stress at baseline compared to wild type animals ^(29,32). In addition to oxidative stress related genes, Nrf1 has been shown to regulate proteasome gene expression. Utilizing brain specific knockouts of Nrf1, neuronal cells exhibit accumulation of ubiquitinated proteins, as well as decreased proteasomal activity and decreased expression of key proteasomal genes ⁽³³⁾. Additionally, liver knockouts of Nrf1 also exhibit significant proteasomal defects ⁽³⁴⁾. This data suggest both an overlapping role of Nrf1 in regulation of antioxidant defense, as well as a unique role in proteasomal regulation.

OXIDATIVE STRESS AND ADIPOSE TISSUE

Nrf1 and Nrf2 play critical roles in the regulation of antioxidant defense, and oxidative stress is of great interest to the study of metabolic syndrome. Exposure to a high fat diet places additional oxidative stress upon a wide variety of tissues within the body ⁽³⁵⁾. For this reason, the study of oxidative stress and

the effects on adipose tissue is of critical importance. For example, oxidative stress has been shown to directly modulate adipose cell differentiation, with low dose hydrogen peroxide resulting in inhibition of adipose cell differentiation, and high dose hydrogen peroxide markedly increasing the differentiation capacity of preadipocyte cells ⁽³⁶⁾. In addition to the use of external oxidants, there are a wide variety of interesting adipose tissue phenotypes which arise from disruption of key antioxidant genes, many of which are under the regulation of both Nrf1 and Nrf2. For example, mice lacking NQO1, which is responsible for the prevention and detoxification of dangerous quinones as well as reduction of chromium (VI) toxicity, present with dramatically reduced adipose tissue mass ⁽³⁷⁻³⁹⁾.

Similarly, studies have shown that there is a protective effect against obesity seen in mice lacking glutathione peroxidase 1 (Gpx1), an important enzyme responsible for protecting cells by catalyzing the reduction of hydroperoxides ⁽⁴⁰⁾. Gpx1 null mice demonstrated decreased adipose tissue mass, a protection against diet induced obesity, and increased oxygen consumption ⁽⁴¹⁾. In this model it is suggested that the increased levels of reactive oxygen species led to increased insulin-stimulated glucose uptake, and this phenotype is supported by data showing that treatment with antioxidants normalized ROS levels and subsequently caused insulin resistance in the mice⁽⁴¹⁾. Not surprisingly, mice lacking GCLM, the regulatory subunit of the glutamate-cysteine ligase enzyme involved in glutathione (GSH) synthesis, also display a resistance to high fat diet induced obesity as well as showing an

increase in energy expenditure, decreased hepatic lipid accumulation and maintenance of insulin sensitivity ⁽⁴²⁾. Finally, it has been shown that during aging, there is an associated decrease in the levels of GSH within the tissues of the body. There are well known detrimental effects due to this age-related decrease in antioxidant defense including cancer, cardiovascular, inflammatory, and neurodegenerative diseases ⁽⁴³⁾. It is important to note that this increase in oxidative stress also results in an inhibition of adipogenesis, resulting in decreased adipose tissue mass ⁽⁴⁴⁾. Taken together, all of these studies demonstrate that while the link between oxidative stress and detrimental disorders is well studied, it cannot be ignored that oxidative stress appears to play an important role in providing protection against obesity through a variety of targets and pathways involved in both development and metabolism.

CNC-BZIP PROTEINS AND OBESITY

A number of studies have also shown a link between Nrf2 and adiposity in mice. For example, administration of potent Nrf2 activators, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl-imidazolide and oltipraz, in mice resulted in protection from high-fat diet-induced obesity, suggesting that activation of Nrf2 promotes a leaner phenotype ⁽⁴⁵⁻⁴⁶⁾. In contrast to these results, mice lacking Nrf2 have been found to demonstrate a lean phenotype. Mirroring the data shown using cell lines, Pi et al. demonstrated that mice deficient in Nrf2 had a marked decrease in adipose tissue mass and adipocyte size and were protected from high-fat diet-induced obesity ⁽⁴⁷⁾. These findings are also supported by two other

studies demonstrating Nrf2 knockout mice were protected from high-fat diet-induced obesity and had a higher sensitivity to insulin and altered metabolic profile with lower circulating glucose, HDL, and leptin concentrations ⁽⁴⁸⁻⁴⁹⁾. While pharmacological activation of Nrf2 appears to promote a leaner phenotype, it is interesting to note that the CDDO-imidazolide study also observed that untreated mice lacking Nrf2 weighed less than their wild-type counterparts when fed a high-fat diet, suggesting an obesogenic role for Nrf2.

THE RELATIONSHIP BETWEEN UNCOUPLED RESPIRATION, ADIPOSE TISSUE AND OBESITY

With such interest focused on the role of oxidative stress in adipose tissue function, there has also been a great deal of interest relating to mitochondrial respiration, which serves as one of the primary sources of reactive oxygen species within the cell. Due to the fact that Nrf1 and Nrf2 regulate many of the critical antioxidant pathways involved in protecting the cell against excessive oxidative damage, understanding all of the facets of mitochondrial respiration is critical to understanding the role of Nrf1 and Nrf2. Energy is produced mainly in the mitochondria via the citric acid cycle coupled with oxidative phosphorylation, in which a proton gradient is established across the inner mitochondrial membrane, creating an electrochemical gradient used primarily for the generation of ATP. However not all of this energy is coupled to ATP synthesis. Some of the energy is dissipated by “proton leaking” in which protons in the inner membrane are able to flow back into the matrix of the mitochondria, bypassing

ATP synthase, by passing through specialized protein structures. This proton leak is termed mitochondrial uncoupling and is a physiologically relevant process accounting for 20-25% of the basal metabolic rate ⁽⁵⁰⁻⁵²⁾.

This mitochondrial uncoupling is mediated by a family of proteins known as the uncoupling proteins (UCPs) which demonstrate a wide variety of tissue specificity as well as functional relevance. The first protein to be extensively characterized and studied, known as UCP1 or thermogenin, is known for its characteristically high expression in brown adipose tissue, expressed at nearly one hundred fold higher levels than that of the other uncoupled protein homologues ⁽⁵³⁻⁵⁵⁾. UCP2 was found to be ubiquitously expressed at low levels throughout the body, while UCP3 was limited almost exclusively to skeletal and cardiac muscle ⁽⁵⁶⁻⁵⁷⁾. Finally, UCP4 and 5 were found to be predominantly expressed in the central nervous system ⁽⁵⁸⁻⁵⁹⁾.

Brown adipose tissue is known as a metabolically active tissue responsible for the generation of heat, known as adaptive thermogenesis, in which energy is dissipated as heat utilizing the UCP1 protein in response to environmental stimuli such as cold exposure and diet ⁽⁶⁰⁻⁶¹⁾. This thermogenic function is facilitated by the mitochondria rich structure of the brown adipose, and generated heat is easily distributed throughout the body due to the highly vascular nature of the tissue ⁽⁶²⁻⁶³⁾. In addition to this environmental stimulation of mitochondrial uncoupling, data has also shown that superoxide, a byproduct of

oxidative phosphorylation, is also capable of activating mitochondrial uncoupling proteins, suggesting a response to oxidative stress as well ⁽⁶⁴⁾. In the past, it was believed that this process of heat generation, while physiologically relevant for small mammals, played little role in the physiology of humans. However there has been extensive demonstrations of metabolically active brown adipose depots in adult humans through the use of PET/CT-scans (Positron Emission Tomography associated with Computed Tomography) ⁽⁶⁵⁻⁶⁷⁾. These depots were mostly observed in the suprarenal, supraclavicular, paravertebral regions, as well as in the neck ⁽⁶⁸⁻⁷³⁾. Consequently, a cascade of new studies has sought to explore the potential therapeutic properties and anti-obesity possibilities of this metabolically significant tissue depot and the process of mitochondrial uncoupling. Studies have shown that mice lacking UCP1 are more susceptible to diet induced obesity ⁽⁷⁴⁾. Conversely, transgenic mice with increased UCP1 expression in WAT were obesity-resistant after being fed a diet rich in saturated fat ⁽⁷⁵⁾. UCP1 has also been ectopically expressed in skeletal muscle of mice, and these animals showed improved glucose tolerance after being fed a high-fat diet, when compared with wild-type mice ⁽⁷⁶⁾. Beside the brown adipose tissue, several studies have shown that brown-like adipocytes (often referred to as “brite” or “beige” adipocytes) occur in some classical white adipose depots as well as skeletal muscle ⁽⁷⁷⁻⁷⁸⁾.

Many studies in recent years have shown that increases in these UCP1 positive brown adipocyte depots within normal white adipose tissue leads to a

resistance to diet induced obesity, suggesting a tantalizing therapeutic aspect for the conversion of white adipose tissue to metabolically active brown adipose tissue. For example, transgenic expression of PRDM16, a transcriptional co-regulator responsible for brown adipose tissue development, in adipose tissue using the aP2 promoter induced the formation of brown adipocytes in subcutaneous white adipose tissue. The transgenic mice exhibited increased energy expenditure, limited weight gain, and improved glucose tolerance in response to a high-fat diet ⁽⁷⁹⁾. Animals lacking hormone sensitive lipase (HSL) also presented with increased UCP1 levels and mitochondrial activity in white adipose tissue ⁽⁸⁰⁾. Amounts of brown adipose tissue in humans have also demonstrated an inverse correlation with body mass index ⁽⁸¹⁻⁸³⁾. Taken together, these studies suggest that brown adipose tissue, and specifically the uncoupling capacity of UCP1, could serve as a promising target for the treatment of obesity and metabolic syndrome.

In this study, we explored the role of Nrf1 and Nrf2 in adipose tissue and mitochondrial function. Specifically, we examine energy expenditure, changes in adipose tissue mass, morphology and gene expression in Nrf2^{-/-} mice fed both a normal and high fat diet. In addition, Nrf2^{-/-} cells were assessed for energy expenditure and gene expression. Our studies are consistent with the hypothesis that oxidative stress modulates mitochondrial function to control fat storage and energy expenditure in Nrf2 knockouts. Additionally we examine changes in adipose tissue morphology and gene expression Nrf1 FatKO mice.

CHAPTER 2

OBJECTIVES

Aim 1:

Determine the metabolic and physiological effects caused by the loss of Nrf2.

Hypothesis:

Loss of Nrf2 results in an increase in metabolic activity, contributing to the lean phenotype.

Rationale:

Previous data from our lab with mixed background Nrf2 knockout animals showed a resistance to obesity. For further confirmation, pure background C57/BL6 wild type and Nrf2 knockout mice would be used. Additionally, several papers have shown that mouse models of glutathione knockout are resistant to obesity with an increase in energy expenditure. Through the use of comprehensive laboratory animal monitoring systems we will test to see if there is any change in energy expenditure in pure background Nrf2 knockout mice.

Approach:

To test this we will expose wild type and Nrf2 knockout animals to a high fat diet. Animals will be subsequently analyzed for changes in histology, weight, activity, oxygen consumption, glucose tolerance, and triglyceride and insulin levels.

Aim 2:

Explore the impact of Nrf2 deficiency on genes involved with respiration and mitochondrial biogenesis.

Hypothesis:

Loss of Nrf2 results in increased expression of genes involved in mitochondriogenesis and respiration.

Rationale:

Nrf2 regulates the expression of key antioxidant enzymes including GCLM and GPX1. It has been shown that mice deficient in these demonstrate increased expression of genes involved in both coupled and uncoupled respiration in the adipose tissue.

Approach:

To test this we will analyze gene expression and protein levels in wild type and Nrf2 knockout adipose tissue. RNA sequencing will be used to obtain global information about differences in gene expression between wild type and Nrf2 mutant animals. RT-QPCR will be utilized to confirm RNA sequencing data.

Aim 3:

Determine the impact of oxidative stress on mitochondrial respiration.

Hypothesis:

Increased oxidative stress from the loss of Nrf2 results in increased uncoupled respiration.

Rationale:

One of the hallmarks of the loss of Nrf2 is a decrease in antioxidant capacity and an increase in oxidative stress. Thus we hypothesize that this increase in oxidative stress brought on by the loss of Nrf2 could help to drive the observed animal and cell phenotype.

Approach:

To test this we will analyze the effects of antioxidants and inducers of oxidative stress on wild type and Nrf2 knockout cells and identify if there are changes in levels of oxygen consumption and uncoupled respiration. Additionally we will test for differences in oxygen consumption in wild type and Nrf2 knockout cells and compare with the data obtained in animals and isolated adipose tissue.

Aim 4:

Determine the impact of Nrf1 deficiency on adipose tissue in vivo.

Hypothesis:

Loss of Nrf1 disrupts normal adipose tissue function and/or structure and result in a lean phenotype.

Rationale:

Nrf1 and Nrf2 exhibit significant similarity in gene targets. Both Nrf1 and Nrf2 are highly involved in the response to oxidative stress and expression of key antioxidant genes. Nrf2 has also been shown to regulate key genes involved in normal adipose tissue development such as Pparg. Nrf2 knockout animals have been shown to be resistant to high fat diet induced obesity and demonstrate increased energy expenditure. Due to these facts we hypothesize that a similar situation could be observed in Nrf1 knockout animals and tissue.

Approach:

In order to test this we will develop an adipose tissue-specific knockout of Nrf1 utilizing the Adiponectin Cre-Lox system, in order to circumvent the lethality of whole animal Nrf1 knockout models. We will then characterize the phenotype of Nrf1^{Lox/Lox} adiponectin Cre animals. We will track animal weights, examine tissue histology and finally we will determine whether there is alteration of genes involved in normal adipose tissue differentiation or function in both white and brown adipose tissue of Nrf1 knockout animals.

Aim 5:

Determine the impact of Nrf1 deficiency in cell function in vitro.

Hypothesis:

Loss of Nrf1 disrupts normal adipose cell differentiation and alters gene expression in vitro.

Rationale:

Similar to Nrf2, Nrf1 regulates a wide variety of antioxidant genes. Loss of these antioxidant genes has been found to induce expression of thermogenic and mitochondrial genes. For this reason we hypothesize that loss of Nrf1 will alter expression key genes involved in bioenergetics.

Approach:

In order to test this hypothesis, we will employ two different cell based models. First we will utilize wild type and Nrf1 knockout fibroblasts and identify if there are any changes in key genes involved in mitochondrial or thermogenesis. Additionally we will develop a stable ShRNA knockdown of Nrf1 in 3T3L1 white adipose precursor cell lines. These cell lines can be used to more closely mirror the results of the Nrf1 knockdown in adipose tissue. These cells can be differentiated into adipose cells and be studied for any variations in lipid accumulation or gene expression.

CHAPTER 3

EXPERIMENTAL DESIGNS

Aim 1 Hypothesis: Loss of Nrf2 will result in an increase in metabolic activity, contributing to the lean phenotype.

Determine the effect of high fat diet on weight gain in wild type and Nrf2 -/- mice

- Weights of wild type and Nrf2 -/- mice were measured prior to treatment and then subsequently monitored over a 6 week period in which animals were fed a high fat diet (60kcal from fat).

- EchoMRI was employed to measure lean and fat mass of animals

- At completion of high fat diet experiment, animals were harvested and the weights of white adipose tissue, brown adipose tissue, liver, heart, and kidney were measured. Harvested tissues were analyzed histologically to identify changes in lipid deposition and lipid size.

Determine if differences exist in energy expenditure and metabolic parameters between wild type and Nrf2 -/- animals.

- Animals were housed in Comprehensive Lab Animal Monitoring System (CLAMS) in order to track a variety of conditions. Food intake, and physical activity was monitored, as well as O₂ consumption and CO₂ generation. From the data, calculations

could be made to determine energy expenditure, as well as respiratory quotient in order to determine the primary energy source used.

- Additional metabolic parameters were measured including serum glucose levels, glucose tolerance, serum insulin levels, serum triglyceride levels.
- Measure oxygen consumption in isolated white adipose tissue explants from wild type and Nrf2 ^{-/-} animals to determine if changes in oxygen consumption are due in part to changes in adipose tissue energy utilization.
- Measure levels of oxygen consumption in wild type and Nrf2 ^{-/-} fibroblasts and determine if Nrf2 re-expression is linked to levels of oxygen consumption.

Aim 2 Hypothesis: Loss of Nrf2 will result in increased expression of genes involved in mitochondriogenesis and respiration.

Determine the effects of loss of Nrf2 on gene and protein expression in white and brown adipose tissue.

- RNA-Seq was performed to identify likely pathways of interest that differ

between wild type and Nrf2 ^{-/-} adipose tissue.

- Quantitative RT-PCR was performed in order to confirm changes in gene expression observed in RNA-Seq data.
- Western blot analysis performed to confirm changes in protein levels in wild type and Nrf2 ^{-/-} adipose tissue.

Aim 3 Hypothesis: Increased oxidative stress from the loss of Nrf2 results in increased uncoupled respiration.

Determine if loss of Nrf2 results in increased oxidative stress

- Measure DCF staining via flow cytometry in order to measure levels of oxidative stress in wild type and Nrf2 ^{-/-} cells.
- Measure glutathione levels to determine oxidative stress in wild type and Nrf2 ^{-/-} white adipose tissue.

Determine if oxidative stress can regulate oxygen consumption levels

- Measure oxygen consumption in wild type and Nrf2 knockout cells
- Treat wild type cells with glucose oxidase to induce stress and measure oxygen consumption
- Treat Nrf2 ^{-/-} cells with antioxidant and measure oxygen consumption levels.

Determine if oxidative stress can regulate UCP1 levels and expression

- Measure UCP1 levels in basal wild type and Nrf2 knockout cells
- Treat wild type cells with glucose oxidase and measure UCP1 protein and

RNA expression.

- Treat Nrf2 ^{-/-} cells with antioxidants and measure UCP1 expression.

Aim 4 Hypothesis: Loss of Nrf1 will disrupt normal adipose tissue function and/or structure and result in a lean phenotype.

Develop an adipose tissue-specific knockout of Nrf1 utilizing the Adiponectin Cre-Lox system and characterize.

- Track body weights of Nrf1 FatKO mice compared to Nrf1 ^{+/+} Cre
- Measure metabolic parameters including glucose levels and glucose tolerance
- Collect tissue and analyze histologically for differences in lipid deposition in white adipose tissue and brown adipose tissue.

Determine the effects of loss of Nrf1 on gene expression in white and brown adipose tissue.

- Examine gene expression in white and brown adipose tissue of Nrf1 FatKO animals, paying special attention to genes involved in mitochondriogenesis and thermogenesis, similar to the Nrf2 model.

Aim 5 Hypothesis: Loss of Nrf1 will disrupt normal adipose cell differentiation and alter gene expression in vitro.

Utilize Nrf1 knockout fibroblasts and Nrf1 knockdown preadipocyte cells to study effects of Nrf1 deficiency.

- Utilize QPCR and identify if loss of Nrf1 results in similar changes in gene expression as seen in Nrf1 deficient adipose tissue.
- Differentiate wild type and Nrf1 deficient 3T3L1 preadipocyte cells and observe levels of lipid accumulation.

CHAPTER 4

EXPERIMENTAL PROCEDURES

Reagents

Dulbecco's modified Eagle's medium (DMEM), alpha minimum essential medium (α -MEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), MitoTracker GreenFM, nanoyl-acridine orange (NaO) and Superscript III reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). Glucose oxidase, butylated hydroxyanisole (BHA), and anti-beta-actin antibody (A1978) was purchased from Sigma (St. Louis, MO, USA). UCP1 (ab23841) antibody was purchased from Abcam (Cambridge, MA). α -Tubulin antibody was purchased from (ab3873) from Cell Signaling (Beverly, MA). Trizol RNA reagent, 2X FastStart SYBR Green Master mix was purchased from Roche (Indianapolis, IN, USA). RNeasy MinElute Cleanup Kit was from Qiagen (Valencia, CA, USA). The enhanced chemiluminescence substrate kit was from Pierce Biotechnology (Rockford, IL, USA). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was purchased from EMD Millipore (Billerica, MA).

Cells

Mouse embryonic fibroblasts (MEFs) were generated as previously described (41), and cultured in DMEM supplemented with 10% FBS, 8.3 mM L-glutamine, 100U/ml penicillin and 100 μ g/ml streptomycin. Oxidative stress was induced in cells by incubating with 25mU/mL glucose oxidase for 6 hours at 37°C, and

antioxidant treatment was accomplished by incubation with 40uM BHA, or with 10uM MnTBAP.

Mice

All experiments were carried out following guidelines of the UCI Institutional Animal Care and Use Committee. Nrf2 knockout mice have been described previously (42), and were backcrossed ten generations to C57BL/6J background to produce near-isogenic lines. Studies were done using age matched 12-16 week old Nrf2^{-/-} and wild type male mice. Mice were housed on 12-h light and dark cycles under controlled environmental settings ($23 \pm 1^\circ\text{C}$), with free access to food and water. Age-matched wild type and Nrf2^{-/-} mice were fed either a standard global rodent diet (Harland Teklad 2020x), or a high fat diet chow containing 60% kcal from fat (Harlan Teklad TD.06414). Fatty acid profile of high fat diet as a percent of total fat consists of 37% saturated, 47% monounsaturated, 16% polyunsaturated fat.

Metabolic and indirect calorimetry studies

Indirect calorimetry to measure oxygen (O₂) consumption, carbon dioxide (CO₂) production, activity, food intake were done using Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH). Mice were individually housed in metabolic chambers maintained at 20–22°C in a 12-h light/12-h dark cycle. After the mice had adapted to the environment of the metabolic chamber for 72 hr, metabolic parameters were assessed. Activity was

monitored by the number of times a mouse moves through infrared beams crossing the cages. Body fat and lean mass were measured using an EchoMRI-100 (Echo Medical Systems, Houston, TX). Glucose tolerance tests were conducted in animals that were fasted overnight. Glucose was administered by intraperitoneal injection at a dose of 1 mg/g body weight. Blood samples were collected from the tail vein at time 0 (prior to the glucose injection), 30, 60 and 120 minutes after glucose loading for blood glucose. Blood glucose concentrations were measured using a TRUE-Test blood glucose meter (Nipro Diagnostics, Fort Lauderdale, FL). Fasting serum triglycerides levels were analyzed with the serum Triglyceride kit according to manufacturer's instruction (Sigma, St Louis, MO), and serum concentrations of insulin were determined using the Ultra Sensitive Mouse Insulin ELISA Kit from Crystal Chem (Downers Grove, IL).

RNA isolation and quantitative real-time PCR

RNA was extracted using Trizol RNA reagent and further purified using RNeasy MinElute Cleanup Kit. cDNA synthesis was generated with a Superscript III first-strand synthesis kit according to the manufacturer's recommendation.

Amplification of cDNA occurred in a Step One Plus PCR machine (ABI) using the FastStart SYBR Green reagent in duplicate 10- μ L reactions. TBP was used as an endogenous control and relative expression was calculated using the equation $2^{(Ct \text{ target gene} - Ct \text{ RPLPO})}$. Fold change in expression was

determined from the difference between the averaged expression levels relative to control.

Western blotting

Protein lysates were prepared using Nonidet P-40 lysis buffer, and protein concentrations were determined using Bio-Rad protein assay reagent with BSA as the protein standard. Samples were electrophoresed on SDS/polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were then blocked in 5% milk at room temperature for 1 h, and incubated with primary antibody overnight at 4 °C. Subsequently, blots were washed and peroxidase-conjugated secondary antibody added and incubated for 1 h. Blots were visualized using a chemiluminescent detection system.

Histology and immunohistochemistry - Tissues were fixed in 10% neutral buffered formalin and imbedded in paraffin. Tissue sections were stained with H&E and visualized using a Nikon microscope equipped with a CCD camera

RNA-seq analysis

From WAT of mice fed a high fat diet, total RNA was isolated using Trizol RNA reagent and further purified using RNeasy MinElute Cleanup Kit. Three independent replicates were used for wild type and Nrf2^{-/-} mice. Integrity and purity of the samples were assessed using Agilent Bioanalyzer, and libraries for RNA-Seq were generated using Illumina Truseq kit v2 per the manufacturer's instructions. RNA-seq libraries were sequenced on an Illumina HiSeq 2500 next

generation sequencer using single read 50bp (SR50) chemistry with a read depth of >10 million reads per sample. Reads are processed and mapped to reference genome (mm10, built name NCBIM37) using ELAND. Transcript abundance quantification are assigned using normalized read counts such as FPKM, and statistical testing for differential expression are performed using Cufflinks. Differentially expressed genes are further analyzed using Ingenuity Pathways Analysis.

Flow Cytometry

For intracellular ROS measurements, cells were incubated with 10 μ M CM-H2DCFDA in PBS for 15 min at 37°C. Fluorescence was analyzed by flow cytometry. Oxidative stress was induced in cells by incubating with 25mU/mL glucose oxidase 6 hours at 37°C prior to CM-H2DCFDA staining. Antioxidant treatment was accomplished by incubation with 100 μ M BHA for 6 hours at 37°C.

Measurement of oxygen consumption in adipocytes and MEF cells using the XF24 Extracellular Flux Analyzer

The Seahorse XF24 Extracellular Flux Analyzer was utilized for measuring the oxygen consumption of adherent MEF cells, as well as extracted adipose tissue explants. The protocol for acquisition of intra-abdominal WAT, preparation of tissue, and usage of XF24 was performed as previously described (43), with minor changes. The sensor cartridge was calibrated with calibration buffer (Seahorse Bioscience) at 37°C the day before OCR measurements. Intra-

abdominal WAT was dissected into small pieces (≈ 5 mg), and washed extensively with XF-Basal Medium containing 25 mM HEPES and 25mM glucose. Two pieces of isolated WAT weighing a total of 10mg were placed into each well of a XF24 Islet Capture Microplate (Seahorse Bioscience, North Billerica, MA), and each well was covered with a capture screen to hold the tissues in place while allowing for free perfusion. XF Basal Medium supplemented with 25mM Glucose, 1mM Sodium Pyruvate, and 2mM L-Glutamine (450 μ l) was then added, and oxygen consumption rate (OCR) was measured in a XF24 extracellular flux analyzer (Seahorse Bioscience) according to manufacturer's protocol. OCR readings were taken over time under basal conditions, and after addition of mitochondrial inhibitors, oligomycin (8 μ g/mL), carbonyl cyanide- p-trifluoromethoxyphenylhydrazone (8 μ M FCCP), and a mixture of rotenone (3 μ M) and antimycin-A (12 μ M), in succession. A minimum of 5 wells was utilized per condition in any given experiment. Plates were saved and tissue protein concentrations were measured by Bradford assay. OCR was determined by Seahorse XF-24 software as a function of time and normalized by protein concentration (picomoles/minute/microgram), respectively. Bioenergetic parameters assessed were basal, ATP linked respiration, proton leak, maximal, and reserve capacity. Basal respiration rate was calculated by subtracting the residual OCR determined after addition of rotenone and antimycin-A. ATP-linked respiration was determined from the difference between basal OCR and OCR following oligomycin addition. The difference in OCR between rotenone and oligomycin represented the amount of oxygen consumed that is due to proton

leak. Maximal OCR was determined by subtracting the OCR after rotenone addition from the OCR induced by FCCP. Lastly, the reserve capacity was calculated by the difference between maximal and basal respiration. All data is expressed as a percentage of the basal OCR to allow comparison between the different genotypes. For MEF cells, 50,000 cells were plated in XF 24 well plates one day prior to analysis. This allowed for 70-90% confluency on the day of measurement. Sensor cartridge was hydrated 24 hours prior to run as indicated by manufacturer's protocol. On the day of the run, cells were washed two times with Seahorse Basal Medium containing 25mM Glucose, 1mM Sodium Pyruvate, and 4mM L-Glutamine, and then covered with 450ul of the same basal medium. Oxygen consumption rate (OCR) was measured in a XF24 extracellular flux analyzer (Seahorse Bioscience) according to manufacturer's protocol. OCR readings were taken over time under basal conditions, and after addition of mitochondrial inhibitors, oligomycin (1uM), carbonyl cyanide- p-trifluoromethoxyphenylhydrazone (1uM FCCP), and a mixture of rotenone (1uM) and antimycin-A (1uM), in succession. A minimum of 5 wells was utilized per condition in any given experiment. Plates were saved and cellular protein concentrations were measured by Bradford assay. OCR was determined by Seahorse XF-24 software as a function of time and normalized by protein concentration (picomoles/minute/microgram), respectively.

Measurement of Oxygen Consumption Utilizing the BD Oxygen Biosensor Plate
Fluorometric measurements of oxygen consumption in adherent mouse cells were done using BD Oxygen Biosensor plates (BD Biosciences, San Jose, CA).

Triplicate wells were seeded with 2×10^5 cells suspended in 25mM Glucose DMEM without phenol red. Plates were scanned every 5 min for 120 minutes in a temperature-controlled (37 °C) plate reader (Thermo, Waltham, MA) with an excitation wavelength of 485 nm and an emission wavelength of 630 nm. Fluorescence values for each well were normalized to their initial value and then to the values of no-cell controls at each time point.

GSH and GSSG assay

Measurements of GSH and GSSG levels were determined using the Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI) following the manufacturers protocol. Tissues were homogenized in 5-10 ml of cold 50mM MES buffer per gram of tissue. Homogenates were spun at 10,000g for 15 minutes and supernatant fractions were collected. Supernatant fractions were assayed for total reduced (GSH) and oxidized (GSSG) glutathione by the standard enzymatic recycling method after deproteination of samples using 5% metaphosphoric acid and addition of triethanolamine. Samples for GSSG determination were first incubated at room temperature with 2 μ l of 4-vinylpyridine (4-VP) per 100 μ l sample for 1 h after vigorous vortexing in order to derivitize endogenous GSH and allow for measurement of GSSG specifically. The GSSG (as GSHx2) was then subtracted from the total GSH to determine actual GSH level and GSH-to-GSSG ratio (GSH/GSSG), which is used as an indicator for oxidative stress (45).

Statistical analysis

Data are expressed as means \pm SEM, and statistical analysis was done using Graphpad Prism software (La Jolla, CA). For simple comparisons of two groups, a two-tailed Student t test for unpaired samples was used. For comparisons of wild type and knockout groups concerning the influence of high fat diet, a two-way ANOVA analysis was used followed by a post-hoc Bonferroni analysis.

Probability (p) values of 0.05 or less were considered statistically significant, and are indicated.

CHAPTER 5

RESULTS

Loss of Nrf2 will result in an increase in metabolic activity, contributing to the lean phenotype when exposed to high fat diet.

Nrf2^{-/-} mice are resistant to high fat diet induced obesity.

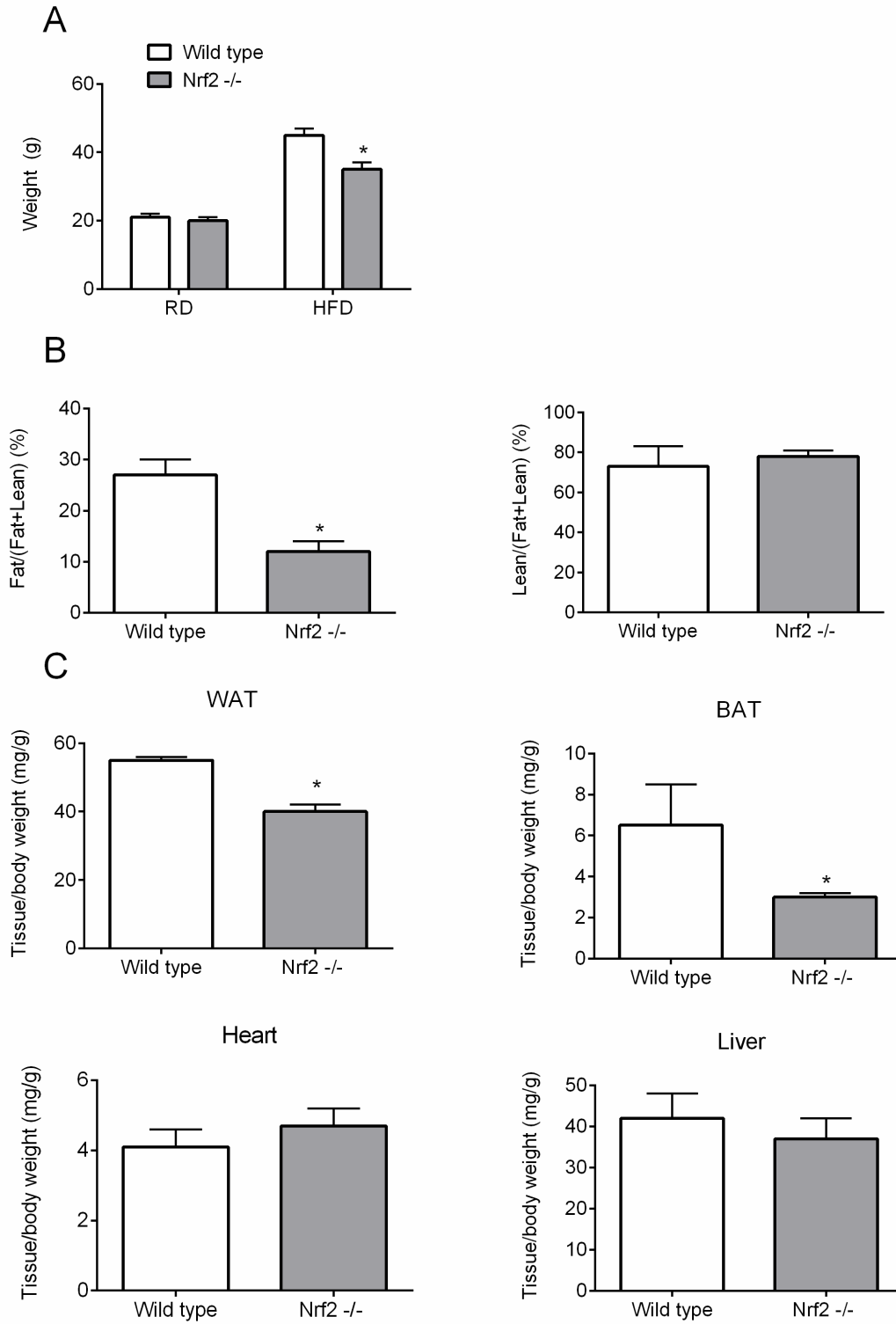
All experiments were performed in the C57BL/6 pure background mice to eliminate possible variation from mixed genetic background. The body weights of wild type and Nrf2^{-/-} male mice were indistinguishable at 12 weeks of age when fed a standard chow diet (Fig. 5-1A). This observation in mice derived from a C57BL/6 background is consistent with our previous results in mice derived from a mixed C57BL/6 x Sv129 genetic background⁽⁴⁷⁾. After exposure to a high fat diet for 6 weeks, the mean body weight of Nrf2^{-/-} mice was 25% less than that of wild type mice (Fig. 5-1A). Wild type mice nearly doubled their starting weight, while Nrf2^{-/-} mice only gained 50% of their starting weight (Fig. 5-1A). A comparison of adipose tissue mass normalized to lean body mass measured by EchoMRI showed that Nrf2^{-/-} mice exposed to high fat diet had 50% less whole-body fat compared with wild type mice. Importantly, there was no significant change in the lean body mass of Nrf2^{-/-} mice compared to wild type mice (Fig. 5-1B). Analysis of white and brown adipose tissue weights normalized to body weight were 30% lower in Nrf2^{-/-} mice compared to wild type mice. Relative weights of liver, heart and kidney were not different between wild type and Nrf2^{-/-}

mice (Fig. 5-1C and data not shown). This data shows that Nrf2 $-/-$ mice are resistant to diet induced obesity and that this decreased body weight is associated purely with a decrease in adipose tissue mass.

Figure 5-1. Nrf2 ^{-/-} mice are resistant to high fat diet induced obesity

(A) Comparison of body weights of wild type and Nrf2^{-/-} mice on a regular diet (RD) and after 6 weeks of high fat diet (HFD). (B) Echo MRI analysis of body compositions of wild type and Nrf2^{-/-} mice. (C) Comparison of white adipose tissue (WAT), brown adipose tissue (BAT), liver and heart weights, normalized to body weights, of wild type and Nrf2^{-/-} mice after 6 weeks of high fat diet.

Figure 5-1



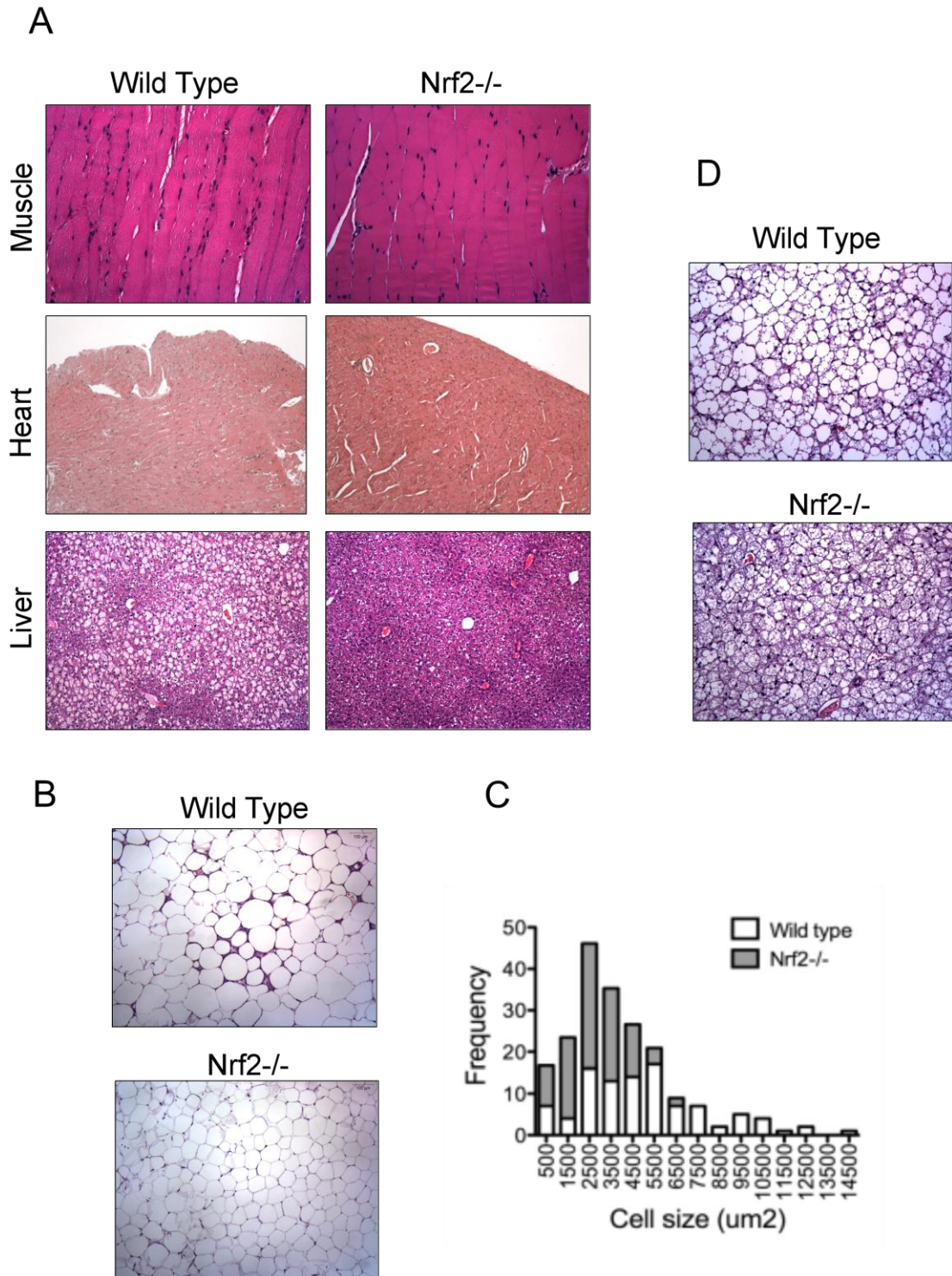
Nrf2^{-/-} mice are resistant to high fat diet induced steatohepatitis and exhibit decreased lipid accumulation in adipose tissue

To rule out possible lipid deposition in other tissues, sections of various tissues were analyzed. Ectopic accumulation of lipids was not observed in heart, or skeletal tissues of wild type and *Nrf2*^{-/-} mice placed on a high fat diet (Fig. 5-2A). However, wild type mice demonstrated significant liver steatosis compared to *Nrf2*^{-/-} mice (Fig. 5-2A). These results indicate that lower fat mass in *Nrf2*^{-/-} mice is not associated with increased lipid accumulation in non-adipose tissues. Along with the reduction in fat mass, intra-abdominal WAT depots of *Nrf2*^{-/-} mice showed smaller unilocular adipocytes compared to wild type mice (Fig. 5-2B). The frequency of smaller fat cells, quantitated from H&E images, was significantly higher in *Nrf2*^{-/-} WAT (Fig. 5-2C). In addition, BAT from *Nrf2*^{-/-} mice showed smaller multilocular lipid droplets (Fig.5-2D). However, strain differences in subcutaneous adipocytes were not observed (data not shown). This data suggests that *Nrf2*^{-/-} mice are protected against hepatic lipid accumulation associated with high fat diet induced obesity.

Figure 5-2 Nrf2 ^{-/-} mice are protected against high fat diet induced hepatic steatosis and exhibit decreased lipid accumulation in adipose tissue.

Representative histological sections of (A) skeletal muscle, heart, liver, (B) peritoneal fat from wild type and Nrf2^{-/-} mice. (C) Histogram depicts the size distribution of the measured white adipocytes in wild type and Nrf2^{-/-} mice. (D) Representative interscapular brown fat from wild type and Nrf2^{-/-} mice. All tissues are taken from mice after 6 weeks of high fat diet feeding.

Figure 5-2

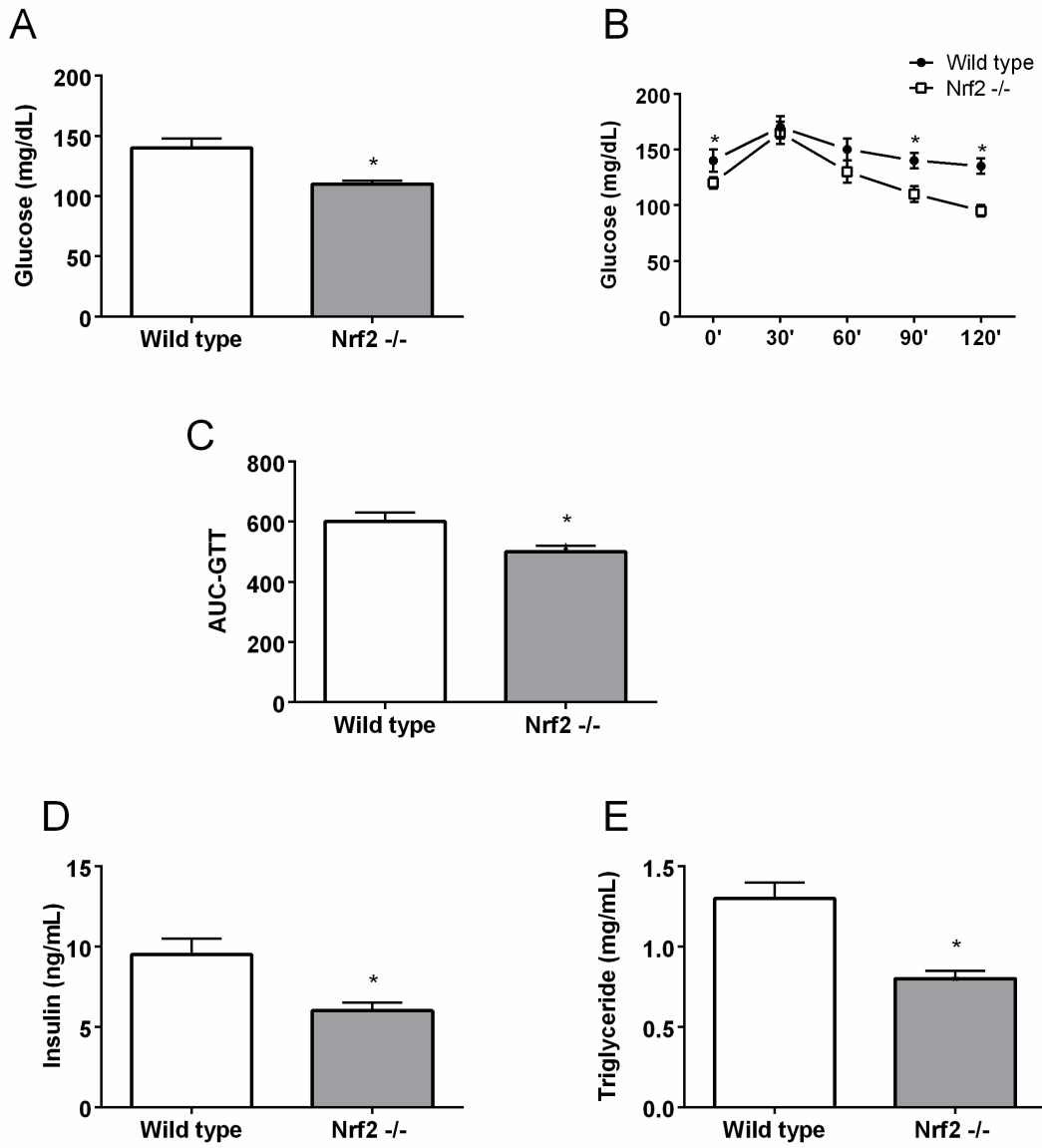


Nrf2^{-/-} mice on a high fat diet showed improved metabolic profiles.

In order to further explore the lean phenotype observed in the Nrf2^{-/-} mice, we sought to measure a variety of factors associated with metabolic syndrome. To test this we examined levels of serum glucose, glucose tolerance, as well as serum insulin and triglyceride levels. Nrf2^{-/-} mice on a high fat diet exhibited decreased fasting glucose levels compared to wild type controls (Fig. 5-3A). Glucose tolerance tests showed significantly lower blood glucose levels in Nrf2^{-/-} mice in 90 and 120 minute time points (Fig. 5-3B). The area under the curve (AUC) 0–120 min of blood glucose was also significantly lower in Nrf2^{-/-} mice than that in wild type controls (Figure 5-3C). The improvement in glucose tolerance in Nrf2^{-/-} mice is not due to increased insulin secretion as plasma insulin levels were lower in Nrf2^{-/-} mice compared to wild type controls (Fig. 5-3D). In addition, serum triglyceride levels were also lower in Nrf2^{-/-} mice (Fig. 5-3E). Taken together, these findings suggest that Nrf2 deficiency provides protection against the deleterious effects of a high fat diet on plasma levels of glucose and triglycerides.

Figure 5-3. Nrf2^{-/-} mice on a high fat diet showed improved metabolic profiles. (A) Fasting glucose levels in wild type and Nrf2^{-/-} mice. (B) Glucose tolerance test. Mice starved for 12 hours were given an intraperitoneal injection of glucose (1 mg/g body weight). Blood samples were taken at the times indicated from the tail vein of the same animals. Glucose was measured as indicated in Methods. (C) Area under the curve (AUC) of blood glucose levels during intraperitoneal glucose tolerance test. (D) Plasma insulin, and (E) triglyceride levels of wild type and Nrf2^{-/-} mice. Experiments were performed after 6 weeks of high fat diet feeding in all groups. Data are expressed as means \pm SEM. All graphs depict mean values \pm SEM, n=8 mice per group and significance was determined by Student's t-test (*p<0.05).

Figure 5-3



Nrf2^{-/-} mice on high fat diet demonstrate increased energy expenditure.

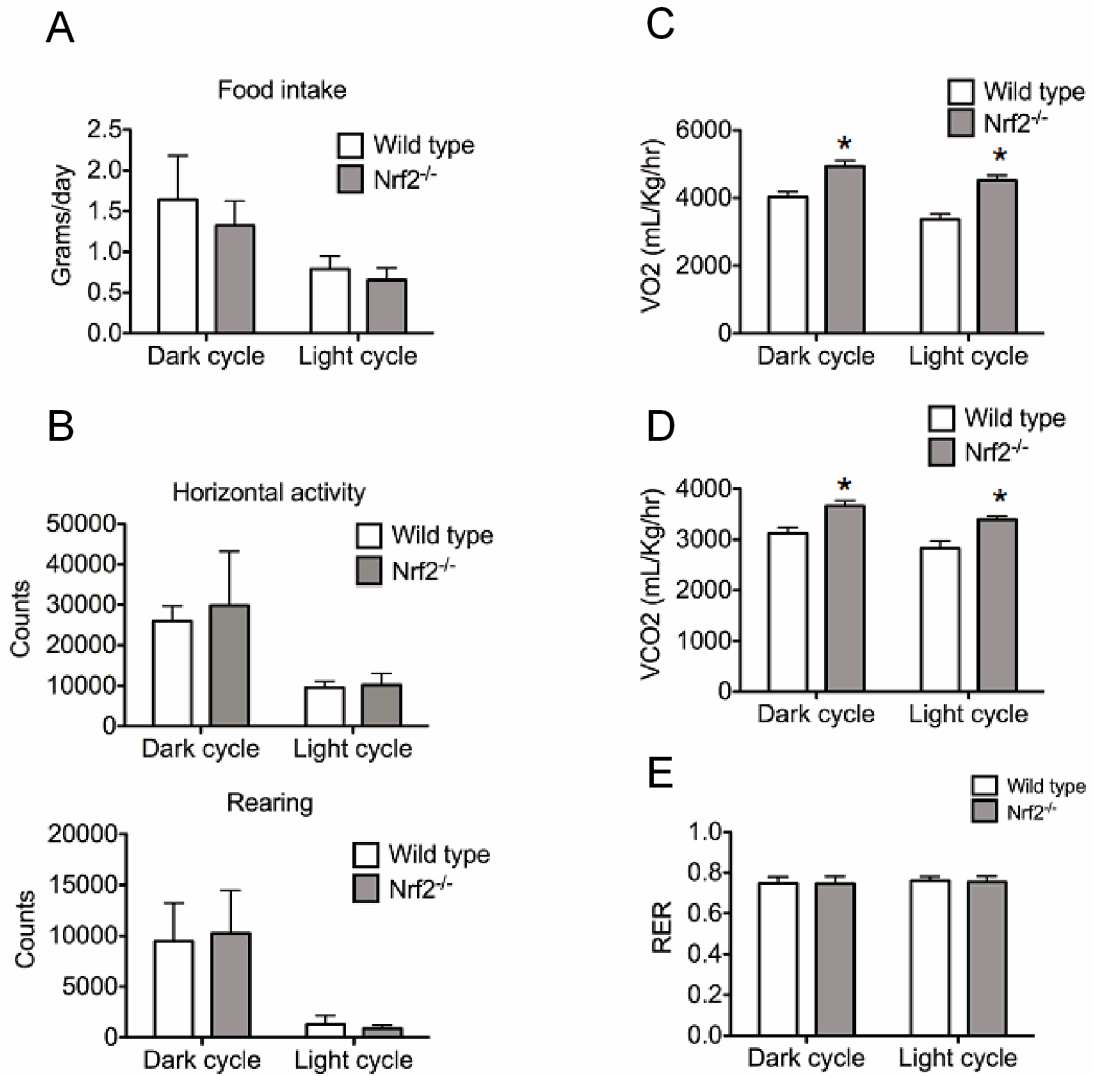
Potential mechanisms including reduced food intake, or increased energy expenditure could contribute to reduced adiposity in *Nrf2^{-/-}* mice. To investigate this, we monitored the food consumption and metabolic activities using Comprehensive Laboratory Animal Monitoring Systems (CLAMS). This system allows for the 24h measurement of a wide variety of parameters, while minimizing investigator interaction with the mice. Food intake per body weight in *Nrf2^{-/-}* mice was comparable to that of wild-type mice fed the same diet (Fig. 5-4A). This suggests that differences in body weight are not associated with changes in food consumption. Activity of mice was also measured for 72-h under a 12-h light-dark cycle. No significant difference was observed in either horizontal activity or rearing behavior between *Nrf2^{-/-}* and wild type mice (Fig. 5-4B). This demonstrates that any changes in metabolic activity or oxygen consumption are not due to a change in physical activity. Although energy expenditure levels in wild type and *Nrf2^{-/-}* mice fed a regular diet were similar (data not shown), O₂ consumption and CO₂ generation were significantly higher in *Nrf2^{-/-}* mice compared to wild type controls under high fat feeding (Fig. 5-4C and D). Taken with previous data, this demonstrates that *Nrf2^{-/-}* mice exhibit higher oxygen consumption that is not associated with an increase in physical activity. Respiratory quotient (RER) levels were identical in *Nrf2^{-/-}* and wild type mice indicating that the utilization of carbohydrate and fat was similar for both groups (Fig. 5-4E). This suggests that despite the increase in metabolic activity, *Nrf2^{-/-}* mice have not shifted their energy source dramatically. When compared with

normal diet RER, both wild type and Nrf2 ^{-/-} animals demonstrated a shift towards utilization of fat as the primary energy source, which is expected due to the high fat percentage present in the diet. Our findings suggest that resistance to obesity of Nrf2^{-/-} mice is due to increased energy expenditure by increasing metabolic rate.

Figure 5-4. Nrf2^{-/-} mice on high fat diet demonstrate increased energy expenditure.

(A) Daily food intake of wild type and Nrf2^{-/-} mice over a 6-day period. (B) Physical activity of wild type and Nrf2^{-/-} mice over a 3-day period measured by horizontal and rearing light beam breaks using the comprehensive laboratory animal monitoring system (CLAMS, Columbia Instrument). Counts of horizontal activity and rearing are shown for both dark and light cycles. Wild type and Nrf2^{-/-} mice were analyzed for (C) oxygen consumption (VO₂), (D) energy expenditure, and (E) respiratory exchange ratio determined by CLAMS over a three-day period. Values from dark and light cycles are shown. Experiments were performed after 6 weeks of high fat diet feeding in all groups. Data are expressed as means ± SEM, n=6 mice per group and significance was determined by Student's t-test (*p<0.05).

Figure 5-4



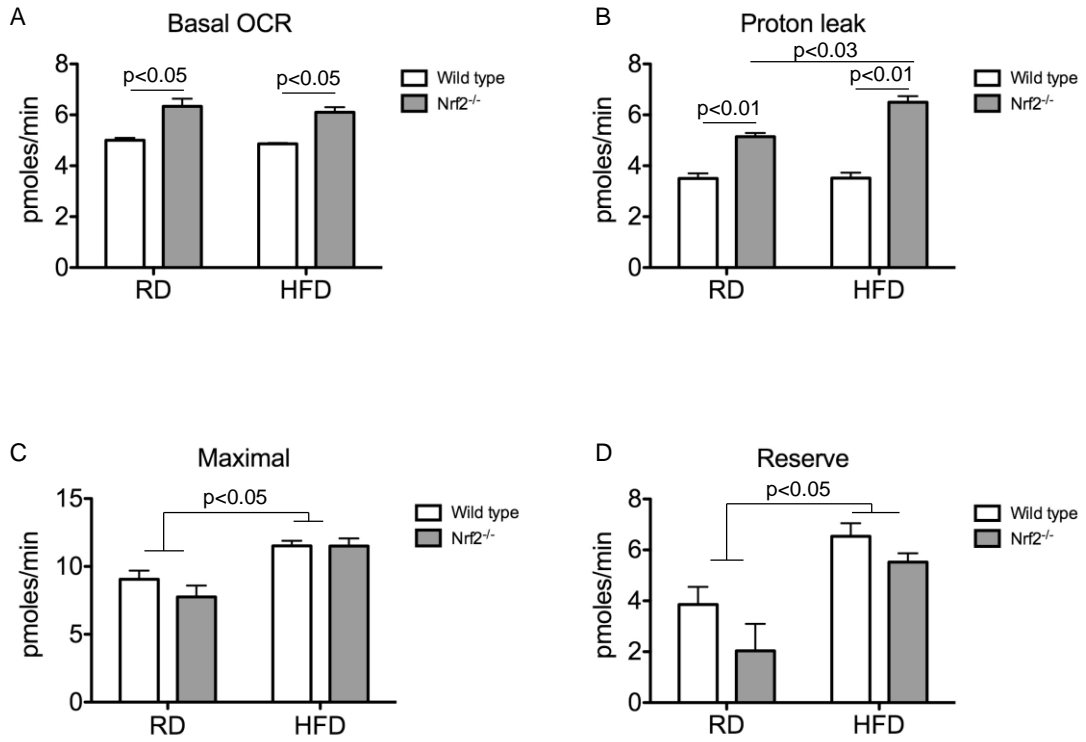
Nrf2 deficient WAT show increased oxygen consumption.

To further substantiate these findings, we investigated whether loss of Nrf2 affects mitochondrial function by comparing oxygen consumption rates (OCR) of adipose tissues from wild type and Nrf2^{-/-} mice. WAT isolated from Nrf2^{-/-} mice fed either a normal or high fat diet showed an increase in basal OCR in comparison to wild type (Fig. 5-5A). Proton leak-dependent respiration measured in the presence of the ATP synthase inhibitor, oligomycin, was significantly higher in WAT of Nrf2^{-/-} mice fed a normal diet, and was further increased in Nrf2^{-/-} mice fed a high fat diet suggesting that mitochondrial coupling was diminished (Fig. 5-5B). Proton leak dependent respiration is associated primarily with the action of uncoupled proteins such as UCP1, UCP2, and UCP3. High fat diet feeding led to an increase in maximal and reserve mitochondrial respiratory capacities in both wild type and Nrf2^{-/-} mice, but no difference were seen between the genotypes (Fig. 5-5C and D). Although WAT of Nrf2^{-/-} mice showed a decrease in reserve capacity, this difference did not reach statistical significance (Fig. 5-5D). Taken together, these results suggest that mitochondrial activity is increased in Nrf2^{-/-} adipocytes, and that this increase in activity is potentially caused by increased proton leak across the mitochondrial inner membrane.

Figure 5-5. Nrf2 deficient WAT show increased oxygen consumption.

Quantitation of (A) basal OCR, (B) proton leak (oligomycin OCR – rotenone/antimycin OCR) (C) maximal rate (FCCP OCR – rotenone/antimycin OCR) (D) reserve capacity (rotenone/antimycin OCR) in WAT of wild type and Nrf2^{-/-} mice. Data were normalized to protein and represented as means \pm SEM, n = 3/group. Significance was determined by two-way Anova and Bonferroni post hoc comparison.

Figure 5-5



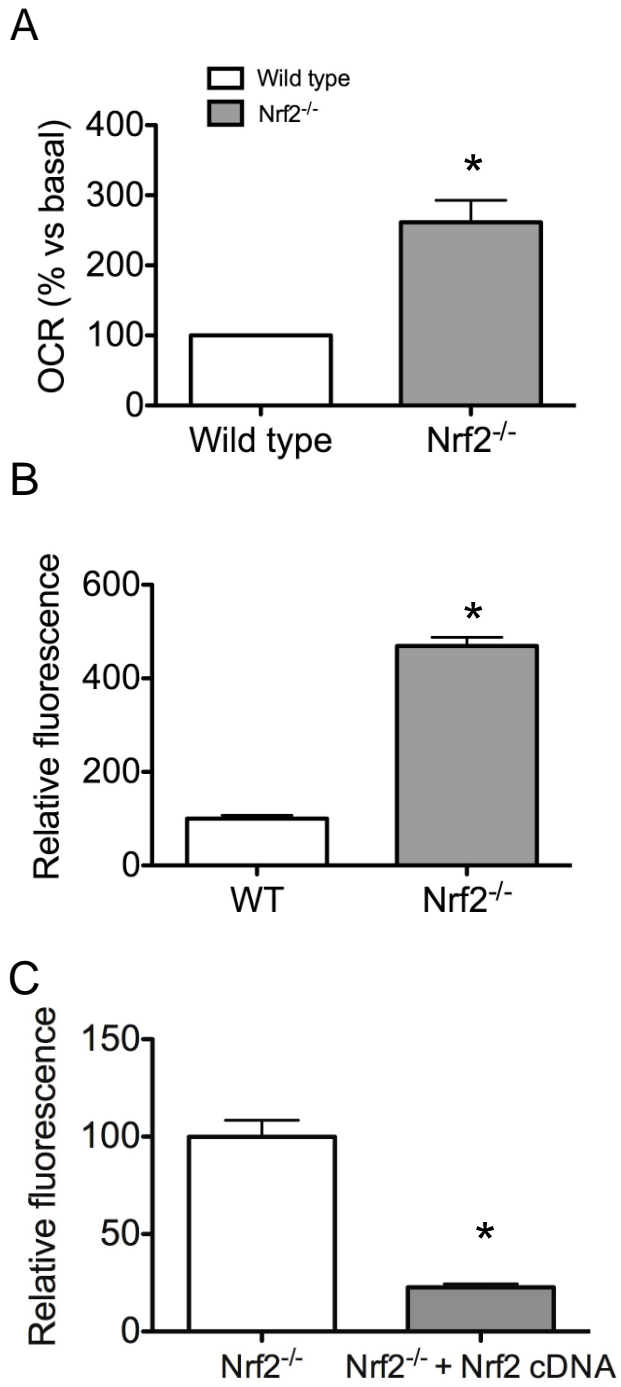
Nrf2 deficient MEF cells show increased oxygen consumption.

To determine whether increased cellular respiration is intrinsic to Nrf2 deficiency, we measured OCR in mouse embryonic fibroblasts (MEFs) utilizing the XF24 extracellular flux analyzer. Compared to wild type MEF cells, basal oxygen consumption rates in Nrf2^{-/-} MEF cells were increased 2.5-fold (Fig. 5-6A). To confirm these results, oxygen utilization of wild type and Nrf2^{-/-} MEFs were also compared using an alternative method utilizing oxygen biosensor plates in which the fluorescent signal from oxygen-sensitive dye is quenched by O₂. In this assay, Nrf2^{-/-} MEF cells showed a similar increase in oxygen consumption compared to wild type MEFs (Fig. 5-6B). To demonstrate that this effect is specific to Nrf2 deficiency, we asked if re-expression of Nrf2 would lower the oxygen consumption rates in Nrf2^{-/-} MEF cells. Retroviral-mediated expression of Nrf2 in Nrf2^{-/-} MEF cells decreased oxygen consumption by greater than 50% (Fig. 5-6C). From this data, we demonstrate that fibroblasts lacking Nrf2 also exhibit increased oxygen consumption, similar to both the whole animal and isolated adipose tissue.

Figure 5-6. Nrf2 deficient MEF cells show increased oxygen consumption.

(A) Seahorse X-24 analysis of basal oxygen consumption rate (OCR) in wild type and Nrf2^{-/-} MEF cells. Data represent means \pm SEM, n=3 per group and significance was determined by Student's t-test (*p<0.05). (B) Oxygen consumption of wild type and Nrf2^{-/-} MEF cells; and (C) Nrf2^{-/-} and Nrf2^{-/-} MEF cells stably expressing Nrf2 cDNA. Quantitative changes in respiratory rate were calculated from oxygen consumption curves measured using BD Oxygen Biosensor plates. Data represent means of maximum fluorescence intensity \pm SEM of 3-4 independent experiments.

Figure 5-6



Loss of Nrf2 will result in increased expression of genes involved in mitochondriogenesis and respiration.

Brown fat markers are up-regulated in white adipose tissues of Nrf2^{-/-} mice.

To determine the molecular basis of the lean phenotype of Nrf2^{-/-} mice, we performed gene expression analysis by high throughput RNA sequencing (RNA seq) of adipocytes derived from intra-abdominal fat pads of wild type and Nrf2 mice fed a high fat diet. Genes that were differentially expressed were analyzed with Ingenuity Pathway Analysis software. The top biological functions and disease networks identified by IPA in Nrf2^{-/-} included cell signaling, small-molecule biochemistry, lipid metabolism, cancer, and cell death (Fig. 5-7A). Overall, the top ranked networks were significantly enriched for pathways involved in lipid metabolism. Of the genes identified in these pathways, Nrf2^{-/-} white adipose tissue showed increased expression of Ucp1 and other brown adipocyte markers. Ucp1 functions as an uncoupler of mitochondrial ATP production, and its up-regulation might account for the change in energetics in Nrf2^{-/-} mice. Additional brown adipose tissue markers such as Pgc1a, Tfam, and Nrf1 are involved in the pathway of mitochondrial biogenesis, which could also lead to increases in changes in energetics. Hence, we focused our attention on these genes to validate the RNAseq data. RT-qPCR analysis showed significant up-regulation of Ucp1, Cox7a1, Cox8b, Sirt3, CideA, Pgc1a, Tfam and Nrf1 (nuclear respiratory factor 1) in WAT of Nrf2^{-/-} mice fed a high fat diet (Fig. 5-7B). Interestingly, expression of some of these genes associated with brown fat was also up-regulated in Nrf2^{-/-} WAT under regular diet conditions (Fig. 5-7B).

However under normal diet conditions, the differences between wild type and Nrf2 gene expression were on average significantly less pronounced than the data observed from high fat diet samples. Western blot analysis showed increased Ucp-1 expression in Nrf2^{-/-} WAT that was further enhanced by high fat diet (Fig. 5-7C, D and E). Taken together, these data suggest that WAT in Nrf2^{-/-} mice gained some BAT-like features, and that this difference is exacerbated upon exposure to a high fat diet.

Brown fat markers are unchanged in brown adipose tissue of Nrf2^{-/-} mice.

Brown adipose tissue is the canonical tissue for expression of genes involved in uncoupled respiration and energy expenditure. In order to determine if the brown adipose tissue also contributes to the increased energy expenditure seen in Nrf2^{-/-} animals, additional RT-QPCR was performed. Nrf2^{-/-} brown adipose tissue showed no increase in any genes tested from either normal diet or high fat diet animals (Fig.5-8 A,B). This data suggests that the changes in energetics seen in Nrf2^{-/-} animals does not stem from an increase in uncoupled respiration in the brown adipose tissue. After identifying this increase in respiration in Nrf2 deficient animals, and subsequently associating it with an increase in uncoupled respiration specifically in the white adipose tissue, we then moved on to try and determine the mechanism behind phenotype.

Figure 5-7. Brown fat markers are up-regulated in white adipose tissues of Nrf2^{-/-} mice. (A) Ingenuity Pathway Analysis of differentially expressed genes in wild type and Nrf2^{-/-} white adipose tissues. Genes that were significantly different between wild type and Nrf2^{-/-} white adipose tissues were used as input for pathway analysis. The top 10 overrepresented canonical pathways of up-regulated genes found in Nrf2^{-/-} white adipose tissues compared with controls are shown. Category names are presented on the y axis. The x axis indicates the $-\log(p)$ value of the overrepresentation analysis. (B) Quantitative RT-PCR analysis of brown fat markers in abdominal white adipose tissues of wild type and Nrf2^{-/-} mice fed a high fat diet (HFD) or regular diet (RD). Expression levels were quantitated relative to Rplpo levels as an internal reference and calculated as $2^{(Ct_{\text{test gene}} - Ct_{\text{RPLPO}})}$. Bar graphs depict mean values \pm S.E. (error bars) of 4–6 different samples of each genotype, and significance was assessed by two-way ANOVA and post-hoc Bonferroni analysis. *, significance between wild type and Nrf2^{-/-} regular diet samples; #, significance between regular diet and high fat diet samples. (C–D) Western blotting analysis of UCP1 protein levels in abdominal white adipose tissues of wild type and Nrf2^{-/-} mice fed a high fat or regular diet, respectively. β -Actin levels were used as a loading control. (E) Bar graph shows quantitation of protein levels; significance was assessed by two-way ANOVA and post hoc Bonferroni analysis.

Figure 5-7

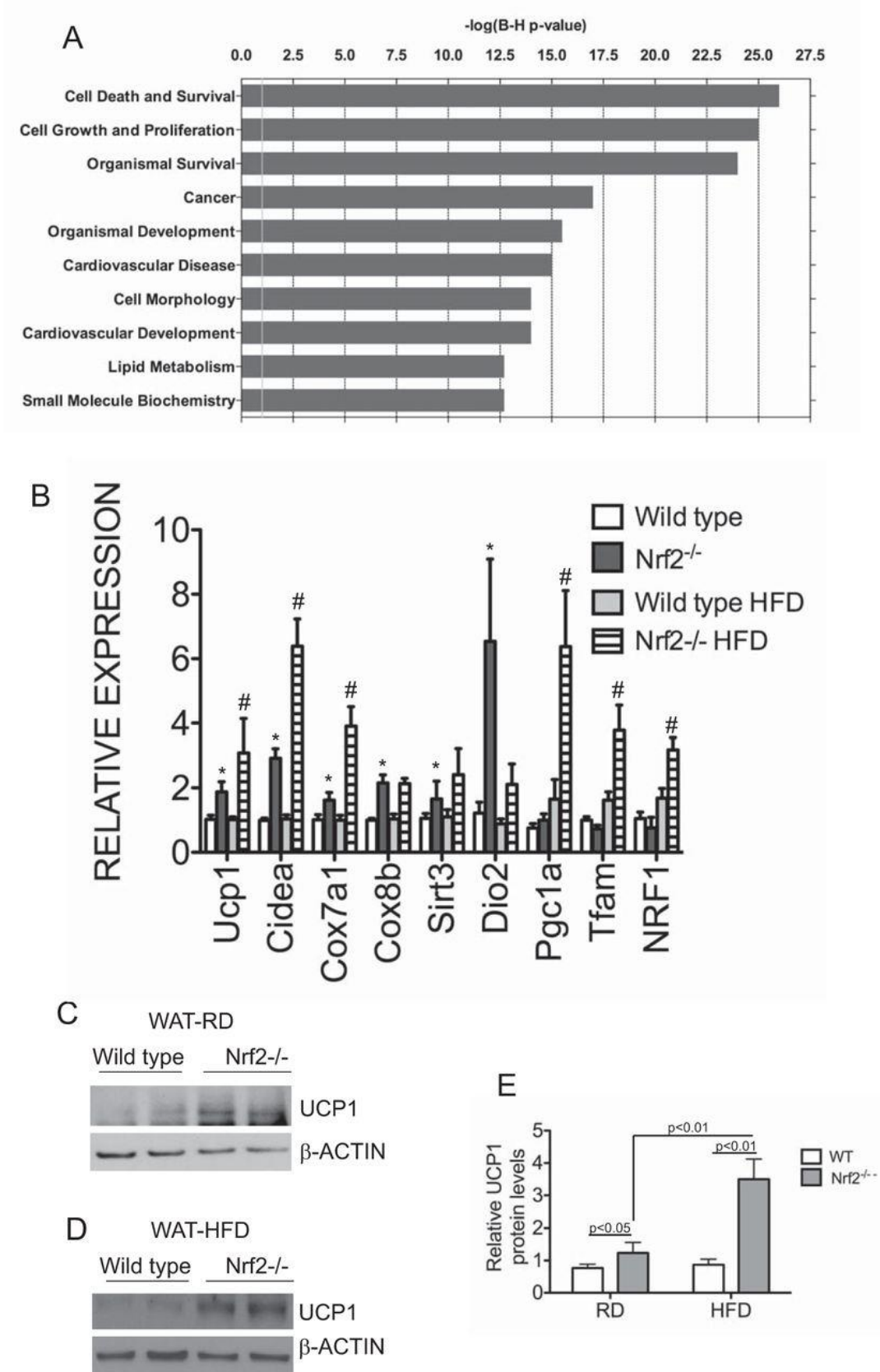
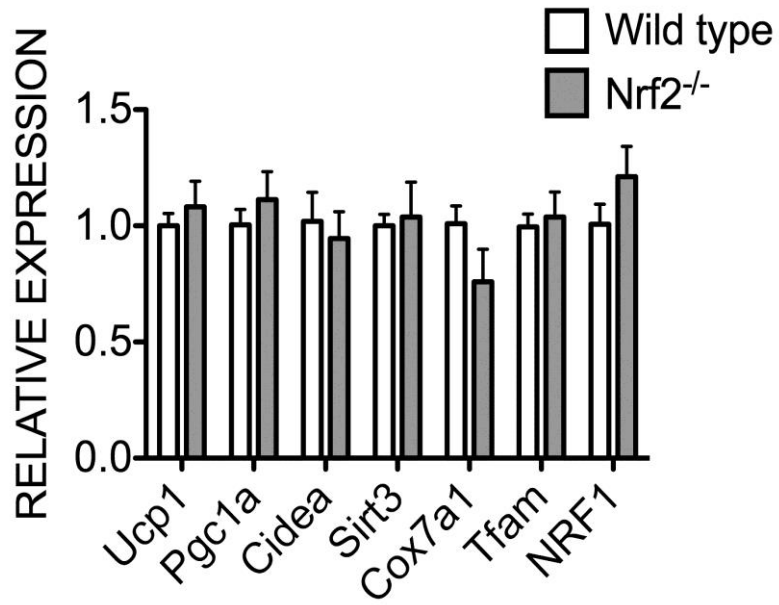


Figure 5-8. Brown fat markers are unchanged in the brown adipose tissue of Nrf2^{-/-} animals

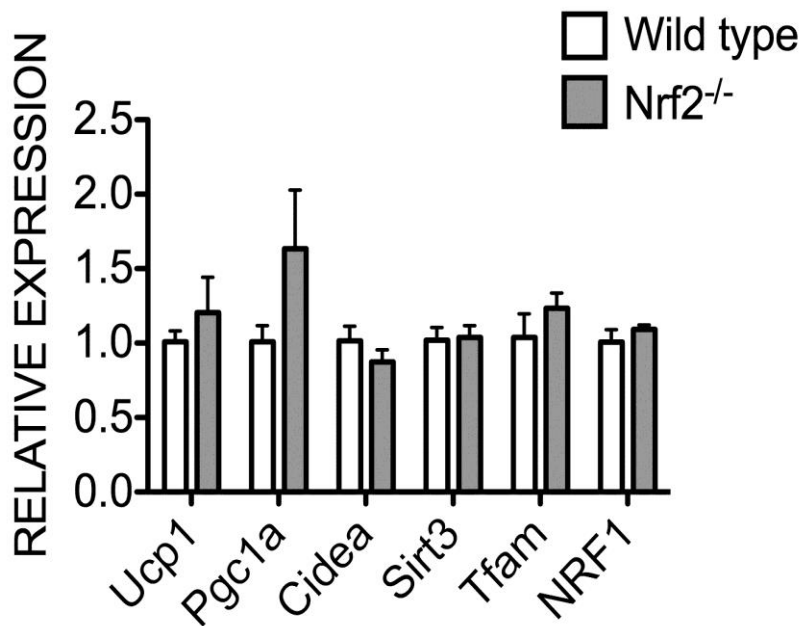
(A, B) Quantitative RT-PCR analysis of brown fat markers in intrascapular brown adipose tissues of wild type and Nrf2^{-/-} mice fed a high fat diet (HFD) or regular diet (RD). Expression levels were quantitated relative to RPLPO levels as an internal reference, and calculated as $2^{(Ct_{\text{test gene}} - Ct_{\text{RPLPO}})}$. Bar graphs depict mean values \pm SEM of 4-6 different samples of each genotype are shown, and significance was assessed by Student's t test, *P < 0.05.

Figure 5-8

A



B



Oxidative stress modulates oxygen consumption and UCP1 expression.

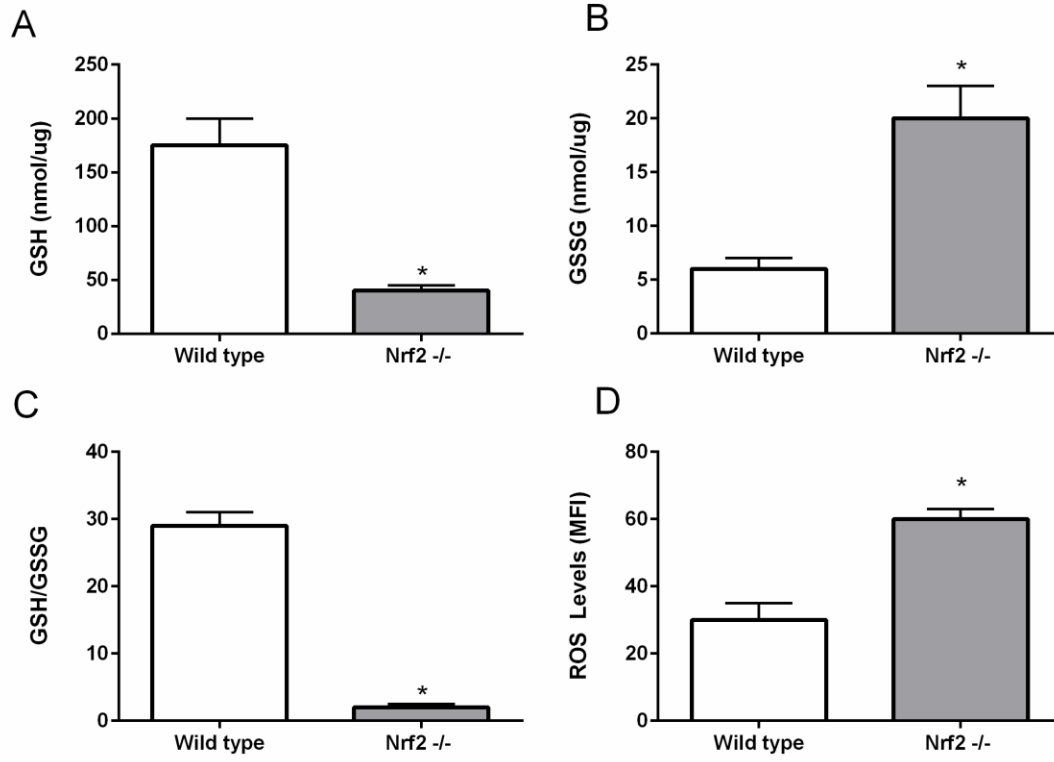
Nrf2 tissues and cells exhibit oxidative stress

Nrf2 plays a pivotal role in oxidative stress response. Hence, we sought to determine if elevated oxygen consumption rates in Nrf2-deficient cells and adipose tissue is potentially linked to oxidative stress. To confirm oxidative stress in our tissue model, we assessed glutathione status in adipose tissues of wild type and Nrf2^{-/-} mice fed a high fat diet. WAT from Nrf2^{-/-} mice fed a high fat diet showed a decrease in GSH with a corresponding increase in GSSG leading to a reduction in GSH/GSSG ratio (Fig. 5-9A, B and C). A decreased GSH/GSSG ratio is indicative of oxidative stress. This result is expected as Nrf2 is one of the major transcription factors regulating glutathione reductase, which is responsible for converting the oxidized GSSG into the functionally active reduced form GSH. So with a decreased expression of glutathione reductase, there is a buildup of GSSG and a concordant decrease in levels of GSH within the tissue. We also observed increased levels of oxidative stress in our Nrf2^{-/-} fibroblast cells as measured by DCF staining and FACS analysis (Fig. 5-9D). The above data suggests that both Nrf2 deficient cells and adipose tissue are under increased levels of oxidative stress compared to wild type.

Figure 5-9. Nrf2 ^{-/-} Tissues and Cells exhibit oxidative stress

(A) GSH (B) GSSG and (C) GSH/GSSG levels of WAT from wild type and Nrf2^{-/-} mice after 6 weeks of high fat diet feeding. Data represent means (n=3/group) ± SEM; *p < 0.05. (D) ROS levels in wild type and Nrf2^{-/-} MEF cells as measured by DCF fluorescence levels.

Figure 5-9



Oxidative stress levels influence oxygen consumption

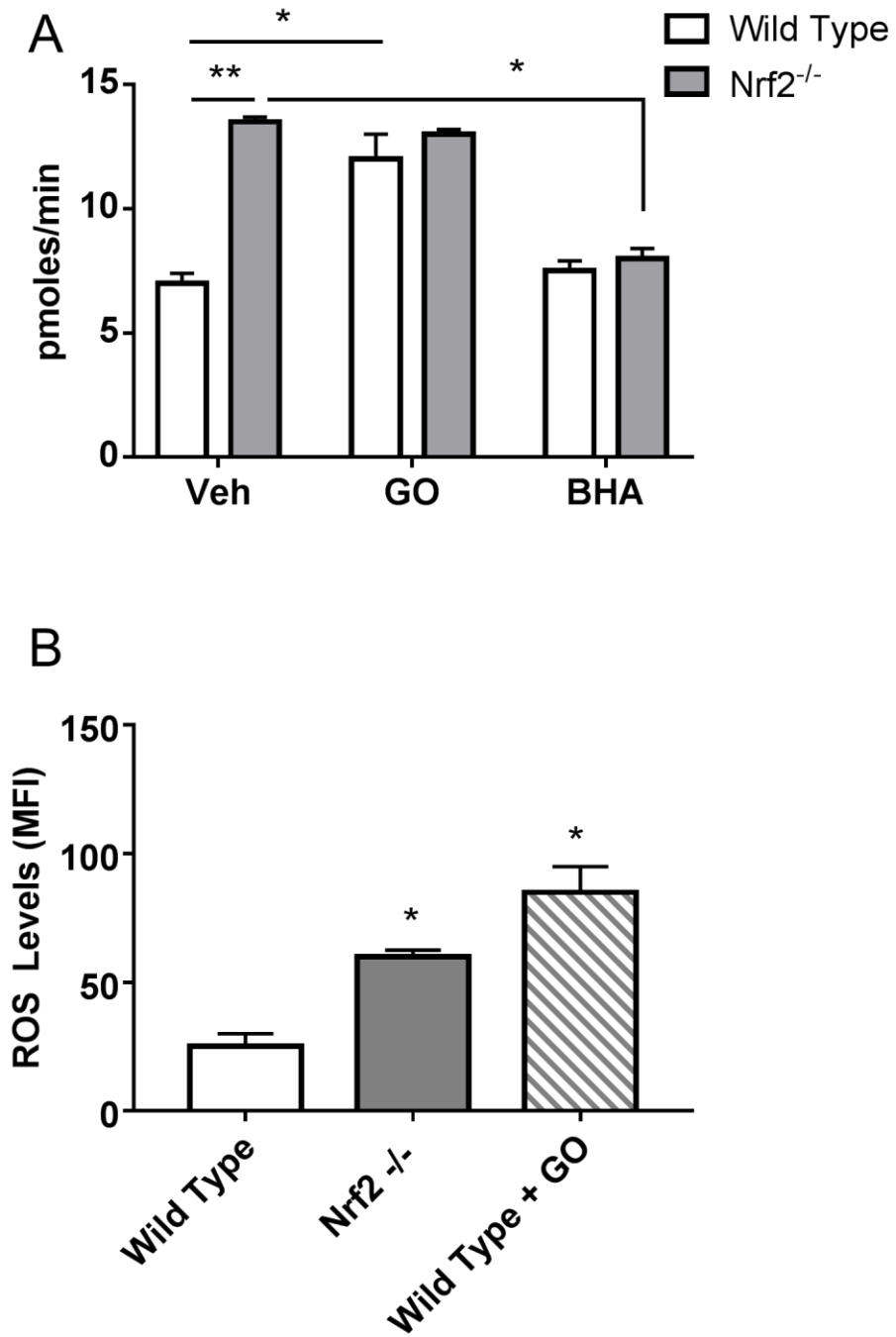
To further test the effects of oxidative stress on cellular respiration, we compared oxygen consumption rates of wild type and Nrf2^{-/-} fibroblasts after the addition of glucose oxidase, an inducer of oxidative stress. Oxygen consumption rates were 2-fold higher in Nrf2^{-/-} cells compared to wild type under basal conditions (Fig. 5-10A). In order to further identify oxidative stress as the driving cause of the change in oxygen consumption, we treated wild type cells with glucose oxidase. Glucose oxidase is an oxido-reductase that catalyzes the oxidation of glucose to hydrogen peroxide and D-glucono- δ -lactone. This produces a consistent low dose of hydrogen peroxide. Wild type cells treated with glucose oxidase showed a 2-fold increase in oxygen consumption compared to non-treated wild type cells (Fig. 5-10A). However, glucose oxidase treatment did not lead to further enhancement of oxygen consumption in Nrf2^{-/-} cells. As expected, wild type cells treated with glucose oxidase demonstrated an increase in oxidative stress as measured by DCF staining (Fig. 5-10B). The data suggests that induction of oxidative stress can increase oxygen consumption. In order to strengthen the argument that oxidative stress can induce oxygen consumption, we then examined the effects of butylated hydroxyanisol (BHA), a free radical scavenging antioxidant, on oxygen consumption in Nrf2^{-/-} cells. Oxygen consumption in Nrf2^{-/-} cells was reduced by nearly 50% following BHA treatment (Fig. 5-10A). BHA treatment had no effect in wild type cells. Taken together, this data suggests that oxidative stress can drive an increase in oxygen consumption, and that conversely, antioxidant treatment can decrease levels of oxygen consumption.

Figure 5-10. Oxidative stress levels influence oxygen consumption

(A) Seahorse X-24 analysis of basal oxygen consumption rate in wild type and Nrf2^{-/-} MEF cells with and without treatment with glucose oxidase or BHA. Data were normalized to protein and represented as means ± SEM (n=6/group).

Significance was assessed by two-way ANOVA and post-hoc Bonferroni analysis; *p<0.05, **p<0.001. (B) DCF fluorescence levels in wild type, Nrf2^{-/-}, and wild type cells treated with glucose oxidase. Data represents means (n=3/4/group) ± SEM, *p<0.05.

Figure 5-10



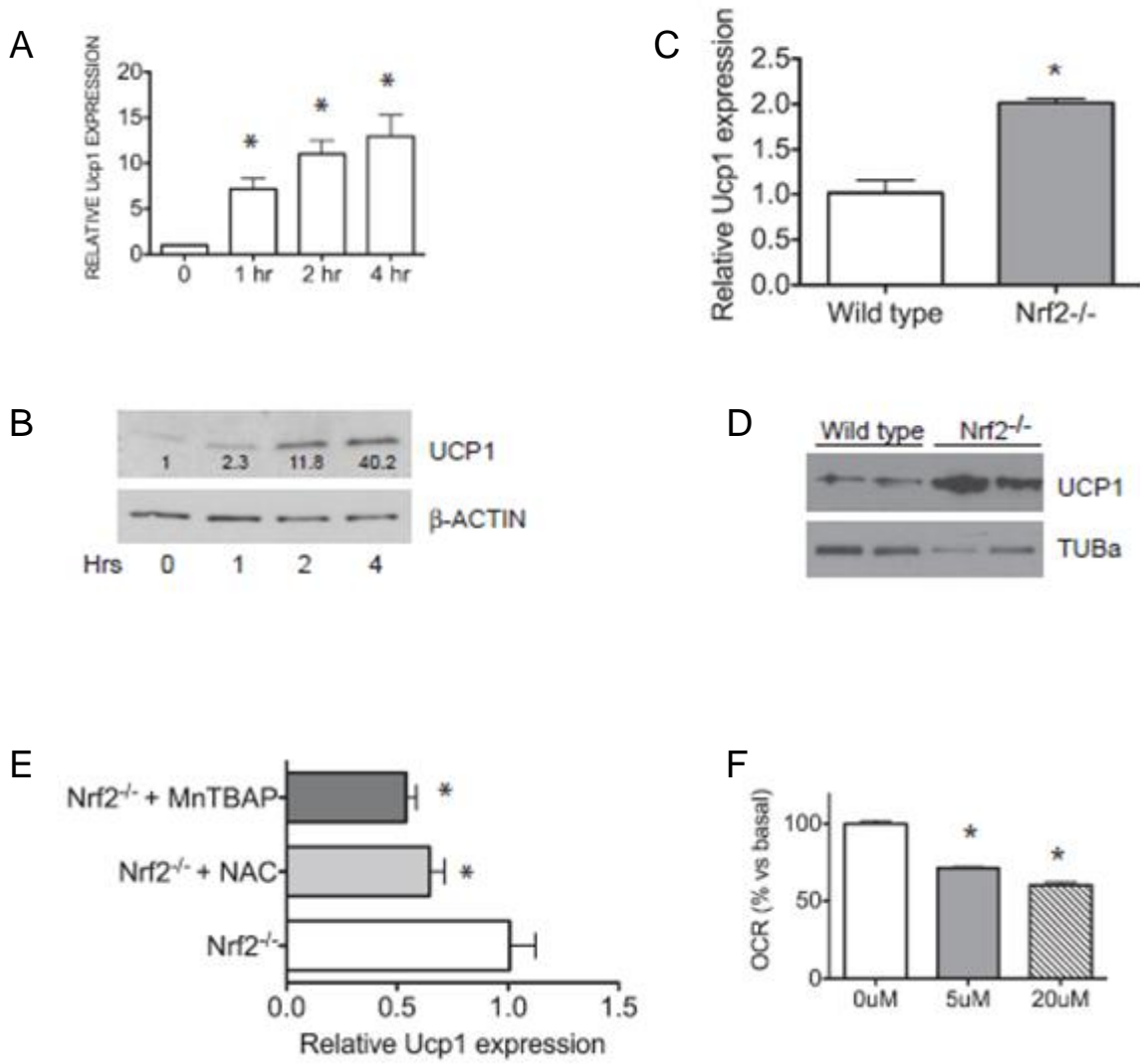
Oxidative stress modulates UCP1 expression

After observing the effects of oxidative stress on oxygen consumption, we examined the effects of oxidative stress on UCP1 expression. We had observed increased levels of oxidative stress in Nrf2 ^{-/-} adipose tissue, as well as increased levels of UCP1 expression and oxygen consumption. We wanted to confirm that oxidative stress could regulate UCP1 levels. We treated wild type MEF cells with glucose oxidase and observed a marked induction of Ucp1 mRNA and protein (Fig. 5-11A,B). This was consistent with previous data that showed an increase in oxygen consumption upon glucose oxidase treatment. Nrf2 ^{-/-} cells were shown to be under higher levels of oxidative stress and similarly Nrf2 ^{-/-} MEF cells showed increased Ucp1 mRNA and protein compared to wild type cells (Fig. 5-11C,D). Finally, treatment with antioxidants—N-acetylcysteine, a free radical scavenging compound, or MnTBAP, a cell permeable superoxide dismutase (SOD) mimetic—led to a decrease in Ucp1 mRNA in Nrf2 ^{-/-} MEF cells (Fig. 5-11E). Finally, to confirm that changing levels of UCP1 can have dramatic effects on cellular oxygen consumption we used SB203580, a p38 MAPK inhibitor known to suppress Ucp1 expression ⁽⁸⁴⁾. SB203580 treatment resulted in a dose-dependent decrease in oxygen consumption rate in Nrf2 ^{-/-} MEF cells (Fig. 5-11F). Taken together, this data suggests that Nrf2 deficiency in MEFs results in oxidative stress, which in turn up-regulates Ucp1 expression and cellular respiration.

Figure 5-11. Oxidative stress modulates UCP1 expression

(A) Expression of Ucp1 mRNA in wild type MEF cells treated with glucose oxidase to induce oxidative stress for the indicated time points. (B) Western blot analysis of UCP1 protein in wild type MEF cells treated with glucose oxidase for the indicated time points. Densitometric quantitations of band intensities are shown. (C) Basal expression of Ucp1 mRNA levels in wild type and Nrf2^{-/-} MEF cells. Results represent means (n=3/group) ± SEM, *p<0.05. (D) Western blot analysis of basal UCP1 protein in wild type and Nrf2^{-/-} MEF cells (E) Expression of Ucp1 mRNA levels in Nrf2^{-/-} MEF cells treated NAC or MnTBAP. Bar graphs represent mean values ± SEM (n=3), *p<0.05. (F) Seahorse X-24 analysis of basal oxygen consumption rate (OCR) in Nrf2^{-/-} MEF cells treated with SB203580. Data represent means ± SEM, n=3 per group and significance was determined by Student's t-test (*p<0.05).

Figure 5-11



Loss of Nrf1 disrupts normal adipose tissue function and/or structure.

Loss of Nrf1 in mouse adipose tissue leads to decreased body weight, decreased serum glucose, and improved glucose tolerance

In order to study the effects of Nrf1 in the adipose tissue, a conditional knockout mouse was generated due to the fact that germ line Nrf1 knockout is lethal during gestation. The cre-lox system was used to create a tissue specific knockout. We used a previously generated mouse which contained a loxP-flanked Nrf1 allele, which was constructed by flanking the terminal exon of the nrf1 gene with loxP sequences ⁽⁸⁵⁾. We then crossed the Nrf1 flox mice with Adipoectin-Cre transgenic mice. In this model, Cre recombinase expression is dependent on the Adipoq gene promoter, which is present in both white and brown adipose tissue. Adipo-Cre has been shown to be expressed specifically in adipose tissue ⁽⁸⁶⁾. For analysis, Nrf1 flox/flox::AdipoCre (Nrf1 FatKO) were used as mutants and Nrf1 +/-::AdipoCre animals were used as controls. RT-qPCR analysis showed that Nrf1 expression was significantly decreased in both the white and brown adipose tissue of Nrf1 FatKO animals when compared with wild type animals (Fig. 5-12A).

Control and Nrf1 FatKO mice were monitored for any abnormalities from weaning until 12 months of age. While no differences were observed at weaning, by three months of age, male Nrf1 FatKO mice were noticeably leaner than wild type littermates, weighing on average 30% less (Fig. 5-12B). While differences were striking in male animals, female animals showed little difference between

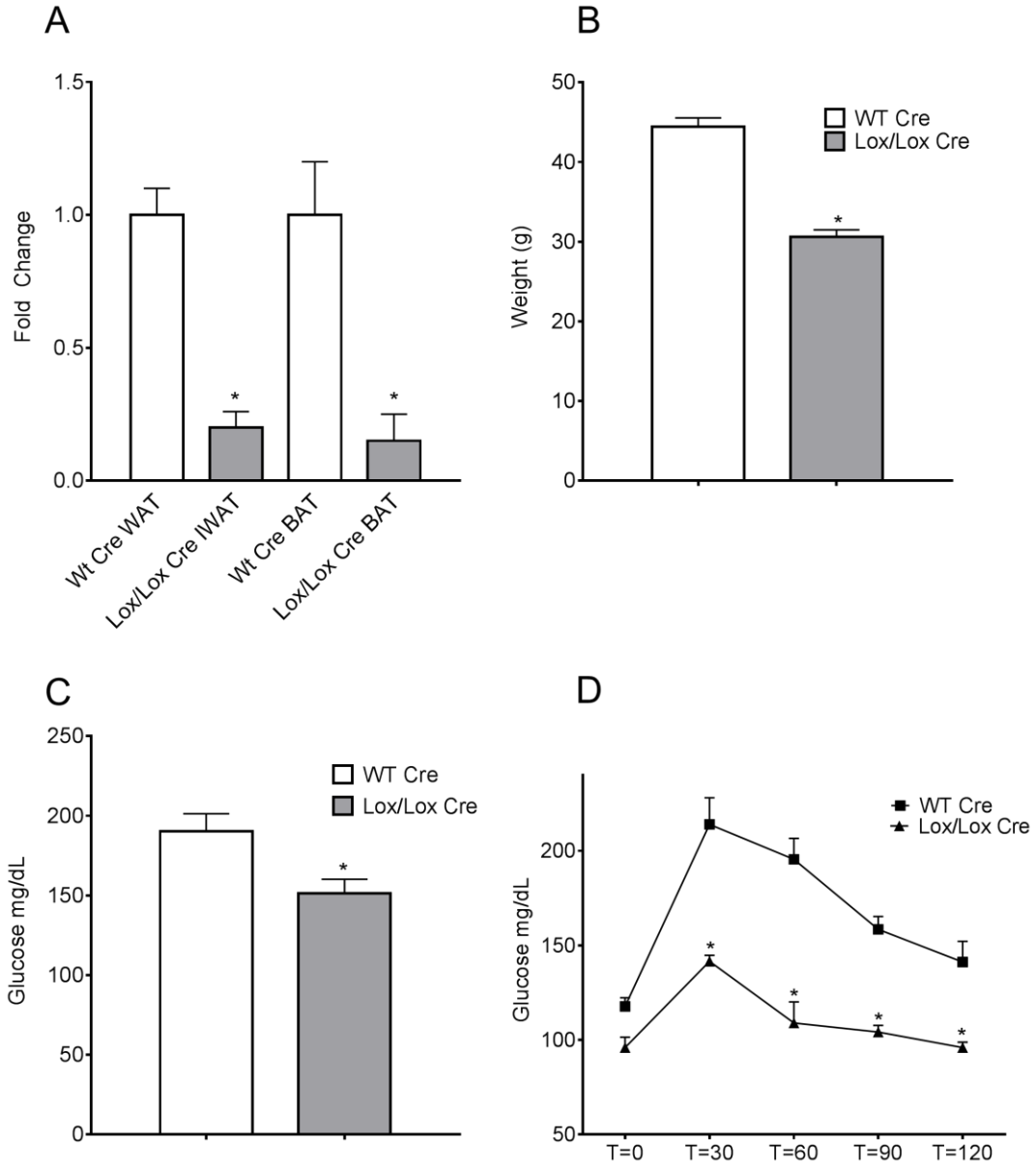
wild type and Nrf1 FatKO weights (Data not shown). Additionally, Nrf1 FatKO animals demonstrated decreased basal glucose levels as well as improved glucose tolerance (Fig.5-12 C,D). This data demonstrates that a successful adipose tissue knockout of Nrf1 had been achieved and resulted in decreased overall body weight and improvement of glucose levels.

Figure 5-12. Nrf1 Fat Specific knockout results in decreased white adipose tissue weight and improved glucose profile

(A) Quantitative RT-PCR analysis of Nrf1 levels in wild type and Nrf1 Fat KO white and brown adipose tissue. (B) Comparison of body weights of three month old male animals fed a normal chow diet. (C) Basal glucose measurements from wild type and Nrf1 FatKO animals on normal chow. (D) Glucose tolerance from wild type and Nrf1 FatKO animals on normal chow after fasting 12 hours.

Normalized Data represent means (n=3-5/group) \pm SEM; *p < 0.05.

Figure 5-12



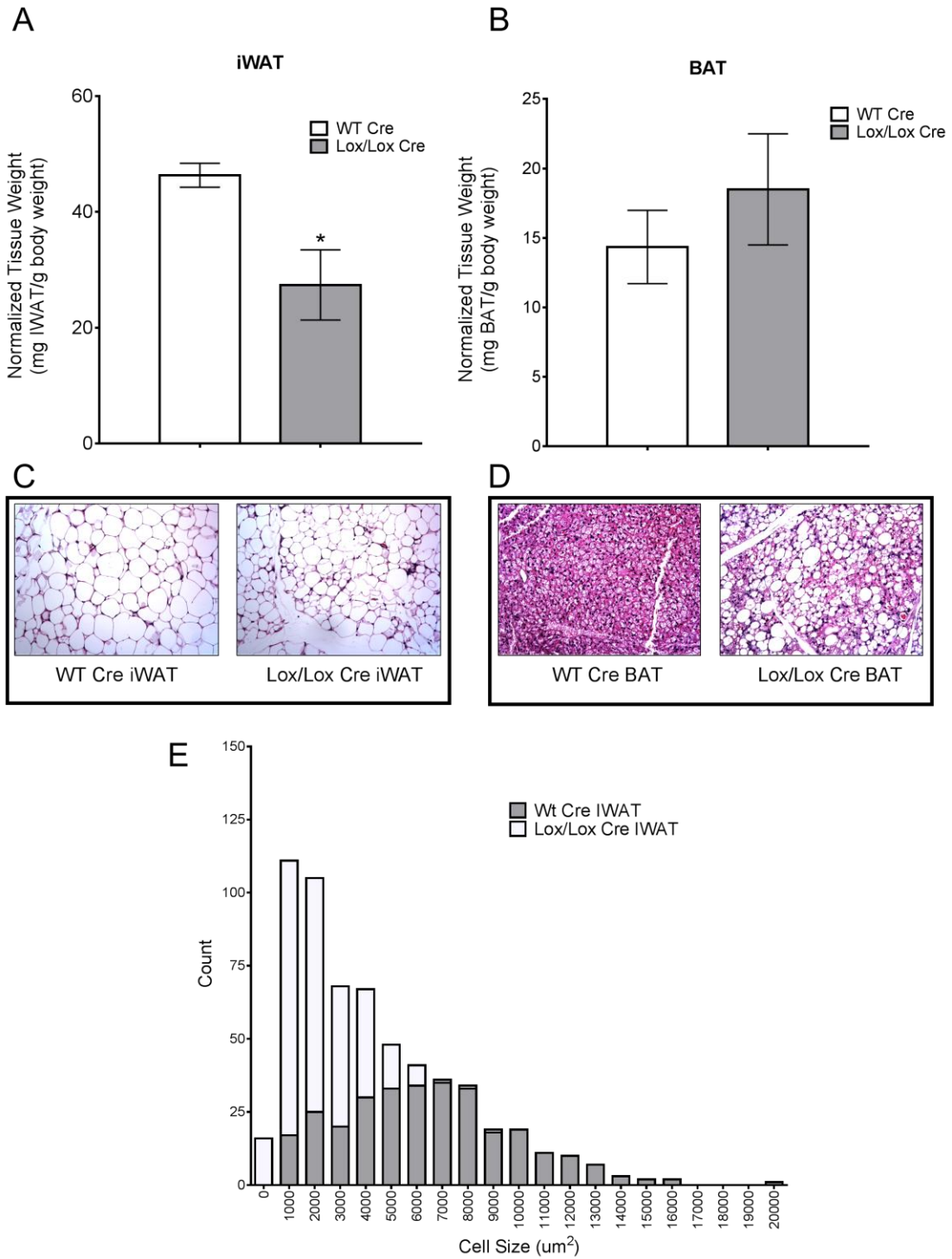
Nrf1 FatKO mice demonstrate decreased lipid accumulation in WAT, but increased lipid accumulation in BAT

Following the observation of decreased adiposity in Nrf1 FatKO mice, examination of the individual adipose tissue depots was conducted. Consistent with the previous weight data for whole animals, weight of white adipose tissue of male Nrf1 FatKO was significantly lower than that of wild type animals (Fig 5-13A). Curiously, this decrease in white adipose tissue weight seen in Nrf1 FatKO mice was not observed in brown adipose tissue (Fig. 5-13B). In order to further explore this difference in adipose tissue weight between wild type and Nrf1 FatKO mice, histological examination of tissue was conducted. It was found that lipid droplet size was significantly decreased in the Nrf1 FatKO white adipose tissue (Fig.5-13C,E). This is consistent with the gross tissue weight that was observed. Contrary to the results observed in whole animal weight and isolated white adipose tissue, the brown adipose tissue of Nrf1 Fat KO mice actually demonstrated an accumulation of lipids (Fig. 5-13D). The brown adipose tissue of mutant animals exhibited not only a dramatic increase in accumulation of lipids, but the distribution of lipids was in large unilocular droplets, more indicative of white adipose tissue. Neither wild type nor Nrf1 FatKO mice exhibited ectopic lipid deposition in the muscle or liver (Data not shown).

Figure 5-13. Nrf1 FatKO mice demonstrate decreased lipid accumulation in WAT, but increased lipid accumulation in BAT

(A) Comparison of normalized WAT weights of WT and Nrf1 FatKO on normal diet (B) Comparison of normalized BAT weights of WT and Nrf1 FatKO on normal diet (C) Representative histological sections of white adipose tissue. (D) Representative histological sections of brown adipose tissue (E) Histogram comparison of lipid droplet size of Wt and Nrf1 FatKO white adipose tissue.

Figure 5-13



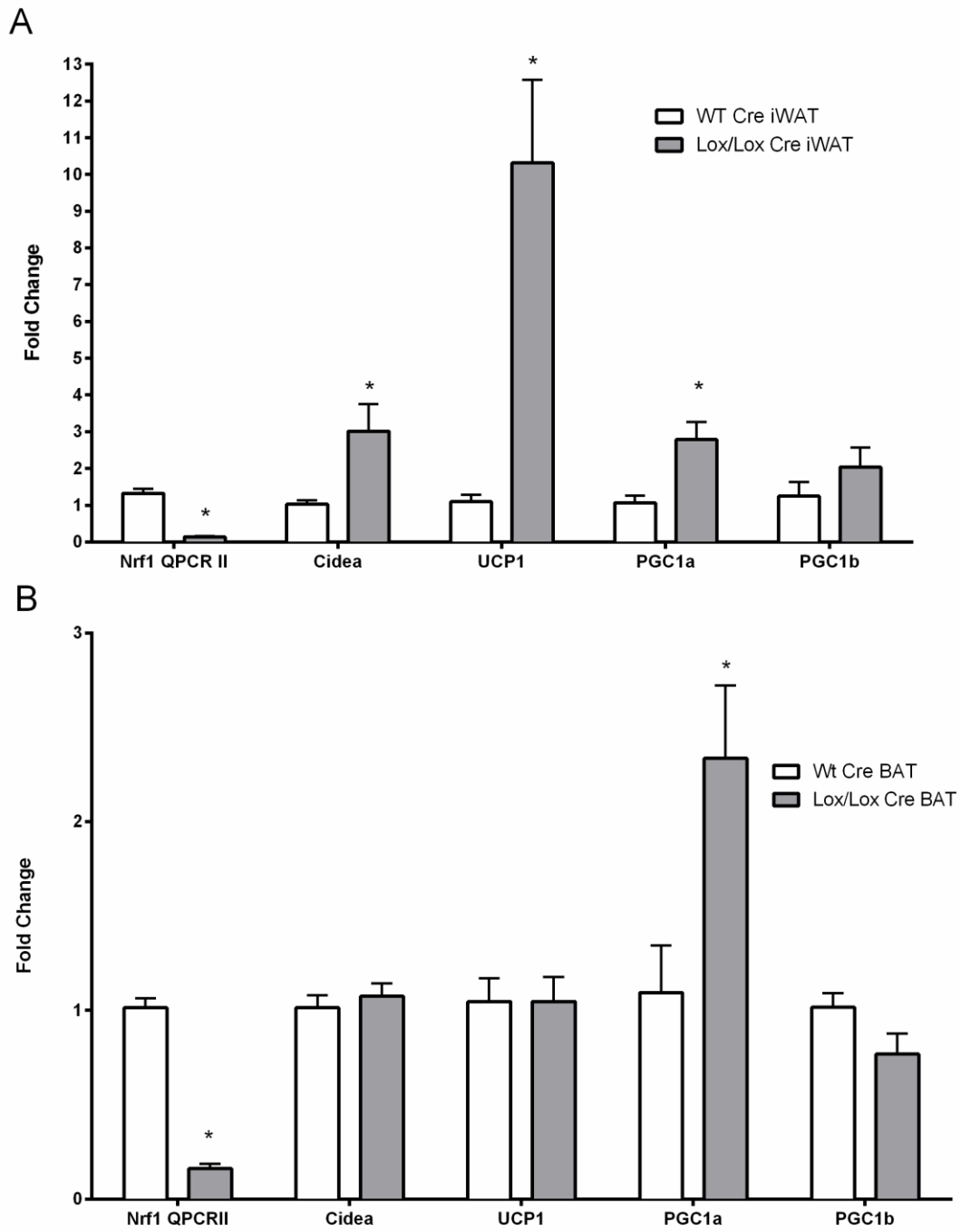
Brown fat markers are upregulated in the white adipose tissue of Nrf1 FatKO mice

After observing the overall lean phenotype of the Nrf1 FatKO animals, we sought to see if there were any changes in gene expression that could account for the observed phenotype. Previous work conducted on the Nrf2 knockout animal model found that there was an increased expression of genes involved in uncoupled respiration and mitochondriogenesis in the white adipose tissue of Nrf2 knockout animals. Due to the similarities between Nrf1 and Nrf2, we hypothesized that there may be upregulation of similar genes in the Nrf1 model that we observed in the Nrf2 model. We examined both white adipose tissue and brown adipose tissue, to see if we could find any explanation for either the decreased lipid accumulation in the mutant white adipose tissue, or the increased lipid accumulation in the mutant brown adipose tissue. We found that the white adipose tissue of Nrf1 FatKO animals showed upregulation of several key genes normally seen in brown adipose tissue including *ucp1*, *pgc1a*, and *cidea* (Fig.5-14A). This is similar to the data seen in the Nrf2 knockout model. The brown adipose tissue however did not demonstrate a similar increase in gene expression, with only *pgc1a* demonstrating a significant increase (Fig.5-14B). Looking at this data as a whole, we hypothesize that the loss of Nrf1 in the adipose tissue results in a lean phenotype due in part to the increased expression of genes involved in uncoupled respiration in white adipose tissue.

Figure 5-14. Brown fat markers are upregulated in the white adipose tissue of Nrf1 FatKO mice

(A, B) Quantitative RT-PCR analysis of brown fat markers in abdominal white and brown adipose tissues of wild type and Nrf1 Fat KO mice fed a normal diet. Expression levels were quantitated relative to RPLPO levels as an internal reference, and calculated as $2^{(Ct_{\text{test gene}} - Ct_{\text{RPLPO}})}$. Bar graphs depict mean values \pm SEM of 4-6 different samples of each genotype are shown, and significance was assessed by Student's t test, *P < 0.05.

Figure 5-14



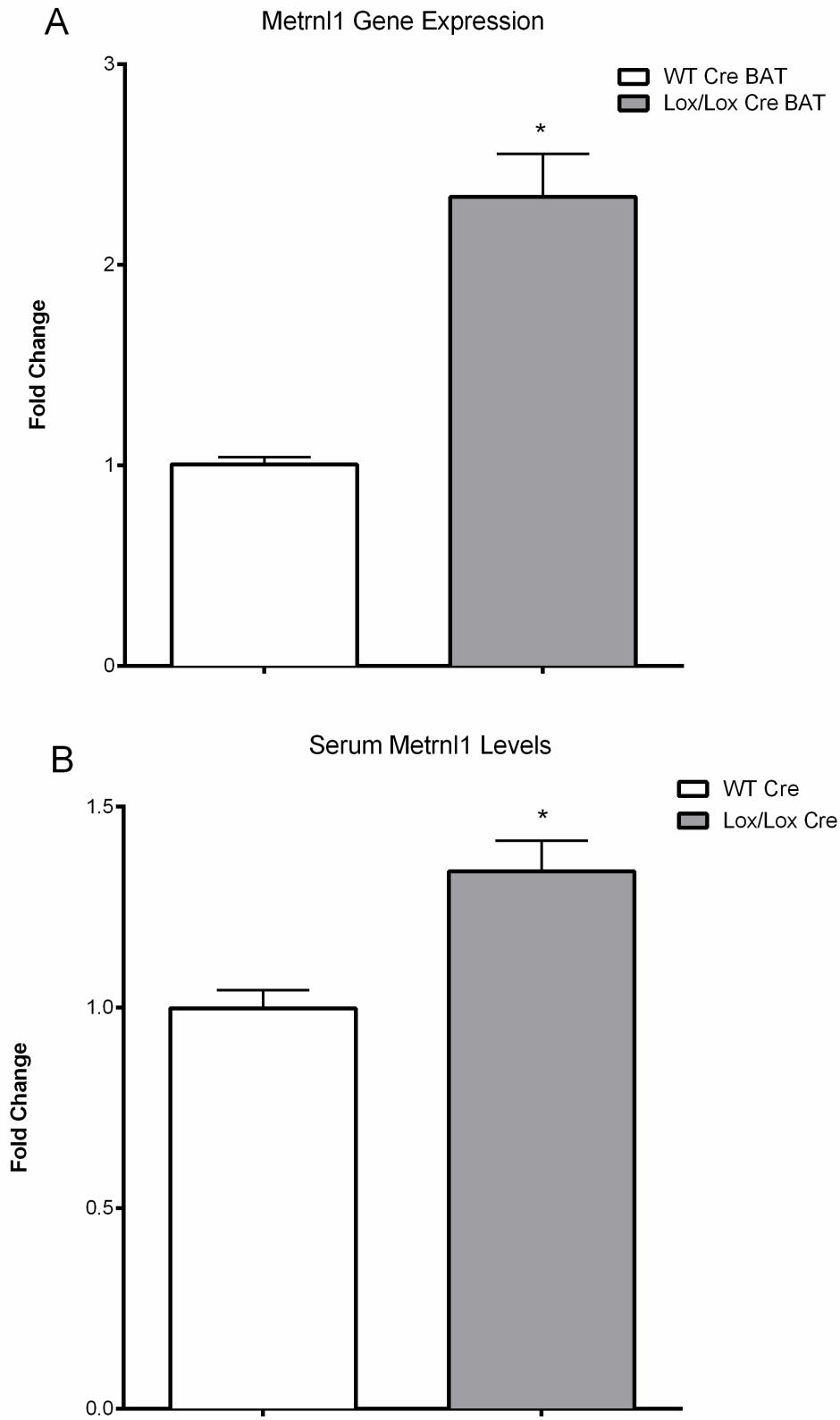
Nrf1 FatKO mice demonstrate increased Metrnl1 gene expression in BAT and increased serum Metrnl1 levels

In order to further explore the gene expression seen in the white adipose tissue of Nrf1 FatKO mice, we explored pathways responsible for regulating Ucp1 levels. Ucp1 is regulated by a wide variety of external environmental stimuli such as cold exposure as well as a number of pathways including Pgc1a⁽⁸⁷⁻⁸⁸⁾. Additionally, recent publications have identified a circulating factor known as meteorin like factor 1 (metrnl1), whose expression is driven by pgc1a, and has been found to strongly increase expression of Ucp1⁽⁸⁹⁾. Curious whether Metrnl1 played any role in our Nrf1 FatKO model, we examined gene expression. We found no difference in Metrnl1 expression levels in the white adipose tissue (Data not shown). However we saw a significant increase in Metrnl1 expression in the brown adipose tissue of Nrf1 FatKO mice (Fig.5-15A). In order to identify if this increase in expression of metrnl1 in the brown adipose tissue, resulted in an increase in circulating levels of metrnl1 we utilized a serum ELISA kit. Using this we found that Nrf1 FatKO mice exhibit a significant increase in the level of metrnl1 in the serum (Fig.5-15-B). Taken together, we hypothesize that the increase in expression of metrnl1 in the brown adipose tissue leads to an increase in circulating metrnl1 in the serum. This increase in circulating metrnl1 levels could then contribute to the increased expression of ucp1 in the white adipose tissue, and contributes to the lean phenotype observed in Nrf1 FatKO animals.

Figure 5-15: Nrf1 FatKO mice demonstrate increased Metrnl1 gene expression in BAT and increased serum Metrnl1 levels

(A) Quantitative RT-PCR analysis of *metrnl1* expression in brown adipose tissues of wild type and Nrf1 Fat KO mice fed a normal diet. Expression levels were quantitated relative to RPLPO levels as an internal reference, and calculated as $2^{(Ct_{\text{test gene}} - Ct_{\text{RPLPO}})}$. Bar graphs depict mean values \pm SEM of 10 different samples of each genotype are shown, and significance was assessed by Student's t test, *P < 0.05. (B) Serum insulin levels of wild type and Nrf1 FatKO mice fed a normal diet. Bar graphs depict mean values \pm SEM of 5-7 different samples of each genotype are shown, and significance was assessed by Student's t test, *P < 0.05.

Figure 5-15



Loss of Nrf1 disrupts normal adipose cell differentiation and alters gene expression in vitro.

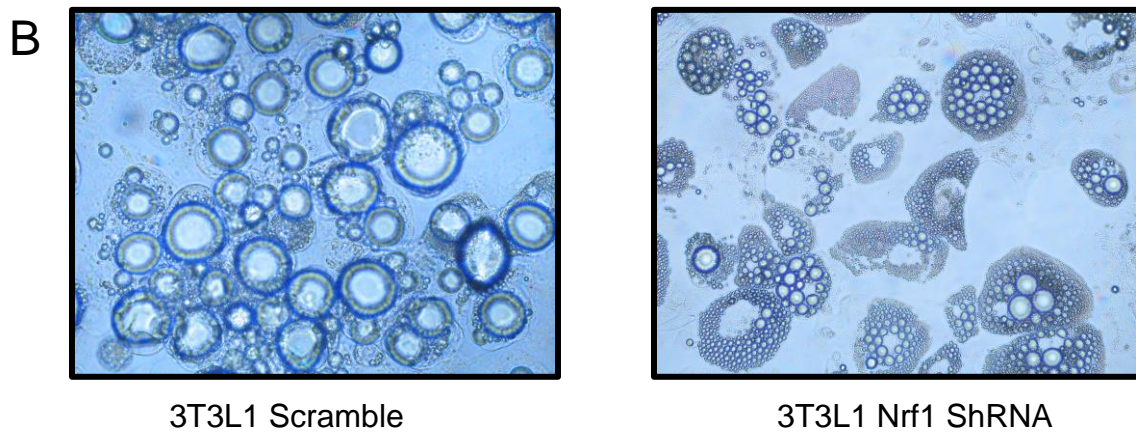
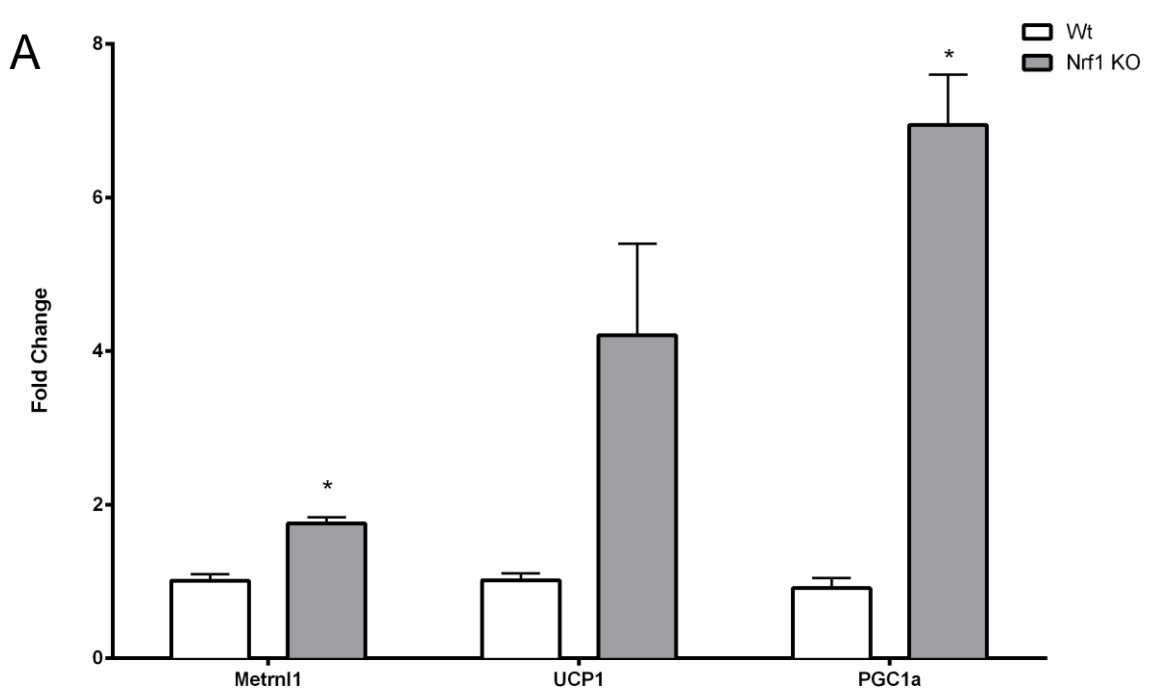
Nrf1 knockout cells exhibit increased Ucp1, Pgc1a, and Metrn1 expression and demonstrate decreased lipid accumulation when differentiated into adipose cells.

In order to examine the effects of Nrf1 deficiency in vitro, we employed two different cell models. Firstly, primary mouse embryonic fibroblasts were taken from wild type and Nrf1 knockout embryos. Secondly, 3T3L1 pre-adipocyte cell lines were treated with Nrf1 ShRNA to create a stable knockdown of Nrf1 in an adipose cell line. Primary MEF cells lacking Nrf1 were found to have increased expression of Ucp1, Pgc1a and Metrn1, mirroring the results found in the adipose tissue of Nrf1 FatKO animals (Fig.5-16A). While Nrf1 deficient MEF cells provide one method of analysis, 3T3L1 preadipocyte cells would more closely mimic the knockdown model seen in our Nrf1 FatKO mice. When 3T3L1 cells containing a ShRNA knockdown of Nrf1 were exposed to a cocktail of drugs used to differentiate cells into white adipose tissue cells, they exhibited decreased lipid accumulation which mirrored the decreased lipid accumulation in Nrf1 FatKO white adipose tissue (Fig.5-16B). Taken together, these in vitro experiments provide similar results to those seen in the in vivo studies, and suggest that loss of Nrf1 results in expression of thermogenic genes and results in decreased lipid accumulation.

Figure 5-16: Nrf1 knockout cells exhibit increased Ucp1, Pgc1a, and Metrn1 expression and demonstrate decreased lipid accumulation when differentiated into adipose cells.

(A) Quantitative RT-PCR analysis of thermogenic gene expression wild type and Nrf1 KO MEF cells. Expression levels were quantitated relative to RPLPO levels as an internal reference, and calculated as $2^{(Ct_{\text{test gene}} - Ct_{\text{RPLPO}})}$. Bar graphs depict mean values \pm SEM of 10 different samples of each genotype are shown, and significance was assessed by Student's t test, *P < 0.05. (B) Representative images from wild type 3T3L1 cells and 3T3L1 Nrf1 knockdown ShRNA cells differentiated into adipose cells.

Figure 5-16



CHAPTER 6

DISCUSSION

Obesity and its related disorders are leading causes of morbidity and mortality worldwide. Various treatment modalities for obesity have been met with limited success. Hence, identification of novel targets that can potentially be employed for prevention and treatment of obesity is of great interest. In these studies we have demonstrated that loss of either Nrf1 or Nrf2 can have dramatic consequences on adipose tissue gene expression and provide protection against obesity. Specifically, loss of Nrf2 protects mice from high fat diet-induced obesity. Nrf2^{-/-} mice exhibit increased energy expenditure not associated with an increase in physical activity. We also found that this increase in energy expenditure is seen in isolated white adipose tissue of Nrf2^{-/-} mice, which exhibits increased oxygen consumption. Additionally, white adipose tissue from Nrf2^{-/-} mice express brown adipose tissue genes involved in mitochondriogenesis, as well as coupled and uncoupled respiration. Loss of Nrf2 in cells results in increased oxygen consumption and expression of UCP1, and we have shown that oxidative stress can modulate UCP1 expression and oxygen consumption in cells. Taken together, these results suggest that increased uncoupled respiration due to increased oxidative stress brought on by the loss of Nrf2, helps to contribute to the lean phenotype of Nrf2^{-/-} animals fed a high fat diet. Additionally, we have found that animals lacking Nrf1 specifically in the adipose tissue are also leaner than wild type mice. Similar to the Nrf2 knockout mice, these fat specific Nrf1 knockout mice express increased levels of brown

adipose tissue genes in the white adipose tissue. Specifically there is increased expression of those genes involved in uncoupled respiration such as Ucp1 as Pgc1a. In agreement with the animal model, Nrf1 deficient cells also exhibit increased expression of these same genes, and demonstrate decreased lipid accumulation when differentiated into adipose cells. As a possible explanation behind the lean phenotype observed in Nrf1 deficient mice, we observed an increase in Metrn1 expression in the brown adipose tissue, as well as increased serum levels of Metrn1. Metrn1 has been shown to drive an increase in Ucp1 levels and could help explain the changes seen ⁽⁸⁹⁾. Taken together, we can see that modulation of either Nrf1 or Nrf2 can have profound effects on bioenergetics, and could possibly serve as exciting future targets for the treatment of obesity and its related disorders.

Nrf1 and Nrf2 both play important roles in the oxidative stress response by regulating the basal and inducible expression of a large battery of antioxidant genes. It is interesting to note that a deficiency in various Nrf1 and Nrf2 target genes leads to a similar decrease in adiposity and improved metabolic parameters observed in the Nrf1 and Nrf2 knockout models. Knockouts of Gclm and Gpx1, which are genes involved in antioxidant defense and glutathione synthesis, result in mice with decreased adipose tissue mass, and protection from high fat diet-induced obesity. In addition, these mice show increased energy expenditure, decreased serum insulin levels, and increased insulin sensitivity ⁽⁴²⁾. Conversely, it has been shown that transgenic mice overexpressing Gpx1 are

insulin resistant and obese ⁽⁴¹⁾. Another pathway of potential interest involves thioredoxins, which are cellular antioxidants that protects against oxidative stress by promoting the reduction of other proteins via cysteine thiol-disulfide exchange reaction. Thioredoxins are kept in the reduced state via thioredoxin reductase 1 (Txnrd1). Thioredoxin interacting protein (Txnip) on the other hand can increase ROS by inhibiting Txnrd1. It has been demonstrated that Nrf2 suppresses Txnip gene expression. Thus loss of Nrf2 results in increased Txnip expression, and a subsequent increase in oxidative stress ⁽⁹⁰⁾. Interestingly, Txnip knockout mice also demonstrate increased adiposity when exposed to high fat diet feeding ⁽⁹¹⁾. With many examples of antioxidant deficiency demonstrating similar lean phenotypes to the Nrf2 and Nrf1 knockout model, the data suggests that oxidative stress and the regulation there of can play an important role in the bioenergetics of the body. However, when discussing the regulation of oxidative stress as a potential treatment for obesity, care must be taken. Data suggests that the severity of oxidative stress plays a critical role in the effect that is observed. For example, oxidative stress has been shown to regulate expression of sirtuins, which are highly conserved NAD dependent deacetylases which are critical to modulating metabolic processes and expression of antioxidant genes ⁽⁹²⁾. Data shows that mild levels of oxidative stress result in increased expression of Sirt1,2 and 3, which results in increased expression of genes such as Pgc1a, responsible for driving both antioxidant gene expression as well as genes involved in mitochondrial biogenesis ⁽⁹³⁾. Our data, along with data such as this, suggests that low levels of oxidative stress activate a battery of protective and

antioxidant genes, such as Pgc1a and the Sirtuins, in order to combat these cellular stressors, but that activation of these genes also carries with it profound bioenergetics changes as well. Our findings suggest that the lean phenotype in Nrf2^{-/-} mice demonstrates a novel link between the oxidative stress caused by loss of Nrf2 and the upregulation of key genes such as Pgc1a and Ucp1 as a direct result of this oxidative stress. While the data that supports this is strong, Nrf2 affects the expression of many genes beside just those involved in oxidative stress and it is possible that multiple mechanisms are contributing to the ability of Nrf2 knockouts to attenuate diet induced weight gain and accumulation of body fat. Additionally our model represents a whole animal knockout of Nrf2. The possibility that Nrf2 deficiency in non-adipose tissue may contribute to the observed phenotype will require further experimentations using adipose tissue specific knockout models of Nrf2 deficiency. While substantial work was done looking into the role of oxidative stress in the Nrf2 knockout model, much less is known regarding the levels of oxidative stress in Nrf1 deficient cells and tissues. Due to the fact that Nrf1 regulates many of the same antioxidant genes as Nrf2, it is possible that oxidative stress may play a role in the Nrf1 knockout lean phenotype, however further experiments need to be conducted.

Key to the lean phenotype of both the Nrf1 and the Nrf2 knockout is the expression of UCP1. UCP1 uncouples the proton motive force generated in the mitochondria from ATP production to produce heat, and UCP1 has been shown to play an important role in energy homeostasis and obesity. Mice deficient in

UCP1 are shown to be susceptible to weight gain; while over expression of UCP1 provides protection against diet-induced obesity⁽⁷⁴⁻⁷⁵⁾. UCP2 and UCP3, which are homologs of UCP1, also have uncoupling function in the mitochondria, and serve as potential targets for increased metabolic capacity. UCP2 is ubiquitously expressed at low levels throughout the body, while UCP3 is limited to skeletal and cardiac muscle⁽⁵⁶⁻⁵⁷⁾. Mice overexpressing UCP3 in muscle are lean and have increased energy metabolism as well as decreased levels of serum glucose⁽⁹⁴⁾. Additionally, polymorphisms in the UCP2 gene have been associated with obesity and decreased metabolic rates in humans⁽⁹⁵⁻⁹⁷⁾. In this study, we found that Nrf2^{-/-} mice fed a high fat diet have an elevated metabolic rate in both whole animals and isolated white adipose tissue that is associated with an up-regulation of UCP1 in their abdominal white adipose depots. Similarly, the basal oxygen consumption rate in Nrf2 deficient cells is increased, and UCP1 expression is induced. However, up-regulation of UCP2 and UCP3 expression was not observed in Nrf2 deficient fat tissues (data not shown). Additionally, we observed decreased levels of UCP3 in muscle tissues of Nrf2^{-/-} mice (data not shown) that is consistent with data demonstrating direct regulation of UCP3 by Nrf2⁽⁹⁸⁾. Thus, UCP2 and UCP3 do not appear to provide additional mitochondrial uncoupling in the adipose tissue of Nrf2 knockout animals. These findings suggest that UCP1-dependent thermogenesis is a mechanism by which energy expenditure in Nrf2^{-/-} mice is up-regulated. Similar to the Nrf2 model, the fat specific Nrf1 KO mice exhibited increased UCP1 expression in the white

adipose tissue. Additional testing should be performed in order to determine if either UCP2 or UCP3 play any role in the Nrf1 FatKO phenotype.

There is a wide range of external stimuli including, cold exposure, diet, and exercise that can control UCP1 expression^(87,99). These factors are not likely to play a role here with either the Nrf2 or Nrf1 knockout animals, as mice in these studies were housed and maintained in a controlled environment. As shown previously, wild type and Nrf2^{-/-} mice showed similar food intake and physical activity. The same was seen in Nrf1 FatKO animals (Data not shown). In addition to these external stimuli, there are a number of genes responsible for controlling expression of UCP1. For example, RIP140 and LXR are both negative regulators of UCP1 expression⁽¹⁰⁰⁻¹⁰²⁾. However these genes were not altered in Nrf2^{-/-} adipose tissues (data not shown). Additionally it has been demonstrated that expression of Ucp1 is activated by oxidative stress⁽⁶⁴⁾. Consistent with this, we show that loss of Nrf2 results in increased levels of oxidative stress in both cells and tissues, and that deficiency of Nrf2 results in an increase in UCP1 expression. Additionally, glucose oxidase-induced oxidative stress in MEF cells up-regulates Ucp1 expression, and antioxidant treatment down-regulates Ucp1 expression in Nrf2^{-/-} MEF cells. Further experiments need to be conducted to see if Nrf1 knockout tissues and cells exhibit similar increases in oxidative stress. It is indeed possible that oxidative stress plays a role in the Nrf1 phenotype as well. Further exploring additional regulators of Ucp1 expression we found that Pgc1a expression is increased in WAT of Nrf2^{-/-} mice fed a high fat diet, as well

as in both WAT and BAT of Nrf1 knockout mice. It has been shown that oxidative stress increases the expression of Pgc1a, and Pgc1a interacts with other nuclear hormone receptors in white adipose cells to enhance mitochondrial biogenesis and the expression of Ucp1^(35,103-104). Hence, we surmise that acquisition of brown fat features by WAT depots in Nrf2^{-/-} mice is driven in part by oxidative stress induction of Pgc1a expression. We found that mice lacking Nrf1 specifically in adipose tissue also demonstrate increased expression of Pgc1a and Ucp1, however our data suggests that it is possibly through an alternative pathway to the one elucidated in the Nrf2 model. In Nrf1 FatKO animals we observed an increase in serum levels of a hormone known as Meteorin-like 1. Induced by Pgc1a, Metrnl1 is a hormone which is able to activate a wide variety of thermogenic genes in adipose tissue, including Ucp1. Groups have shown that Metrnl1 produced from the muscle in response to strenuous exercise is capable of activating a wide number of thermogenic genes within adipose tissue⁽⁸⁹⁾. Nrf1 FatKO animals also exhibited an increase in circulating Metrnl1, which in our model was associated with increased expression of Metrnl1 from the brown adipose tissue. It is not known at this time why the adipose tissue specific deficiency in Nrf1 leads to the increased expression of Metrnl1. We hypothesize that the increase in Pgc1a expression also observed in Nrf1 FatKO may be the driving force for the increased Metrnl1. Similar to the Nrf2 model, increased oxidative stress due to Nrf1 deficiency may be increasing Pgc1a expression, which may account for the changes seen. In order to test this, additional experiments must be performed to confirm oxidative stress in the Nrf1 deficient

adipose tissue. Similarly, it would be of interest to examine the levels of *Metrn1* in the *Nrf2* knockout model, due to the large role that oxidative stress plays in driving the *Pgc1a* expression. Taken as a whole, *Nrf1* and *Nrf2* deficiency result in remarkably similar white adipose tissue lean phenotypes which provide further evidence that regulation of oxidative stress could provide key insight into adipose tissue energetics. This “browning” of white adipose tissue is an area of great interest, and any possible pathways involved are of great interest in combating obesity.

Data obtained previously showed that *Nrf2*^{-/-} mice of C57/129 mixed genetic background are resistant to high fat diet-induced obesity⁽⁴⁷⁾. In agreement with this, our results also show that *Nrf2*^{-/-} mice derived on a C57/BL/6 background are resistant to diet-induced obesity. Here we demonstrated that *Nrf2*^{-/-} mice have increased energy expenditure and acquisition of brown-fat-like gene expression in their WAT depots. Additionally, we show that fat specific deficiency of *Nrf1* also results in increased expression of brown-fat-like genes in WAT depots and provides a similar protection against obesity. There is growing evidence that the acquisition of brown-like fat cells in WAT protects against both genetic and diet-induced obesity through increased metabolic activity, and this “browning” phenomenon has become a subject of considerable interest for possible treatment of obesity and metabolic syndrome⁽¹⁰⁵⁻¹⁰⁷⁾. Browning of WAT has been shown to occur in mouse models of prolactin receptor deficiency, IκB kinase epsilon deficiency, COXII overexpression, and

increased prostaglandin synthesis ⁽¹⁰⁸⁻¹¹¹⁾. In these instances, mice displayed a protection against diet-induced obesity, as well as increased oxygen consumption. Additionally, FGF21 has been shown to exert anti-obesogenic effects, and ameliorates insulin and leptin resistance in ob/ob mice ⁽¹¹²⁾. It has been shown that mice lacking Nrf2 demonstrate protection against diet-induced obesity, maintain insulin sensitivity, as well as demonstrate increased circulating levels of FGF21 and expression of FGF21 in liver and WAT ⁽⁴⁸⁾. However, no difference in FGF21 expression was found between wild type and Nrf2 deficient white adipose tissue or liver in our experiments (data not shown). The basis of this discrepant result is not known currently, however our high fat diet duration of 6 weeks was significantly shorter than the 180 days tested in the above study. We propose that the metabolic and gene expression changes observed in both our Nrf1 and Nrf2 -/- models are due in part to increased levels of oxidative stress, caused by a combination of decreased antioxidant capacity through the loss of Nrf1 or Nrf2 function, as well as the additional oxidative stress provided through exposure to high fat diet. Currently, we cannot rule out the possibility that increased UCP1 expression in Nrf1 and Nrf2-/- mice is through modulation of alternative downstream target genes, independent of oxidative stress effects. Previous data has shown that deficiency of Nrf2 results in down-regulation of PPAR γ ⁽⁴⁷⁾. PPAR γ knockout mice demonstrate decreased adipose tissue mass, protection against diet-induced obesity, and increased expression of UCP1 in white adipose tissue ⁽¹¹³⁻¹¹⁴⁾. It is thought that decreased PPAR γ activity drives increased expression of prostaglandin E synthase, which in concert with COXII,

drive the formation of prostaglandin, which is hypothesized to be important in the formation of brown adipose tissue within white adipose tissue depots ⁽¹¹³⁾. It is likely that the phenotype observed in Nrf1 and Nrf2^{-/-} mice is due to a complex interplay of a variety of pathways. Further adding to the complicated nature of the Nrf1 and Nrf2 knockout phenotypes, is the disparate effects Nrf1 deficiency appears to have on white adipose tissue compared with brown adipose tissue. Deficiency in Nrf1 and Nrf2 both result in decreased white adipose tissue mass and an increase in brown adipose tissue thermogenic gene expression within the white adipose tissue. Additionally, deficiency of Nrf1 and Nrf2 appears to have a much more muted effect on the expression of thermogenic genes in the brown adipose tissue. However deficiency of Nrf2 results in decreased lipid accumulation in brown adipose tissue, but conversely our results showed that Nrf1 deficiency in brown adipose tissue leads to increased lipid accumulation. Presently we cannot explain this brown adipose tissue phenotype. While there are extensive publications on the “browning” of white adipose tissue, few papers have observed the “whitening” of brown adipose tissue. Recently it was found that capillary rarefaction through adipose tissue specific knockout of Vegf-a results in brown adipose tissue adopting many of the physical characteristics of white adipose tissue ⁽¹¹⁵⁾ While the physical appearance of the Nrf1 FatKO brown adipose tissue appears similar to the Vegf-a knockout brown adipose tissue, the gene expression profiles are very different. Vegf-a knockouts exhibit decreased levels of thermogenic genes such as Ucp1 and Pgc1a, while the Nrf1 FatKO does not show this. Taken as a whole, it is likely that both the Nrf1 and Nrf2

adipose tissue phenotypes are due to complex interactions between many pathways and oxidative stress may simply be one of those pathways.

Further supporting the idea of this complex interplay of pathways, recent studies have shown that over expression of Nrf2 through the use of graded knockdown of Keap1 actually provides protection against the onset of diabetes in genetic models of diabetes in mice ⁽¹¹⁶⁾. This suggests a beneficial role for Nrf2 in the protection against the development of diabetes with increased expression of Nrf2 resulting in decreased blood glucose and protection against obesity in a db/db mouse background. However it is worth noting that in addition to the protective effects of Nrf2, the same group also found that mice lacking Nrf2 demonstrate decreased serum glucose levels compared to control mice, which correlates with data from our model of Nrf2 deficiency. In mice doubly deficient in Keap1 and leptin, there was a resistance to high fat diet induced obesity and a decrease in adipose tissue weight that was also associated with impaired insulin signaling, hyperglycemia, and glucose intolerance ⁽¹¹⁷⁾. Additional studies utilizing the ob/ob genetic model of obesity have yielded varied results. Nrf2 knockout animals crossed into the ob/ob mouse background, using either a whole animal, or fat specific Nrf2 knockout demonstrated that loss of Nrf2 resulted in decreased adipose tissue mass, yet aggravated insulin resistance, hyperglycemia, and hypertriglyceridemia ⁽¹¹⁸⁾. This seems to demonstrate that in genetic models of obesity and diabetes, the results of Nrf2 modulation can vary drastically depending on the specific conditions of the model. In order to most closely

compare results with our own, it is important to look at systems similar ours, in which the experiments are performed looking at strictly diet-induced models of obesity, rather than genetic models of obesity and diabetes. For example, Keap1 knockdown mice in the C57BL/6 background without crossing to any genetic models of obesity or diabetes, gained significantly more weight than wild type animals as well as developed glucose intolerance, increased inflammation, and hepatic steatosis when placed on a high fat diet ⁽¹¹⁹⁾. As mentioned previously, other groups have also shown that Nrf2 knockout mice were found to be resistant to long-term high fat diet induced obesity ⁽⁴⁸⁾. These results coincide with our data and seem to suggest increased Nrf2 levels lead to increased obesity, and that Nrf2 deficiency may provide protective benefits in a diet induced obesity model. These varied results in the literature suggest a complex interaction between Nrf2, obesity and diabetes, and further research will likely provide insight.

In summary, Nrf2 deficient mice are protected from diet-induced obesity and exhibit increased energy expenditure associated with the up-regulation of brown adipose tissue specific genes in white adipose depots. Additionally, adipose tissue specific deficiency of Nrf1 also results in a lean phenotype and upregulation of thermogenic genes in the white adipose tissue. Cell based experiments demonstrated loss of Nrf2 resulted in increased UCP1 expression, and that this increase in Ucp1 expression is linked with an increase in oxidative stress. In the Nrf1 knockout model, the increase in Ucp1 expression is associated with an increase in Metrnl1 expression. These observations suggest a

promising model for increasing energy expenditure in white adipose tissue through both Nrf1 and Nrf2, and the possibility of pharmacological modulation of Nrf1 or Nrf2, or regulation of oxidative stress as treatments for obesity and its related disorders.

CHAPTER 7

CONCLUSIONS AND SIGNIFICANCE

Members of the CNC-bZIP family of transcription factors including Nrf1 and Nrf2 are known to be important in the regulation of ARE-driven genes involved in the protection against oxidative stress. This report presents data on the involvement of Nrf2 and oxidative stress on the upregulation of UCP1 as a contributing factor to the protection against diet induced obesity observed in Nrf2-/- animals. Additionally we present data on the involvement of Nrf1 and upregulation of UCP1 through increased expression of Pgc1a and Metrnl1. These findings provide insight into possible therapeutic strategies targeting both the Nrf1 and Nrf2 pathway in order to combat obesity.

Excessive oxidative stress has long been linked with certain negative aspects of metabolic syndrome such as insulin resistance and diabetes.⁽³⁵⁾ However, antioxidant treatments have met with only marginal success in combating obesity⁽¹²⁰⁾. Our data along with others demonstrates that disruption of antioxidant genes, and mild increases in oxidative stress can actually confer a protection against obesity. Here, we have demonstrated two additional examples of disruption of antioxidant genes that results in lean phenotypes. We have found that Nrf1 and Nrf2 deficient white adipose tissue gain increased thermogenic capacity and this provides yet another exciting avenue for study against obesity. Pharmacological modulation of both Nrf1 and Nrf2 could serve as powerful methods for controlling obesity, and warrant further research.

CHAPTER 8

SUGGESTIONS FOR FUTURE RESEARCH

- *To further explore the mechanism of Nrf2/Oxidative Stress/UCP1 mediated protection against diet-induced obesity.* Knockout of Nrf2 results in protection against diet induced obesity in our mouse model, however the field is hotly contested. We have suggested that upregulation of UCP1 due to increased oxidative stress is one of the driving factors behind the lean phenotype. In order to completely prove that UCP1 is critical to the Nrf2^{-/-} lean phenotype, animals lacking UCP1 should be crossed to Nrf2^{-/-} animals and exposed to a high fat diet. We hypothesize that loss of UCP1 in the Nrf2^{-/-} animal would result in a loss of the lean phenotype, and accumulation of lipids similar to wild type animals. This is supported by groups which have studied UCP1 knockout animals and shown them to be susceptible to diet induced obesity.
- *Examine the effects of oxidant and antioxidant treatment on wild type and Nrf2^{-/-} mice.* We saw similar phenotypes of oxidative stress, oxygen consumption, and UCP1 expression between wild type and Nrf2^{-/-} mice, tissue, and cells. However treatment with oxidants and antioxidants were limited to cells. Expanding the treatment experiments to wild type and Nrf2^{-/-} animals could generate additional data. It would be beneficial to see if increasing levels of oxidative stress in adipose tissue could help wild type animals to resist obesity. Conversely, if antioxidant treatment abolished the lean phenotype of Nrf2^{-/-} animals our data would be further

strengthened. Exposure of Nrf2 ^{-/-} mice to antioxidants such as BHA or NAC could further solidify the idea of oxidative stress as the driving factor in the lean phenotype.

- *Explore if loss of Nrf2 specifically in adipose tissue is sufficient to cause the lean phenotype.* We examined the effects of loss of Nrf2 in a whole mouse model, and focused specifically on the effects of the loss on the white adipose tissue. However, it cannot be ignored that systemic loss of Nrf2 could, and most likely does, cause dramatic changes in a wide variety of tissues. If adipose tissue specific knockout models exhibited similar phenotype to the whole mouse knockout model, it would suggest alternative causes for the phenotype, aside from systemic increase in oxidative stress.
- *Explore the Nrf1 deficient brown adipose tissue phenotype.* The phenotype observed in the brown adipose tissue of Nrf1 FatKO mice is strikingly different from that of Nrf2 knockout mice, and lends itself to a unique role of Nrf1 in brown adipose tissue structure and function. This “whitening” of brown adipose tissue is seen in rare other cases, and should be further explored.
- *Examine levels of oxidative stress in Nrf1 FatKO mice and cells.* Due to the overlapping control of antioxidant genes by Nrf1 and Nrf2, it would be beneficial to test for levels of increased oxidative stress in Nrf1 deficient cells and tissues. If stress is present, it offers a possible explanation for

the increased expression of brown adipose tissue genes in the Nrf1 FatKO white adipose tissue, which would be similar to the results seen in the Nrf2^{-/-} model.

- *Examine the role of proteasome dysfunction in the Nrf1 FatKO adipose tissue phenotype.* Previous work in our lab has shown significant proteasomal defects in Nrf1 deficient cells and tissues. In keeping with this, we also identified decreased expression of key proteasomal genes in the adipose tissue of Nrf1 FatKO animals. The role of the proteasome in adipose tissue warrants further study.
- *Further explore the role of Nrf1 deficiency on Metrn1 and Pgc1a expression.* Nrf1 FatKO animals exhibit increased levels of Pgc1a, which is shown to drive expression of Metrn1, which in turn can increase expression of Ucp1. While all of the above have been observed in the Nrf1 KO animals and cells, we do not know why the loss of Nrf1 specially causes these changes. Experiments have shown that Nrf1 does not appear to directly regulate Metern1 gene expression, however further work should be done to see if Nrf1 could possibly directly regulate Pgc1a levels.

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