

Control of Uncoupling Protein-1 (UCP1) by Phosphorylation and the Metabolic Impact of
Ectopic UCP1 Expression in Skeletal Muscle of Mice

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ABSTRACT:

UCP1 is a member of the mitochondrial transmembrane anion carrier protein superfamily and is required to mediate adaptive thermogenesis in brown adipose tissue (BAT). Once activated, UCP1 uncouples mitochondrial respiration from ATP synthesis, thereby wasting the protonmotive force formed across the mitochondrial inner membrane as heat. It is hypothesized that proton leaks through UCP1 could be a molecular target to combat certain forms of obesity. Although it is well established that UCP1 is regulated by allosteric mechanisms, alternative methods such as post-translational modification still remain to be explored. The aims of the present study were to confirm the phosphorylation of UCP1 and the physiological relevance of this modification. Using isoelectric focusing, we confirmed that UCP1 displayed acidic shifts consistent with phosphorylation in BAT mitochondria isolated from cold exposed versus warm acclimated mice. A mouse model that ectopically expressed UCP1 in skeletal muscle was used to explore the link between the mitochondrial redox status and UCP1 function. Our results show that the expression of UCP1 in skeletal muscle led to decreases in body and tissues weights. In contrast, glucose uptake into skeletal muscle, food intake and energy expenditure was increased with the expression of UCP1. Finally, proton leaks through UCP1 were determined to be increased in isolated mitochondria from transgenic versus wild-type mice. Taken together these results indicate a complex interplay between mitochondrial redox status, post-translational modification and UCP1 function. Elucidation of novel mechanisms regulating UCP1 offers alternatives strategies that can be explored in order to modulate BAT thermogenesis.

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LIST OF ABBREVIATIONS

A

ADP-adenosine diphosphate
ATF2- activating transcription factor 2
AMPK- AMP activated protein kinase
ANOVA- analysis of variance
ANT- adenine nucleotide translocase
ANT A- antimycin A
ATP-adenosine triphosphate

B

β -oxidation- Beta oxidation
BAT- brown adipose tissue
BMI- body mass index
BSA- bovine serum albumin
 β_3 -AR- beta-3- adrenergic receptor
cAMP- cyclic adenosine monophosphate
BMR- basal metabolic rate

C

$^{\circ}\text{C}$ - degrees Celsius
CE- cold exposed
cDNA- complementary deoxyribonucleic acid
Complex I- NADH-coenzyme Q reductase or NADH dehydrogenase
Complex II- succinate:quinone oxidoreductase or succinate dehydrogenase
Complex III- cytochrome *c* oxidoreductase or cytochrome *bc1* complex
Complex IV- cytochrome *c* oxidase
Complex V- F_0F_1 ATP synthase
CRE- cAMP response element
CREB- cAMP response element-binding protein

D

ddH₂O- deionized distilled water
DNA- deoxyribonucleic acid
DIT- diet induced thermogenesis

E

E-epinephrine
EE- energy expenditure
EDTA- ethylenediamine tetraacetic acid

EGTA- ethelenglycol tetraacetic acid
i.e.- id est (that is)
EI- energy intake
ETC- electron transport chain
ETF- electron-transfer flavoprotein
ETF-QO- electron-transfer flavoprotein coenzyme Q oxidoreductase

F

FADH/FADH₂- flavin adenine dinucleotide
FA- fatty acids
(¹⁸F-FDG)- ¹⁸F-fluorodeoxyglucose
(¹⁸F-THA)- ¹⁸F-fluoro-6-thia-heptadecanoic acid (FTHA)
FLD- fatty liver disease
FCCP-carbonylcyanide-p-trifluoromethoxyphenylhydrazone

G

µg- microgram
g- gram
ΔG-Gibbs energy change
GDP- guanosine diphosphate
GLUT- glucose transporter
GSH- reduced glutathione
GSSG- oxidized glutathione
GSIS- glucose stimulated insulin secretion
GTP- guanosine triphosphate
gWAT- gonadal white adipose tissue

H

HEPES- hydroxyethyl piperazine ethane sulphonic acid
4-HNE- 4-hydroxy-2-nonenal
HM- homogenization media
h- hour

I

IL-1- interleukin 1
IL-6-interleukin 6
IL-10- interleukin 10
IRS- insulin receptor substrate

K

K₂HPO₄- dipotassium phosphate

KCl-potassium chloride
kDA- kilodalton
KH₂PO₄- potassium dihydrogen phosphate

L

L- litre
LPL- lipoprotein lipase

M

μM- micromole/litre
mTFA- mitochondrial transcription factor
M- moles/litre
mg- milligrams
MgCl₂- magnesium chloride
MIM- mitochondrial inner membrane
min- minute
ml- millilitre
mM- milimoles/litre
Mom- mitochondrial outer membrane
mRNA- messenger ribonucleic acid

N

NAD⁺/ NADH- nicotinamide adenine dinucleotide
NADP⁺/NADPH- nicotinamide adenine dinucleotide phosphate
NE- norepinephrine
NEFA- non-esterified fatty acids
NRF- nuclear respiratory factor

O

Ob/Ob- obese mouse model (Leptin deficient)
OLA-oleic acid
O₂⁻- superoxide
O₂- molecular (diatomic) oxygen

P

ΔpH- pH gradient
ΔP- mitochondrial protonmotive force
P_i- inorganic phosphate
PAGE- polyacrylamide gel electrophoresis
PET- positron emission tomography

PGC-1 α - peroxisome proliferator-activated receptor- γ coactivator alpha
pI- isoelectric point
PI3K-phosphatidylinositol-3 kinase
PKA- protein kinase A
PKB- protein Kinase B
PMF- protonmotive force
PPAR- γ - peroxisome proliferator-activated receptor gamma
PRDM16- PR domain zinc finger protein 16
PPRE- peroxisome proliferator response element
PTM- post-translational modification

R

RA- retinoic acid
RER- respiratory exchange ratio
RNS- reactive nitrogen species
ROS- reactive oxygen species
RXR- retinoic acid receptor sites

S

SDS- sodium dodecyl sulphate
SEM- standard error of the mean
SIRT3- silent mating type information regulation 2 (S.cerevisiae, homolog) 3
SOD- superoxide dismutase
State 3- ADP stimulated respiration
State 4- basal respiration (absence of ADP)
State 4_o- oligomycin induced respiration (ATP turnover)
SUV- specific uptake values
SVF- stromal vascular fraction

T

TCA- tricarboxylic acid
TG- triacylglycerol or triacylglyceride
TNF α - tumor necrosis factor alpha
TRE- thyroid response element
TRL-triglyceride rich lipoprotein
T2DM- type 2 diabetes mellitus

U

UCP- uncoupling protein
UCP1-KO- uncoupling protein 1 knockout mice
UCP1-TG- uncoupling protein 1 transgenic

V

v/v- volume per volume

V-volts

W

WAT- White adipose tissue

w/v- weight per volume

X

xg - centrifugal force

W

WA-warm acclimation

WAT- white adipose tissue

Symbols

~ - approximately

$\Delta\Psi_m$ - mitochondrial membrane potential

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GENERAL INTRODUCTION:

Obesity:

The etiology of obesity is complex and obesity currently ranks highly amongst diseases that are known to cause major health problems. Currently, 34% of adults in the United States are considered obese which has made obesity a major contributor to disease burden in the United States (22, 107, 108). In addition, between 2007-2009 it was estimated that approximately 24.1% of Canadians are obese (143). Obesity is commonly assessed by measuring an individual's body mass index (BMI) which is calculated by dividing mass measured in kilograms by height measured in meters squared (kg/m^2). Furthermore, obesity is characterized by an excessive accumulation of visceral and subcutaneous adipose tissue as well as accumulation of triacylglyceride (TG) in liver, muscle and heart tissue (75, 178, 213, 214). This buildup can lead to impairments of cellular and whole body function. The etiology of obesity is complex; a myriad of genetic, nutritional, and environmental factors coalesce to affect adiposity (85, 213). However, at a fundamental level obesity can be described as an imbalance between energy intake and energy expenditure (**Figure 1.1**) (100). Specifically, in terms of energy imbalance obese persons have a positive energy balance, which is defined as energy intake (EI) exceeding energy expenditure (EE) (223, 232). Over time this prolonged state of energy imbalance leads to the storage of excess calories as adipose tissue triglycerides. The excessive accumulation of adipose tissue as well as accumulation of intramyocellular and intrahepatic lipids is associated with several comorbidities such as cardiovascular diseases, colon cancer, type 2 diabetes mellitus (T2DM), hypertension, chronic inflammation and osteoarthritis (22, 120, 142, 219).

Currently, non-pharmacological treatments for obesity include hypocaloric diets, increased physical activity and bariatric surgery, which to date seem to be the most effective interventions. However, considerable variability exists in the response to physical activity and changes in diet (95). These differences in response have prompted the development of a limited repertoire of pharmacological agents to ameliorate obesity. Pharmacological treatments available involve appetite suppression (Belviq and Qsymia), or reduced fat absorption (Orlistat) (43, 165). However, both pharmacological and non-pharmacological treatments are limited by program adherence, adverse health effects and long-term success rates (22, 165). Hence, other molecular targets are drawing interest in combination with the current therapies to prevent and treat obesity. As described in detail below, the recent identification of brown adipose tissue (BAT) in adult humans (51, 227, 230, 236) has stimulated interest in the future development of pharmacologic agents that could activate BAT to treat some forms of obesity.

Adipose Tissue:

Adipose tissue is subdivided into two types: white adipose tissue (WAT) and BAT. Briefly, WAT functions as both a storage depot for fat and as an endocrine tissue. On the other hand, when adrenergically activated, BAT releases large amounts of energy as heat by oxidizing fatty acids. Structurally, mature white adipocytes are single nucleated cells consisting of a large lipid droplet that makes up a significant proportion of the cell size (98). In addition, WAT contains several other cell types such as preadipocytes (progenitors), pericytes, mast cells, fibroblasts, endothelial cells, and adipose tissue-derived macrophages; collectively these cells are referred to as the stromal vascular fraction (SVF) (81). Functionally, WAT is thought to participate in two major physiological roles, lipid storage and release of endocrine factors. Adipocytes store lipids

in the form of TG in circumstances of positive energy balance by the process of lipogenesis and TG biosynthesis (192). In contrast, in circumstances of energy deficiency TG stored within WAT are mobilized via lipolysis. Liberated fatty acids (FA) are then transported to the peripheral tissues such as liver, skeletal muscle, and heart for β -oxidation and the generation of ATP by mitochondria. (38, 192).

TG biosynthesis and degradation for energy storage and release respectively, are tightly regulated processes and their disruption has been implicated in the onset of obesity, T2DM and fatty liver disease (FLD) (117, 144, 191). For instance, disruption in the capacity to properly store fats in WAT can lead to an accumulation of non-esterified fatty acids (NEFA), diacylglycerol, ceramides and acyl-CoA in muscle, which in turn activates several kinases such as protein kinase C, c-Jun N-terminal kinases, and I κ B kinase to promote the hyper-phosphorylation on serine and threonine residues of insulin receptor substrates (IRS) and the disruption of insulin signaling and glucose uptake (58, 188). In normally functioning skeletal muscle cells insulin induces the autophosphorylation of tyrosine residues on insulin receptor activating a cascade of specific phosphorylating events involving insulin receptor substrate-1 (IRS1), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (PKB) (77). PKB subsequently induces the recruitment of glucose transporters (Glut) to the plasma membrane that facilitate the diffusion of glucose into the cell. However, accumulation of intramyocellular lipids as described above can disrupt this process leading to improper glucose transport via a blunting of glucose transporter 4 (GLUT4) activity (58, 162, 172, 188). Consequently, hyperglycemia ensues leading to insulin resistance and over time, this results in some of the pathologies of T2DM (172). Additionally, increased lipid species accumulation from the disruption of WAT metabolism leads

to improper IRS signaling in liver, as described above, and over time can lead to lipid accumulation, FLD and insulin resistance (196).

In addition to its TG storage function, WAT also fulfills various endocrine functions. Specifically, WAT in a healthy individual is responsible for a diverse array of processes including blood pressure control, bone metabolism, inflammatory response, and glucose homeostasis (38, 98, 117, 191, 192). These processes are modulated by a specific set of peptide hormones and cytokines secreted by WAT called adipokines. The disruption in secretion of hormones and adipokines from WAT has also been implicated in the development of obesity and T2DM (191, 192). Peptide hormones and adipokines secreted by WAT include leptin, adiponectin, tumor necrosis factor alpha (TNF α), interleukins 1 and 6 (IL-1 and IL-6), amongst others (98, 117, 191, 192). Leptin was the second hormone found to be released by WAT in 1994 following the discovery of TNF α a year earlier (76, 90, 170). Leptin is secreted by WAT and functions to maintain body weight homeostasis through the suppression of energy intake and promotion of energy expenditure (76, 90, 98, 170, 192). Moreover, mutations in the leptin gene or its receptors located in abundance in the hypothalamus lead to obesity and hyperphagia (98, 191, 225).

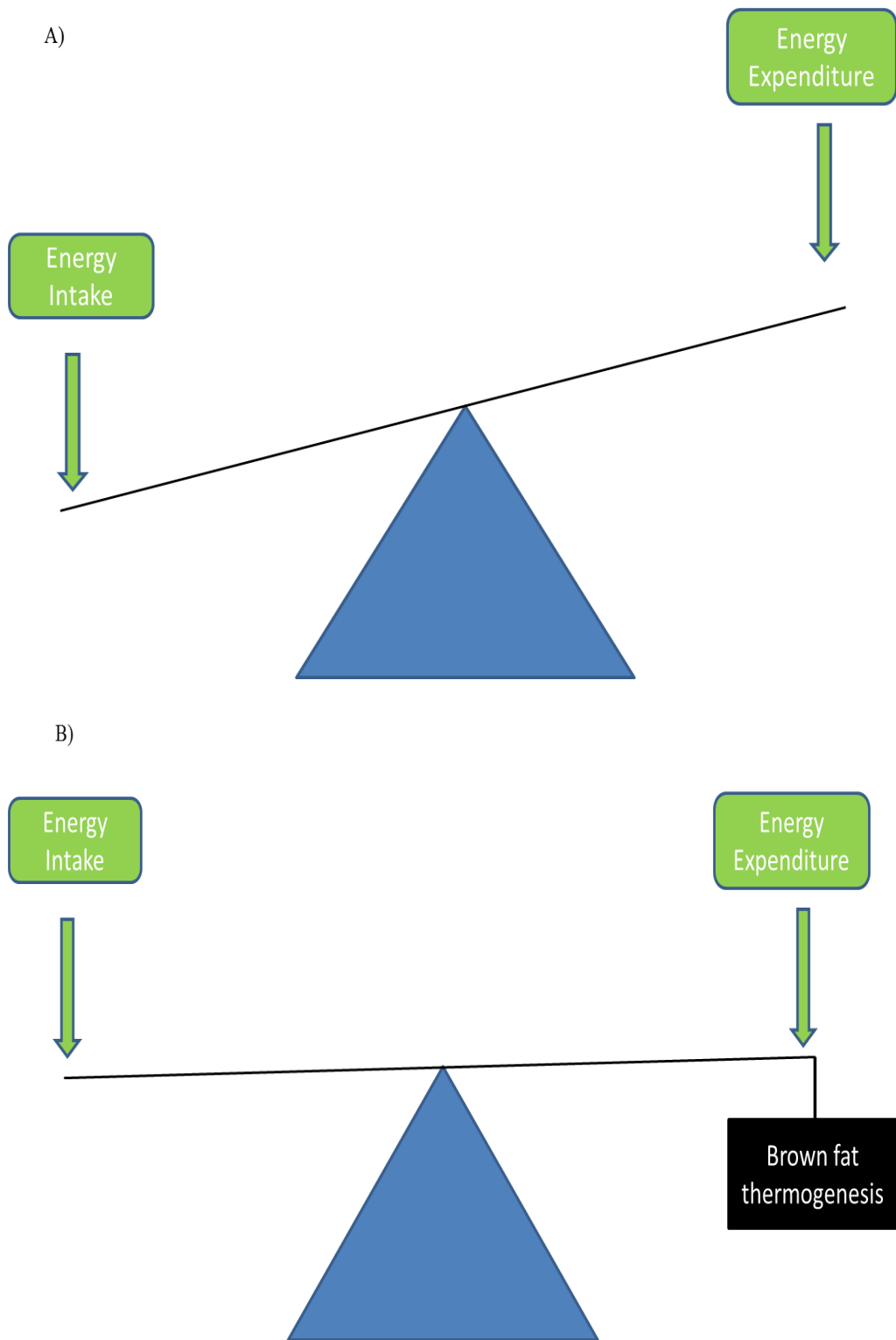
Consequently, leptin injections in ob/ob mice (a leptin deficient mouse model of obesity) reverses hyperglycemia and hyperphagia (54, 76, 90, 225). It also has been shown that leptin improves glycemia and insulin sensitivity in muscle through activation of AMP-activated protein kinase (AMPK), which in turns helps dissipate intramyocellular lipid content (146). Although leptin administration reduces caloric intake and promotes energy expenditure it is of note that in obese individuals circulating leptin levels remain high (44, 99). In addition WAT secretes

adiponectin, an adipokine that also has an impact on body weight regulation (228). Adiponectin expression is inversely correlated to body weight, while its expression is associated with protection against insulin resistance and an overt inflammatory response (98, 171, 192).

In contrast, impaired WAT metabolism and deregulated hormone release have been linked with increased inflammation and the development of obesity. In fact, certain types of obesity are being described as a result of chronic low grade inflammation (23, 24, 60). In certain cases of obesity, inflammation is promoted by improper WAT function and a disruption in the secretion of IL-6 and IL-10; this results in macrophage infiltration (203). Furthermore, high levels of TNF α , IL-6 and IL-10 are correlated with comorbidities of obesity such as insulin resistance and hyperglycemia (203). Over time, increased adipokine secretion promotes macrophage and immune cell infiltration in WAT (12, 132, 203). The precise mechanisms leading to macrophage infiltration and inflammation are still not clear. However, it is believed that bone marrow derived macrophages are recruited to WAT in obese individuals by certain conditions such as hypoxia, adipocyte cell death, and adipocyte hypertrophy (203). Mechanistically, monocytes from the endothelium are recruited in the above-mentioned conditions, leading to the differentiation into macrophages. Once macrophages are present in WAT they begin to form crown-like structures that surround white adipocytes (23, 24). These structures lead to the recruitment of more macrophages, and the secretion of more pro-inflammatory cytokines resulting in insulin resistance (23). Macrophages can be classified as M1 or M2 derived, and a shift from M2 to M1 is thought to initiate the inflammatory response by aiding in the production of IL-6 and TNF α as well as further recruiting other immune cells to WAT (118, 203). Hence, WAT metabolism and regulation are important targets in combating obesity and its comorbidities.

Figure 1.1: Factors that modulate whole body energy balance: Energy balance is defined by two parameters, energy intake and energy expenditure. Energy intake (EI) is defined by the energy acquired through the diet. In contrast, energy expenditure (EE) is defined as energy used to support ATP demanding process in cells throughout the body. An imbalance which results in EI exceeding EE leads to a state of positive energy balance. The excess calories are stored as fat which over time may lead to the development of obesity. **B)** In order to combat obesity it has been postulated that the activation of BAT, which increases EE, could be useful.

Figure 1.1: Factors that modulate whole body energy balance:



Brown Adipose Tissue (BAT):

BAT is a form of adipose tissue that is highly specialized for the maintenance of body temperature in cold environments, *i.e.*, thermoregulation (102, 156). This is achieved through a unique form of adaptive thermogenesis that is attributed to the highly selective expression of uncoupling protein-1 (UCP1) in this tissue(183). UCP1 is a mitochondrial inner membrane protein that, when activated, allows exceptionally high rates of mitochondrial respiration in the absence of ATP production. The heat emanates from all of the oxidative reactions that fuel mitochondrial respiration (25, 101, 154) (Note that the control of UCP1 and other forms of proton leak are discussed in detail below). Thus, due to the presence of UCP1, brown fat mitochondria are used mainly for heat production rather than oxidative phosphorylation (26). In fact, UCP1 knockout mice (UCP1 KO) are unable to thermoregulate when challenged with cold exposure leading to cold intolerance (62).

Functionally, WAT and BAT share several similarities such as TG synthesis, the release of unesterified fatty acids through lipolysis, and the selective expression of certain adrenergic receptors (*i.e.*, β_3 adrenergic receptors) (131). However, brown adipocytes are smaller than white adipocytes, contain multilocular lipid droplets, are highly vascularized and are highly dense with mitochondria (125, 131). Also, due to the presence of UCP1 and relatively low levels of ATP synthase in BAT, brown fat mitochondria produce very little ATP. Instead, once activated, UCP1 uncouples mitochondrial respiration from ATP production to produce heat. This heat is then quickly dispersed into circulation via the arteriovenous anastomoses. ATP production in BAT is reliant on the glycolytic conversion

of glucose to lactate to satisfy ATP demands (25). Structurally, BAT is composed of other cell types beyond mature adipocytes such as precursor cells, preadipocytes, endothelial cells and those of the SVF (25). In addition, progenitors and preadipocytes can be adrenergically stimulated to proliferate and differentiate into mature adipocytes with the use of norepinephrine *in vivo* and *in vitro* (25). As mentioned above, WAT and BAT share several similarities; however it is now well accepted that WAT and BAT originate from distinct cellular lineages (6, 201, 220). In fact, evidence shows that brown adipose precursor cells contain engrailed-1 and Myf5, two transcription factors that are normally associated with muscle cells. This indicates that brown adipose originates from the dermamyotome of the mesoderm layer (6, 61, 201, 220). Indeed, Seale and colleagues showed that the loss of a transcriptional factor called PR domain zinc finger protein 16 (PRDM16) from BAT precursor cells was sufficient to induce the loss of BAT characteristics and promote muscle differentiation (201). The mechanism by which PRDM16 promotes adipogenesis is through the binding of the peroxisome-proliferator-activated receptor- γ (PPAR- γ). This interaction commits precursor cells to the BAT lineage through promotion of adipogenesis and the induction of expression of thermogenic proteins (201). It is important to note though that WAT progenitor cells can also be induced to adopt brown fat characteristics (234). This process is called “browning of white fat” and is characterized by an increase in WAT mitochondrial content and the expression of UCP1. White fat “browning” can be achieved using a β_3 adrenergic selective agonist such as CL316243 compound or through the overexpression of PGC-1 α , a transcriptional coregulator required for UCP1 gene expression (201). Although, these newly generated cells show BAT characteristics, they do not present all the molecular markers of traditional BAT precursor cells, further showing the distinction between WAT and BAT (173, 174, 208). Hence, BAT and muscle precursor cells share common

lineages that are distinct from WAT cells. However, through selective stimulation of WAT progenitor cells it is possible to generate thermogenic “beige” cells.

BAT originally was thought to exist only in human neonates and small mammals. However, with the unequivocal identification of BAT in adult humans (51, 227, 230, 236) there has been a renaissance in BAT research. In adult humans BAT was discovered using ^{18}F -fluorodeoxy glucose (^{18}F -FDG) positron emission tomography (PET) and the immunohistochemistry of biopsied tissue that exhibited high rates of FDG uptake (51, 195, 227, 230, 236). It was previously noted during radiographic scans for metastatic cancer that when patients were cold during these radiographic scans, often clinicians would note FDG uptake into areas in the supra-clavicular region (96). Since BAT uses its mitochondria predominantly for heat production, glycolytic ATP production is needed to satisfy tissue energy needs and thus glucose uptake rates are high when the tissue is active.

The renewed interest in BAT research was catalyzed by three seminal papers published simultaneously in the New England Journal of Medicine. Briefly, Van Marken Lichtenbelt and colleagues tested 10 lean ($\text{BMI} < 25$) and 14 overweight ($\text{BMI} > 25$) adult males for the presence of BAT. BAT activity was measured under room temperature (22°C) and cooler conditions (16°C). The main results in this study show that lean individuals have more BAT activity than overweight individuals when cold exposed.

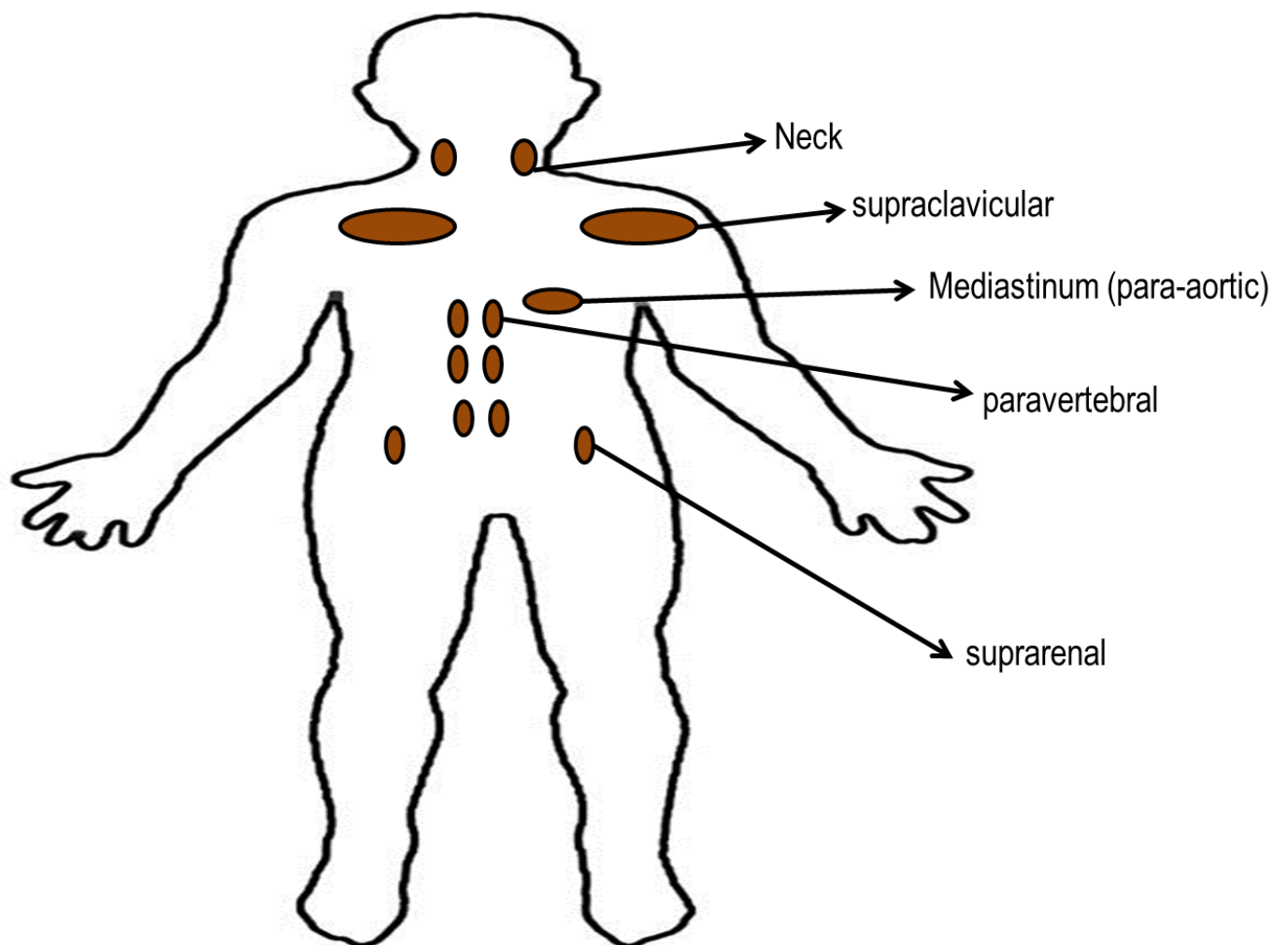
Moreover, BAT activity was inversely correlated with BMI, while being positively correlated with energy expenditure (227). Additionally, Cypess and colleagues corroborated these findings in a separate study and extended the results by showing an increased prevalence of BAT in

women vs. men and that presence the BAT was inversely correlated with age (51). Finally, Virtanen *et al*, (230) confirmed the presence of BAT in adult humans by showing increased mRNA expression of UCP1, deiodinase, PGC1- α , PRDM16, and β_3 -adrenergic receptors in supraclavicular biopsies compared to WAT. Furthermore, Western blot and immunohistochemical analysis of supraclavicular biopsies confirmed UCP1 expression, while morphological analysis displayed multilocular lipid droplets (51, 227, 230).

Recently, oxidative metabolism in BAT was dynamically quantified in lean individuals that were cold exposed by the usage of radio tracers such as ^{18}F -FDG, ^{11}C -acetate, and a fatty acid tracer ^{18}F -fluoro-thiaheptadecanoic acid (^{18}F -THA). The results indicated that uptake of NEFA and glucose were used to support non-shivering adaptive thermogenesis; uptakes in skeletal muscle and subcutaneous adipose tissue were not significant (164). Collectively, the data acquired from the recent publications indicate that the distribution of BAT in adult humans range from the neck, supraclavicular region, mediastinum (para-aortic), paravertebral to the suprarenal region (**Figure 1.2**). It is important to note that the presence of BAT in adult humans seems to vary significantly on an individual basis (154). Possible explanations for this may include an atrophying of the tissue due to lack of exposure to cold temperatures, the insulative effect of WAT in obese individuals, lack of physical activity and general genetic variations. However, activated BAT in humans can contribute up to 5% in basal metabolic rate (231).

Figure 1.2: The locations of brown adipose tissue in adult humans. Adapted from Cannon *et al.* BAT depots were identified by PET-CT (Positron Emission Tomography-Computational Tomography). Sites were identified using [^{18}F] fluoro-2-deoxyglucose (^{18}F FDG). Based on previous anatomical knowledge of BAT expression in rodents and recently generated images in adult humans, BAT depots were shown to be located around the neck, supraclavicular, mediastinum (para-aortic), paravertebral and suprarenal (51, 154, 227, 230) .

Figure 1.2: The locations of brown adipose tissue in adult humans



The excitement around the confirmation of BAT in adult humans is based on the extensive research in rodents that has demonstrated that the activation of BAT leads to a resistance to diet induced obesity, increased lipid and glucose clearance, increased insulin sensitivity and increased whole body energy expenditure (26, 27, 101, 145, 156). In fact, adrenergic stimulation of BAT by cold exposure of mice leads to the up regulation of lipoprotein lipase (LPL) expression, which aids in the release of fatty acid from circulating triglyceride-rich lipoprotein (TRL) (11, 25). Consequently, the liberated fatty acids are then taken up into BAT from circulation for β -oxidation and the generation of heat. Additionally, BAT can also be very effective at glucose clearance from circulation since adrenergic (e.g., β 3-agonists) or other agonists (e.g., insulin) promote glucose uptake and metabolism (45, 210, 211). The adrenergic stimulation of glucose uptake is proposed to involve various members of the glucose transporter protein family (GLUTs) (210, 211). Insulin stimulated glucose uptake in BAT is controlled specifically by GLUT 4 activity (68). Thus, BAT activation leads to the enhanced uptake of fatty acids and glucose since the former is used for thermogenesis and the latter for glycolytic ATP production.

Mice either exposed to cold or ectopically expressing UCP1 in skeletal muscle display significant increases in whole body EE compared to warm acclimated or wild type control mice, respectively (49, 68, 123). Consequent to the increase in UCP1 mediated proton leaks, the uptake and metabolism of glucose and fatty acids increases to support the increase in mitochondrial respiration. Thus, activation of UCP1 has been proposed as a target for the prevention and treatment of obesity and T2DM (50, 222). Mouse studies in which the UCP1 gene has been knocked out (UCP1 KO) confirmed the importance of UCP1 in providing protection from diet-

induced obesity. The original studies employing UCP1 KO mice showed that they were cold-intolerant but did not develop obesity (62). However, Feldmann and colleagues showed that UCP1 KO mice did indeed become obese when fed standard and high fat diets and housed at thermoneutrality (for mice, this is 29°C) (70). They hypothesized that under non-stress related environmental temperature conditions, mice no longer recruit thermogenic processes, thereby suppressing their energy expenditure output. It is also important to note that BAT thermogenesis can be activated by diet, as well as the cold (25, 101). Early studies by Rothwell and Stock indicated that rats fed a cafeteria diet increased their core body temperatures in order to burn excess calories (193). Feldmann *et al* not only confirmed these early observations but also illustrated that diet-induced thermogenesis (DIT) was dependent on the presence of UCP1 (70). However, several other studies indicated that while DIT was present, it was independent of UCP1 and BAT (67, 187, 221, 226).

Cold-induced activation of BAT thermogenesis involves the sympathetic nervous system activated release of catecholamines, such as norepinephrine (NE) and epinephrine (E), which bind to and stimulate β_3 adrenergic receptors (25, 101). As previously mentioned, BAT expresses the adrenergic receptors β_3 and to a lesser extent α_1 , which bind NE and E to stimulate the cyclic adenosine monophosphate (cAMP) pathway. It is of interest to note that the disruption of β_3 adrenergic receptors (β_3 -AR) expression and/or their activity due to genetic mutations are inversely correlated with the incidence of obesity (42, 131, 224, 233). Upon activation of the β_3 -AR, adenylate cyclase is activated leading to increased cellular cAMP levels, and this, in turn, activates protein kinase A (PKA) (55, 131, 180). Subsequently, PKA activates adipocyte triacylglycerol lipases to liberate FA from cellular lipid droplets. FA then activate UCP1 through

allosteric mechanisms that induce conformational changes, and also provide abundant substrates for mitochondrial β -oxidation (**Figure 1.3**) (25, 55, 69).

PKA activation also leads to the phosphorylation of the transcription factor cAMP response element binding protein (CREB), which in turn activates the transcription of UCP1. Briefly, PKA phosphorylates CREB which then binds the cAMP response element (CRE) sequence in the proximal promoter region upstream of the UCP1 gene in order to induce transcription. Another site known to regulate UCP1 transcription is the distal promoter region. This region is located -2500 base pairs upstream of transcriptional start site (TATA box) in mouse and rats, and -3500 in humans (25). These regions contains thyroid hormone receptor element (TREs), peroxisome proliferator-activated receptor element (PRE) and retinoic acid receptor sites (RXR) (52) that are regulated by ligands such as thyroid hormones and retinoic acids (RA). The phosphorylation of CREB also induces the transcription of the peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1- α) which then binds to the peroxisome PPAR γ located in the distal region of the UCP1 promoter sequence. Binding by PGC1- α leads to mitochondrial biogenesis through the activation of the nuclear respiratory factors (NRF 1 and 2) and mitochondrial transcription factor A (mtTFA) as well as inducing adipogenesis (10, 176, 185). Furthermore, the control of the distal promoter site by PGC1- α is thought to be essential in the induction UCP1 transcription as it promotes the recruitment of other factors such as activating transcription factor 2 (ATF2) (33, 176, 185). As such, the loss of PGC1- α has been implicated in decreased oxidative phosphorylation and at the whole body level the onset of obesity and T2DM (127). On the other hand, the release of thyroid hormones by thyroid stimulating hormone leads to the binding of thyroid receptors located at TRE sites in the distal region (25, 91). Finally, RXR can form heterodimer complexes with PPAR γ . These

heterodimer complexes are located at previously mentioned PRE sites, and can be induced by RA to prompt transcription of UCP1 (10, 52)

Control of Brown Fat Thermogenesis by Uncoupling Protein 1:

Mechanistically, UCP1 is inhibited by the physiological concentrations of purine nucleotides such as ADP, ATP, GDP and GTP, and is allosterically activated by FA (**Figure 1.3**) (56, 57, 97). The mechanism by which FA activate UCP1 is still highly debated. Currently, there are three models of FA induced proton conductance. The first is the co-factor model in which FA bind to UCP1 and shuttle protons through the UCP1 dimeric channel via their carboxylic groups (7, 8). The second model consists of UCP1 acting as a FA anion transporter. Briefly, FA bound within the inner membrane of the mitochondria in an ionized state binds protons and flips into the mitochondrial matrix where they are deprotonated. Next, UCP1 is thought to transport FA back to the intermembrane face in order to start the cycle. Finally, the third and more widely accepted model of UCP1 proton conductance involves the binding of FA to induce a conformational change in UCP1, thereby allowing the flow of protons down their concentration gradient (7, 8). Indeed, Divakaruni *et al.* (56), showed that FA activate UCP1 through competitive binding kinetics using a fluorescent GDP analog, 2',3'-O-(N-methylanthraniloyl)-GDP. Federoenko and colleagues not only confirmed the competitive binding kinetics of GDP and FA for UCP1 activation, but also provided direct evidence that UCP1 conducts protons via the third mechanism described above (69). Another method of regulating UCP1 expression is through the control of protein turnover. The half-life of UCP1 is thought to be a few hours and

can be extended to a few days if stimulated by NE. Stimulation with NE leads to the to an inhibition of UCP1 degradation and increase in UCP1 synthesis (101).

Although the regulation of UCP1 at the transcriptional level is well characterized, the post-translational control of UCP1 is still not clear. Several hypotheses have been put forth and include a role for ubiquitination in the control of protein turnover (8, 40). In contrast, Carroll *et al.* (29) showed that UCP1 was phosphorylated in BAT of Wistar rats and cold exposure of the rats increased the levels of phosphorylation. They went further to suggest that Ser³, Ser⁴, and Ser⁵¹ were potential sites of modification based on mass spectrometry analysis. However, apart from these findings, there is very little information on the post-translational modification (PTM) of UCP1 and as such, offers a very intriguing avenue for exploration.

Mitochondria, Oxidative Phosphorylation, and Reactive Oxygen Species (ROS) Production:

Beyond their essential roles in oxidative phosphorylation and brown adipose thermogenesis, mitochondria are also important in processes including calcium homeostasis, apoptosis, and the regulation of nuclear gene transcription (32, 34, 80). However, mitochondria excel at converting ΔG (Gibbs free energy) stored in energy-containing nutrients (glucose, FA, and amino acids) into the universal energy currency of cells, ATP. Mitochondria contain very high concentrations of the necessary enzymatic machinery and protein complexes required for ATP production. The production of the metabolic intermediates such as acetyl-CoA, glyceraldehyde-3-phosphate and

ketone bodies from the processes of glycolysis, β -oxidation and amino acid catabolism is crucial for the generation of ATP. The above-mentioned metabolites can be transported to the mitochondria via specific shuttle systems for entry into the TCA cycle to generate reducing equivalents for ATP production.

The coupling of fuel oxidation to mitochondrial ATP synthesis is referred to as oxidative phosphorylation. Peter Mitchell originally proposed the theory of chemiosmotic coupling for oxidative phosphorylation. To support his theory Mitchell posited that an electrochemical proton gradient, also termed a protonmotive force (PMF), was formed across the mitochondrial inner membrane following energy substrate oxidation. The PMF, which is a temporary form of stored ΔG , is then used to drive ATP synthesis. Note that the PMF is composed of an electrical gradient ($\Delta\Psi$) and a chemical gradient (ΔpH) (147, 148, 150, 212). Also, central to this theory is the functioning of the electron transport chain (ETC), which is composed of four transmembrane enzyme complexes (complexes I, III-V) and one complex embedded in the inner leaflet of the mitochondrial inner membrane (complex II; succinate:quinone oxidoreductase) (134, 197). The ETC is driven by reducing equivalents (electron carriers) such as nicotinamide adenine dinucleotide ($NADH + H$) and flavin adenine dinucleotide ($FADH_2$), which are generated from the oxidation of carbohydrates, fatty acids and amino acids (13, 200). In contrast, an alternative pathway in which reducing equivalents are supplied to the ETC is through the electron shuttle system involving the electron-transfer flavoprotein (ETF). Specifically, electrons derived from the breakdown of fatty acids by various dehydrogenases are shuttled to ETF and then to the electron-transfer flavoprotein coenzyme Q oxidoreductase (ETF-QO) and ultimately to the ETC for the process of oxidative phosphorylation (186). These reducing equivalents generated from

different pathways are oxidized by NADH-Coenzyme Q oxidoreductase (complex I), succinate dehydrogenase (complex II) and cytochrome *c* oxidoreductase or cytochrome *bc1* complex (complex III) (179, 200). The liberated electrons are then passed from I and II to the lipophilic electron carrier ubiquinone (Q) producing a fully reduced ubiquinol molecule (197). Next, coenzyme Q undergoes a complex series of oxidation reactions driven by complex III (cytochrome *c* oxidoreductase, cytochrome *bc1* complex), which results in the donation of electrons to cytochrome C, a peripheral membrane protein bound to the outer leaflet of the mitochondrial inner membrane. Cytochrome C then shuttles electrons to complex IV (cytochrome *c* oxidase). Upon reaching complex IV single electrons are coupled to a terminal electron acceptor, molecular oxygen (O_2), which is reduced to form water (H_2O) (89, 140). The transfer of electrons between complexes I-IV is accomplished through a series of well-positioned prosthetic groups such as iron sulfur clusters (in proteins of complexes I, II, and III), copper sulfur clusters (in complex IV) and flavin groups (complex I) (21, 106). The transfer of two electrons from one $NADH_2$ to O_2 is an exergonic process that involves a redox change of ~ 1140 mV (where the E_h of the NADH and O_2 are -320 mV and +820 mV) and thus a favorable ΔG of -52.44 kcal/mol (20). Thus electron transfer results in a favorable free energy change. This favorable change is coupled to the efflux of protons (H^+) (complex I, III, IV) from the mitochondrial matrix into the intermembrane space to build a PMF. The potential energy stored in the PMF is used to drive the synthesis of ATP by the F_0F_1 ATP synthase (complex V) as H^+ return to the matrix through the F_0 portion of the protein complex. (See **Figure 1.4**) (147, 149). Thus, oxidative phosphorylation is the harnessing of an energetically favorable movement of electrons down their respective gradients to generate a PMF that is then used to drive the activity of ATP synthase.

Figure 1.3: Control of UCP1 activity by fatty acids induces conformational changes leading to the translocation of protons. The stimulation of protein kinase A (PKA) activity by upstream cascades in BAT cells leads to the activation of cellular lipases which in turn liberate free fatty acids (FA) from stored lipid droplets through lipolysis. FA are transported into the mitochondria through the carnitine palmitoyltransferase I&II (CPT1 & CPTII) systems for both β -oxidation and allosteric activation of UCP1. Allosteric activation by FA's is thought to induce a conformational change when bound to UCP1, thereby relieving the purine nucleotide inhibition of adenosine triphosphate (ATP). Furthermore, recent studies showing PKA activation in the mitochondrial matrix may also lead to covalent modification and thus activation of UCP1. This increases proton leaks through the dimeric channel. PM- Plasma membrane; MOM- Mitochondrial outermembrane; MIM- Mitochondrial innermembrane.

Figure 1.3: Control of UCP1 activity by fatty acids induces conformational changes leading to the translocation of protons.

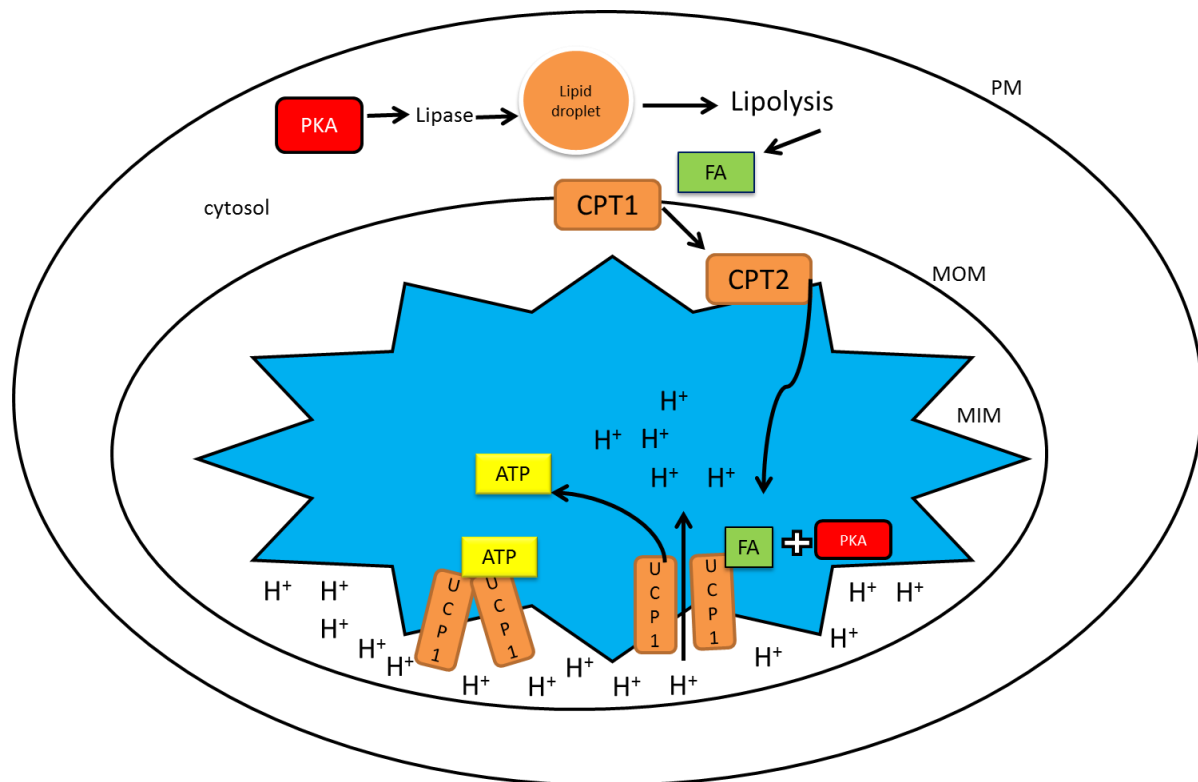
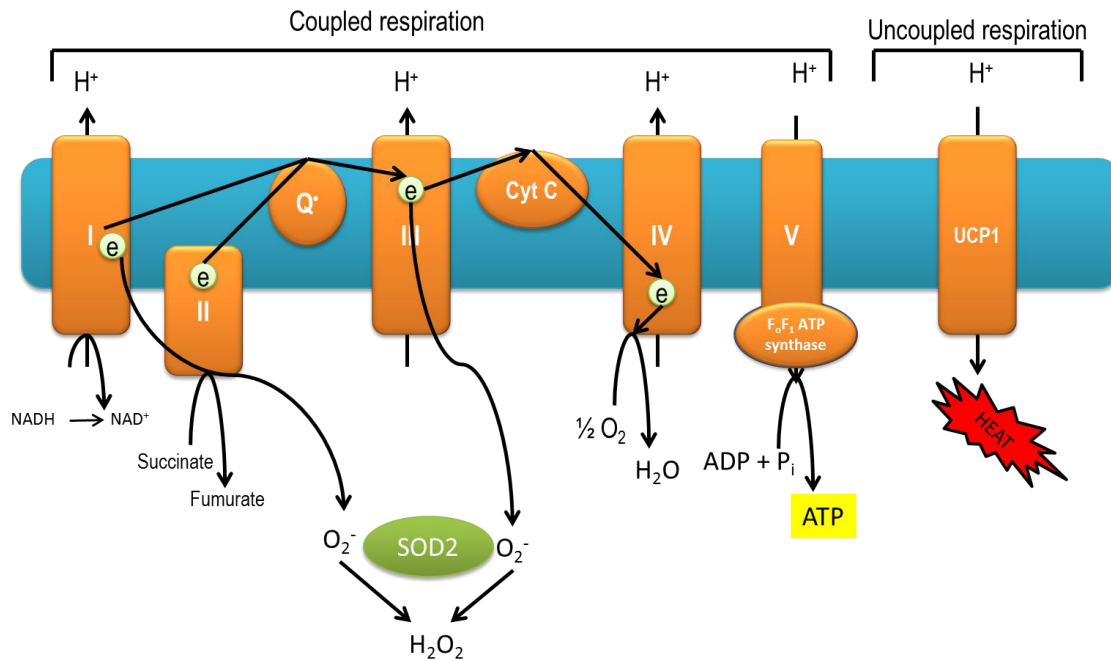


Figure 1.4: Oxidative phosphorylation under “coupled and uncoupled” states. Adapted from (112). Coupled respiration during oxidative phosphorylation involves the use of the electrochemical proton gradient built by the transfer of electrons from reducing equivalents (e.g; $\text{NADH}^+ + \text{H}$) between complexes (I-IV) to drive ATP production by the F_0F_1 ATP synthase. During uncoupled respiration, protons leak back (down their concentration gradient) through uncoupling protein-1 (UCP1) into the mitochondrial matrix. Leaks through UCP1 are required to generate heat (due to the increased substrate oxidation reactions) for thermoregulation in mammals. This process is known adaptive thermogenesis and can be initiated through inducible leak. A by-product of coupled respiration is electron slippage, which subsequently reduces molecular oxygen to generate superoxide ($\text{O}_2^{\bullet-}$) which is dismutated into hydrogen peroxide (H_2O_2) by the matrix enzyme superoxide dismutase (SOD2). Complex (I, II, III, IV) are protein complexes associated with the electron transport chain. (E)- Electrons; P_i - inorganic phosphate; H^+ - hydrogen ions

Figure 1.4: Oxidative Phosphorylation under “coupled” and “uncoupled” states. Adapted from (112).



Although oxidative phosphorylation is a highly favorable process, it is not always efficient. One reason for variability in oxidative phosphorylation efficiency is that electrons can escape the ETC, interact with O₂ and thus produce reactive oxygen species (ROS) (13, 106, 139, 140). This process is termed electron slippage (quantitatively though, the energetic wastefulness caused by proton leak is far greater than electron slippage) (106). Subsequently, when oversupplied with reducing equivalents the mitochondria can be a major source of ROS production, specifically at complex I and III of the ETC (**Figure 1.4**) (9, 137, 139, 140). Other factors affecting ROS production from mitochondria can be the previously mentioned inefficiency of electron transfer through the complexes, or substrate oxidation, ADP availability, and the polarity of the transmembrane potential (21, 106). ROS production occurs with higher frequency when the ETC becomes overly reduced (over supplied with electrons), while the PMF is high and ATP demand is low. Studies using respiratory chain inhibitors such as rotenone (complex I inhibitor, which binds to the ubiquinone binding site) and antimycin A (complex III inhibitor, which binds the Q_i site of the cytochrome C reductase) have shown that complex I and complex III are the major site of ROS production (133). Complex I produces superoxide into the mitochondrial matrix, while complex III produces superoxide into both the matrix and intermembrane spaces (151).

The rate and amount of superoxide production by the ETC are dependent on a number of factors such as the availability of ADP, NADH levels, efficiency of electron flow to O₂, and the PMF. Typically conditions that prevent electron flow to O₂ (such as low ADP or a high PMF) promote superoxide production. While superoxide is the form of ROS that is produced by the ETC, it has a very short half-life and is quickly dismutated into hydrogen peroxide (H₂O₂) either

spontaneously or through enzymatic action (superoxide dismutase –SOD) or it is reduced to hydroperoxyl radical ($\text{HOO}\bullet$) (151, 158, 166, 200). H_2O_2 is a more stable molecule and can transverse the lipid membranes and lead to DNA, lipid and protein damage (140, 151). Importantly there are several other forms of ROS and free radical species, such as hydroxyl radicals and reactive nitrogen species (RNS) respectively. Excessive ROS production can lead to oxidative stress, oxidative damage and the latter are associated with aging, insulin resistance and obesity (14, 158, 217). In order for ROS accumulation to induce oxidative stress the amounts must overwhelm the formidable cellular anti-oxidative defense systems. Upon generation of superoxide, SOD (Cu/ZnSOD in cytosol and MnSOD in mitochondria) converts this highly reactive molecule into H_2O_2 . Following the conversion, glutathione peroxidase, glutaredoxin and peroxiredoxin systems are invoked to limit H_2O_2 levels (140, 158, 200). In addition, catalase mitigates ROS levels by converting H_2O_2 into H_2O and O_2 . However, the anti-oxidative defense system comes with a cost since it depends on the reductive potential stored within the nicotinamide adenine dinucleotide phosphate (NADPH) (140, 158). The latter depends on the cytosolic pentose phosphate pathway and mitochondrial reactions catalyzed by NADP-linked malic enzyme, NADP-linked isocitrate dehydrogenase, and nicotinamide nucleotide transhydrogenase.

It is also important to recognize that ROS have key physiological roles as signalling molecules. In low amounts ROS regulate phosphorylation cascades by inhibiting phosphatases (36, 86, 124, 130, 140, 200). In addition, low levels of ROS (mainly generated from extra mitochondrial sources such as NADPH oxidase) increase insulin and growth factor signalling (36, 124, 130, 140, 200). Hence, the regulation of oxidative phosphorylation plays crucial roles not only in ATP

production, but also in cell fate and signalling. As discussed in greater detail below, ROS also play an important role in cellular function since they activate some uncoupling protein family members.

Mitochondrial uncoupling in other tissues:

Overall, uncoupling of oxidative phosphorylation can occur in two manners basal or inducible. Basal uncoupling is proton leak that is not acutely regulated, and is believed to be present in mitochondria of all tissues (21, 93, 106). This basal leak is associated with a substantial proportion of basal metabolic rate (BMR) of an organism. In rat hepatocytes and perfused skeletal muscle basal leak accounts for 20-40% and 52% of resting oxygen consumption respectively, while at the whole body level it accounts for approximately 25% of BMR (160, 190). It worth noting that basal proton leak is highest when an organism is resting and ATP demands are low since mitochondrial PMF is highest under these conditions. Bioenergetic experiments have established that a large fraction of basal leak is due to adenine nucleotide translocase (ANT) and that the amount of basal leak is directly related to ANT content (205). It has been postulated that basal leaks are required to prevent high PMF in very metabolically active tissues to control mitochondrial ROS production (21). Work has established that leaks through ANT can diminish mitochondrial ROS production (152).

In contrast, inducible proton leak is the mechanism by which mitochondrial targeted activators can induce proton translocation across the mitochondrial inner membrane through members of the mitochondrial anion carrier protein family (e.g., ANT and UCPs) (93, 106). The known

activators of inducible proton leaks range from fatty acids, ROS, reactive alkenes and high membrane potential (69, 169). In contrast, known inhibitors range from purine nucleotides (e.g., ADP and GDP), to exogenous small molecules such as carboxyatractyloside, and bongkreikic acid (78, 97, 119, 183). The proposed physiological functions of inducible leak include the control of EE (UCP1 and UCP3), ROS and cellular signaling (UCP2, UCP3, and to a lesser extent UCP1) (131, 135, 137, 153). Hence, mechanisms that regulate inducible proton leak may offer therapeutic targets in the treatments of metabolic disorders.

The Novel Uncoupling Proteins and their Physiological Functions:

The five known uncoupling proteins (UCPs 1-5) belong to the anion carrier protein super family (4). The first UCP to be discovered was UCP1 or thermogenin, a ~32kDa protein, which exists almost exclusively in BAT (19, 97, 157, 182-184). In 1997 UCP2 and UCP3 were discovered and shown to share a sequence homology of ~73% between each other and of ~58% to UCP1 (18, 74, 122, 181, 229). The expression of UCP2 is almost ubiquitous with higher abundance in tissues such as pancreas, spleen, thymus, as well as certain immune cells, while UCP3 is expressed in skeletal muscle and BAT (47, 65, 181). In addition, UCP 4-5 transcripts are expressed in the brain and to a lesser extent skeletal muscle, heart, lung, liver and kidney; their functions have not been extensively studied (4, 5, 116, 235, 237).

UCP1 protein accounts for ~8-10% of all mitochondrial proteins in BAT (194). Upon catecholamine activation of BAT, lipolysis is stimulated generating free FA that allosterically activate UCP1 (56, 69, 97, 106, 159). As described above, this leads to the conductance of protons back into the mitochondrial matrix without being coupled to ADP phosphorylation (55,

106, 168). The augmented oxidation of fuel substrates results in the production of heat, and this is quickly dispersed through the highly vascularized BAT and into the circulation (101, 102, 159).

In contrast to UCP1, proton leaks through UCP2 and UCP3 are not used for adaptive thermogenesis but are required to control the emission of ROS from mitochondria (46, 135). ROS production occurs, as described above, with the over reduction of the ETC thereby leading to electron slippage. It has been proposed that increased electron slippage which lead to increased ROS production activates UCP2 and UCP3. Upon activation, UCP2 and UCP3 decrease the PMF and thus reduce the amount of ROS generated by mitochondria. It is of note that proton leak through UCP1 has been implicated in ROS control (155, 161). However, these results are still highly debated (136). Interestingly, the control of ROS by UCP2 and UCP3 has been documented to have several physiological implications in various tissues. In fact, UCP2 has been associated with glucose-stimulated insulin secretion (GSIS) (138). Increases in UCP2 expression has been linked to drug resistance of certain cancer cells by sequestration of ROS (94, 135). Indeed, this mechanism of UCP2 conferring chemoresistance to tumor cells was shown by selectively inhibiting UCP2 expression by genipin, while simultaneously treating cells with ROS producing agent menadione (135). The inhibition of UCP2 in drug resistant cells sensitizes cells to the toxic effects of ROS accumulation (135).

Compared to UCP2, UCP3 has a much more restricted pattern of tissue expression; it is highly selectively expressed in skeletal muscle and BAT. The proposed function of UCP3 is mainly thought to be involved in ROS control with implications also for obesity, since uncoupling in

skeletal muscle can have an overall effect on EE at the whole body level (16, 39, 137). Indeed, two fold overexpression of UCP3 protein in muscle of mice mitigated diet induced obesity (47, 48). In addition, UCP3 has been shown to be related to the rate of weight loss in obese women. In a study by Harper et al (95), obese women in a clinical dietary weight loss program who lost weight rapidly had increased UCP3 mRNA expression versus those who showed resistance to weight loss. Also, UCP3 is indirectly associated with enhanced fatty acid uptake, transport and metabolism. Indeed, chronic exposure to fatty acids can up regulate both UCP3 mRNA and protein content in rodents and humans (37, 103). Furthermore, two fold overexpression of UCP3 in skeletal muscle of mice is associated with a decreased respiratory exchange ratio and increased markers for complete fatty acid oxidation (16). The importance of UCP3 in relation to muscle insulin sensitivity and T2DM stems from its indirect (and as yet poorly understood) role in enhancing fatty acid metabolism. Of note also is the fact that mRNA levels of UCP3 are decreased in patients with T2DM, while UCP3 protein expression is decreased by 50% in *vastus lateralis* samples of patients with T2DM (31, 121, 198, 199).

Although UCP2 and UCP3 have been implicated in a wide variety of roles the physiological regulation of these proteins *in vivo* is still not clear. Indeed, there is controversy as to whether purine nucleotides such as ADP inhibit the proton leak functions of UCP2 and UCP3, and whether they are allosterically activated by FA in the same manner as UCP1. Alternatively, other methods of regulating UCPs such as glutathionylation have been demonstrated by Mailloux and colleagues (138, 141). They showed that unlike UCP1, UCP2 and UCP3 are covalently regulated by reversible glutathionylation, which consists of the addition of glutathione to highly reactive

cysteine groups (141). Hence, the novel uncoupling proteins have numerous roles within the cell and are regulated through different mechanism.

In summary, the uncoupling proteins are a family of proteins with significant roles in mitochondrial bioenergetics, metabolism, cell signaling and metabolic diseases. As discussed, their expression patterns differ greatly from tissue to tissue (181). While UCP1 is exclusively found in BAT and is used for thermogenesis, the UCP1 paralogs are found in various tissues and function in the control of mitochondrial ROS production. Curiously, leaks through UCP1 do not seem to curtail mitochondrial ROS production in BAT (this will be addressed extensively in the Discussion). Studies in mice have shown that the overexpression of UCP3 can lead to protection from weight gain, better insulin sensitivity, and improvements in numerous metabolic parameters such as circulating cholesterol, complete fatty acid metabolism and better glucose handling (4, 16, 93, 126). On the other hand, knock-outs or decreased expression of UCPs have been linked to obesity, T2DM, decreased insulin secretion, while making certain tumor cells sensitive to chemotherapeutics (46, 70, 135, 138, 215). The recent discovery of BAT in adult humans has led to a resurgence of interest into this tissue and its potential as a target for obesity treatment. Despite much research the mechanisms of post-translational control of UCP1 function are poorly understood, and this propelled the research described herein. Indeed, the results discussed in this thesis are an attempt to characterize and validate the potential role phosphorylation and redox status on UCP1 regulation and function.

The first chapter of my thesis explores the hypothetical phosphorylation of UCP1 *in vivo* and the implications for BAT bioenergetics. The second chapter investigates the impact of the ectopic

expression in mice of UCP1 in skeletal muscle on redox and ROS in that tissue and on whole body energy metabolism.

HYPOTHESIS AND RESEARCH OBJECTIVES:

Overall Aims:

The **overall aims of my project** are to confirm and explore the implications of UCP1 phosphorylation in BAT metabolism, and to explore the effects of ectopic UCP1 expression on redox and ROS in skeletal muscle and on whole body energetics in mice.

Hypotheses:

Given limited but exciting evidence in the literature, I hypothesized that UCP1 phosphorylation controls UCP1 function in BAT. Specifically, I expected that upon cold exposure in mice, UCP1 would be phosphorylated and would augment proton leak respiration. Moreover I hypothesized that the ectopic expression of UCP1 in skeletal muscle in mice would lead to changes in the mitochondrial redox environment such as glutathione levels and ROS production, and would result in mice that were leaner than wild-type control mice.

RESEARCH OBJECTIVES:

1. Confirm that UCP1 is covalently modified by phosphorylation and determine if this modification is physiological (*e.g.*, activated by cold exposure in mice)
2. Delineate the bioenergetic implications of UCP1 covalent modification by analyzing *ex vivo* preparations of BAT mitochondria

3. In transgenic mice that ectopically express UCP1 in skeletal muscle, assess the bioenergetic, redox and oxidative stress implications in skeletal muscle and the metabolic implications at the whole body level

MATERIALS AND METHODS:

Mice:

C57BL/6J mice were used for cold exposure experiments. Mice were acquired from the Jackson Laboratory (Jackson Laboratory; Bar Harbor, ME) for all experiments involving proteomics analysis and mitochondrial bioenergetics. Mice were housed at room temperature (22-23°C) and given free access to a standard chow diet-44.2% carbohydrate, 6.2% fat and 18.6% crude protein (Harlan Laboratories; Mississauga, ON) and water. For proteomics and mitochondrial bioenergetics studies, some mice were exposed to 4°C for 24 h (cold exposure; CE) in single cages without any housing materials; other mice were housed at normal room temperatures and were referred to as warm acclimated (WA). All experiments were approved by the Animal Care committee at the University of Ottawa in accordance with the guidelines of Canadian Council of Animal Care and were conducted on mice at ages of 7-12 weeks.

Isolation of Mitochondria from Brown Adipose Tissue:

WA or CE mice were weighed using a Mettler PE 1600 analytic balance (Mettler Toledo; Columbus, OH) and sacrificed by decapitation. BAT mitochondria were isolated by differential centrifugation essentially as described in (206). The protocol used is described as follows. All tissue extraction and mitochondrial isolation steps were performed on ice or at 4°C. Interscapular

BAT was dissected and cleaned of any observable connective tissue, hair, and white fat and then minced on a Teflon plate. Minced BAT was then placed in ~30 mL of homogenizing media (HM; 250mM sucrose + 0.2% (w/v) defatted bovine serum albumin (BSA)) and homogenized using an ice-cold glass/Teflon Potter-Elvehjem tissue grinder for a minimum of 10 passes. The BAT homogenate was then centrifuged at 8500 xg for 9 min to pellet mitochondria while keeping debris and white fat in the supernatant. The solution was decanted and the inside of the tubes were wiped down to ensure that any residual fat was removed. The pellet was then resuspended in ~30 mL of ice cold HM and the suspension was centrifuged at 800 xg for 9 min to remove cellular debris, the nuclear and endoplasmic reticulum fraction. The supernatant was then removed and centrifuged 8500 xg for 9 min to pellet mitochondria. The pellet was re-suspended in reaction buffer A, which contained 125 mM sucrose, 20 mM Tris-Base, 2 mM MgCl₂, 4 mM KH₂PO₄, and 1 mM EDTA (pH 7.2). All spins were conducted using the Sorvall ST-16R centrifuge (Thermo Scientific; Waltham, MA). The protein concentration of the mitochondrial suspension was determined using the Bradford method. A standard curve was developed using 200µg/ml of BSA reagent (Sigma; Mississauga, ON) and samples were measured at an absorbance of 595 nm using the Synergy Mx microplate reader (BioTek; Winooski, VT).

Mitochondrial Bioenergetics:

All mitochondrial bioenergetics measurements were performed by assessing oxygen consumption using the Seahorse XF24 analyzer (Seahorse Biosciences; North Billerica, MA) **(see Figure 1.5)**. The Seahorse XF24 analyzer allows rapid real-time measurements of oxygen (O_2) consumption rates (OCR) of adhered cells or mitochondria by positioning O_2 -sensitive fluorescent probes 200 *microns* above the sample (72). This creates a 7 μ L microenvironment above the sample allowing sensitive detection of changes in O_2 levels. Note that measurement of O_2 levels in a medium is a proxy measure for aerobic respiration since O_2 is the terminal electron acceptor of the respiratory chain. The Seahorse XF24 was originally developed to measure mitochondrial energetics in intact cells (72). However, recently our group has adapted this technique to measure OCR in isolated mitochondria (138, 189). Following isolation, BAT mitochondrial suspension was diluted to 0.05 mg/ml in reaction buffer B which contained 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 1 mM EGTA, 10 mM pyruvate, 2 mM malate, 0.2% (w/v) defatted bovine serum albumin (BSA), 10 mM KH_2PO_4 , and 5mM $MgCl_2$ (pH 7.2, filter sterilized using 0.2 *micron* filters). Mitochondrial suspensions were plated in the 24 well XF24 Seahorse assay plate (Seahorse Biosciences; North Billerica, MA) in volumes of 50 μ l and attached to the plate surface by centrifugation at 2000 xg for 20 min at 4°C using the Sorvall ST-16TR centrifuge (Thermo Scientific; Waltham, MA). Following centrifugation, mitochondria were supplemented with 350 μ l of reaction buffer B and incubated for ~10 min at 37°C.

Measurements of oxidative phosphorylation were carried out as follows; OCR was first measured under state 4 conditions (substrate only; 10 mM pyruvate/2 mM malate) and then mitochondria were treated with 0.1 mM adenosine diphosphate (ADP) to evaluate phosphorylating or ADP-stimulated respiration (to assess state 3), 2.5 μ g/mL (to assess state 4o

or proton leak-dependent respiration), 8 μ M carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; to assess maximal respiratory capacity), and 4 μ M antimycin A (Ant A; to assess any residual O₂ consumption after the inhibition of respiration by this complex III inhibitor) (see **Figure 1.5B**). Note that all respiration values were corrected for any O₂ consumption independent of the respiratory chain (Ant A) and in the absence of mitochondria.

Proton leak through UCP1 in isolated mitochondria was determined as follows; following measurement of state 4 respiration, mitochondria were sequentially treated with 2.5 μ g/mL oligomycin (state 4o), then titrated with guanosine diphosphate (GDP, a UCP1 inhibitor used at concentrations from 0.5 mM to 1 mM), and 4 μ M Ant A to measure respectively, proton leak-dependent respiration, proton leak-dependent respiration associated with UCP1, and O₂ consumption independent of the electron transport chain.

To determine if UCP1 can be phosphorylated and activated by protein kinase A (PKA), mitochondria were treated with 8-bromo-3',5'-cyclic adenosine monophosphate (8Br-cAMP). The experiment was conducted as follows; following measurements of state 4 respiration, mitochondria was treated with 20 μ M 8Br-cAMP, then treated with 2.5 μ g/mL oligomycin, 1 mM GDP and 4 μ M Ant A.

Figure 1.5: Oxygen consumption measurements using the XF24 extracellular flux analyzer.

A) Mitochondrial bioenergetics is assessed through the sequential titration of known inhibitors. Briefly, in order to assess uncoupled respiration, isolated mitochondria were treated with oligomycin (oligo 2.5µg/mL), guanosine diphosphate (GDP-0.5-1mM), and antimycin A (Ant A-4µM). First, basal respiration (state 4) is measured in the absence of ADP, followed by ATP synthase inhibition by oligomycin (state 4o). The change in respiration is thus the respiration associated with ATP turnover, while the residual respiration is associated with proton leaks (uncoupled) and non-mitochondrial respiration. Next, UCP1 proton leaks are titrated using the inhibitor GDP. Finally, non-mitochondrial respiration is assessed with the addition of Ant A which inhibits complex III of the ETC. **B)** Coupled respiration was assessed by the addition of adenosine dinucleotide (ADP-100µM; state 3 respiration), followed by oligomycin. FCCP (8µM) is injected to measure the maximal capacity or spare respiratory capacity of the ETC. Finally non-mitochondrial respiration is assessed using Ant-A. Oxygen consumption rate is assessed using the XF24 Extra cellular flux analyzer and is measured in pmoles/min normalized to protein content.

Figure 1.5: Oxygen consumption measurements using the XF24 extracellular flux analyzer.

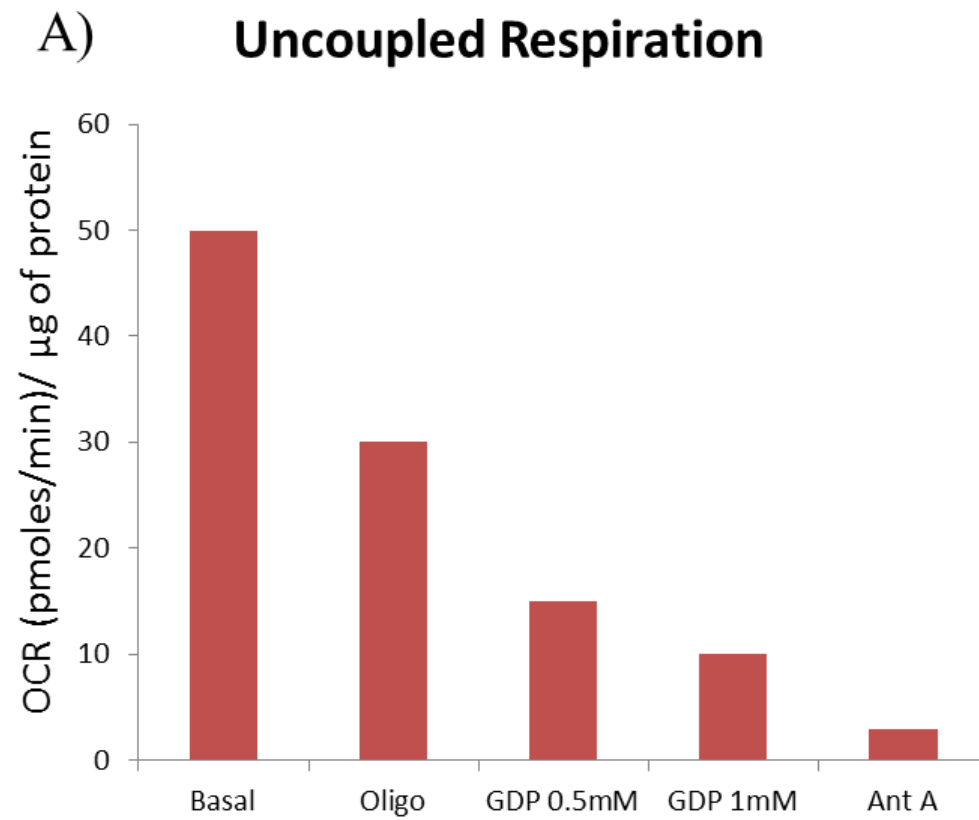
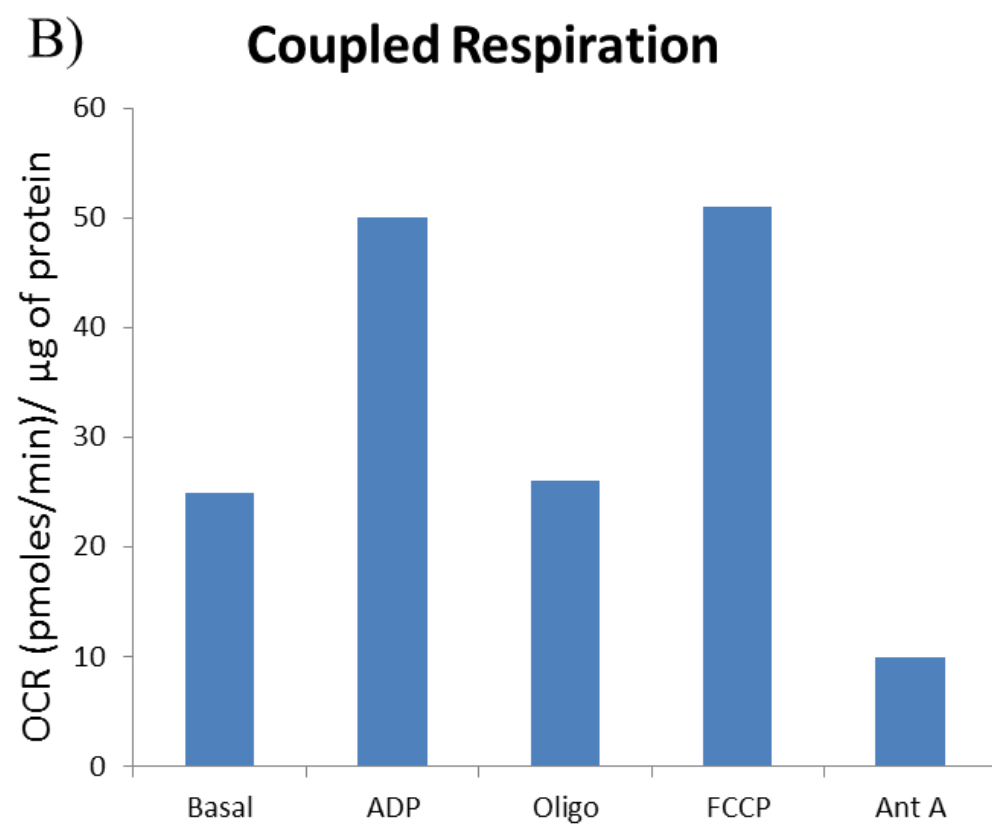


Figure 1.5: Oxygen consumption measurements using the XF24 extracellular flux analyzer.



2-Dimensional Gel Electrophoresis:

Isolated mitochondria (250-350 μ g) were subjected to a clean-up protocol before first dimension analysis as follows: 2% n-dodecyl- β -D-Maltoside (DDM) and 1% amidosulfobetaine-14 (ABS-14) were added for 2h at room temperature with constant agitation. Samples were then centrifuged at 12,000 xg for 10 min at 4°C using an Eppendorf centrifuge (Fischer Scientific; Waltham, MA). The supernatant was collected and treated with 1 M 4-vinyl pyridine (4-VP) for 1h in the dark. Subsequently, the reaction was stopped with equimolar amounts of dithiothreitol (DTT) and then subjected to isoelectric focusing as follows. A 7-11 cm immobilized pH gradient (IPG) strip (pH 3-10, Bio-Rad; Mississauga, ON) was carefully stripped of its covering and placed face down (IPG gel side down) in the Protean IEF cell (Bio-Rad; Mississauga, ON). Samples were diluted by addition of equal volume of rehydration buffer (8 M Urea, 0.5% CHAPS, 10 mM DTT, 0.2% (w/v) Bio-Lytes and 0.001% bromophenol blue), and then 250 μ l of sample was carefully pipetted onto the IPG strip. After samples were loaded 2-3 mL of mineral oil was laid over the IPG strip to prevent evaporation and electrophoresed at 50V for 12h. Furthermore, the voltage was gradually increased to 200V for 1h, then 500V for 1h, then 1000V for 1h, then 8000V for 6h, and then decreased to 500V for 5h. After isoelectric focusing (IEF) the IPG strips were carefully lifted and blotted on Whatman filter paper to remove residual mineral oil. Next, the IPG strips were placed in an equilibration tray and proteins were reduced by adding 5 mL of equilibration buffer I (6 M Urea, 2% sodium dodecyl sulfate (SDS), 375 mM Tris-HCL pH 8.8, 20% glycerol, and 130 mM DTT) for 10-15 min at room temperature with constant agitation. Equilibration buffer I was discarded and the IPG strips were again carefully blotted and dried. Next, IPG strips were alkylated with equilibration buffer II containing 6 M

urea 2% SDS, 375 mM Tris-HCl pH 8.8, 20% glycerol and 135 mM iododacetamide to prevent protein re-oxidation for 10-15 min at room temperature in the dark with periodic agitation. IPG strips were then cleaned of any residual buffer and overlaid onto a 12 % isocratic SDS gel (40% acrylamide/bis-acrylamide, 1.5 M Tris-base (pH 8.8), 0.4% SDS, 10% ammonium persulfate (APS), and 50 μ l of N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)) as well as encased in warm 0.5% agarose. Samples were electrophoresed at 150V for ~2h. The transfer of proteins and detection of UCP1 was achieved as described below (see Immunoblot section, below), with the notable exception of the use of the rabbit polyclonal anti- UCP1 antibody at a dilution of 1/1000, and the secondary goat anti-rabbit horseradish peroxidase antibody at a dilution of 1/1000.

Generation of Anti-UCP1 Protein A Bead Conjugate for Immunoprecipitation:

Anti-UCP1 protein A bead conjugates were produced using Dynabeads Protein A, as described by the manufacturer (Life Technologies; Burlington, ON). Following resuspension of protein A beads, 50 μ l of the suspension was transferred to a 1.5 mL minitube and then placed on a dynaMag magnet (Life Technologies; Burlington, ON). The supernatant was discarded and dynabeads were then resuspended in 200 μ l of antibody binding and washing buffer (Ab&Wb; phosphate buffered saline (PBS) with 0.01% Tween-20) containing 5 μ g of rabbit polyclonal anti-UCP1 antibody (Sigma Aldrich; St. Louis, MO). The antibody-bead complex was then incubated for 30 min at room temperature with constant mixing. After the incubation period, the minitubes were placed on the dynaMag and the supernatant was discarded. The tubes were then removed from the dynaMag, washed with 200 μ l Ab&Wb and placed back onto the magnet. The

supernatant was discarded and the beads were either frozen at -20°C or used immediately for immunoprecipitation of UCP1.

Immunoprecipitation of Antigen:

Briefly, 1-3 mg/ml of isolated mitochondrial protein was diluted in lysis buffer (50 mM Tris-HCL, 5 mM EDTA, 100 mM NaCl, 0.5% NP-40, 0.1% SDS, 0.5% sodium deoxycholate pH 7.5). Protein samples were sonicated using the Branson Digital Sonifier 250 (Fisher Scientific; Waltham, MA) for 30 s with a 3 s pulse on, and a 3 s pulse off protocol on ice. Following sonication, protein samples were centrifuged at 8000 xg at 4°C for 10 min to pellet cellular debris and the supernatant was kept. Protein samples were then supplemented with anti-UCP1 protein A dynabeads and incubated at 4°C overnight with constant agitation. After overnight incubation samples were placed on the dynaMag and washed 3x with washing buffer. Supernatant was discarded and the pellet was gently resuspended in fresh new 100 µl washing buffer (PBS) and subsequently transferred to clean minitubes. Following washing protein samples were eluted from the anti-UCP1 dynabead complex by placing the samples back on the dynaMag, removing the supernatant, and then resuspending the beads in 50 µl of elution buffer. Protein complexes were gently eluted for 20 min at 4°C with constant agitation. Subsequently, both beads and supernatant were separated and kept for further analysis. Samples were treated with 1 M Tris-base pH 7.5 to neutralize the acidic nature of the elution buffer, as well as Laemmli sample buffer (SB) containing 50 mM Tris-Cl pH 8, 2% (w/v) SDS, 100 mM DTT, 10% glycerol and 1% bromophenol blue, in preparation for SDS page.

SDS-Page and Silver Staining:

Immunopurified UCP1 from CE and WA mouse mitochondria were diluted in SB and boiled for 5 minutes at 95°C prior to loading onto the NuPage 4-12% Bis-tris precast gels (Life Technologies; Burlington, ON). The gel was electrophoresed at 160V for 90 min and then placed in a fixing solution containing 50% (v/v) methanol and 2.5% (v/v) acetic acid for 30 min with constant agitation at room temperature. Following addition of fixing solution, gel slabs were washed 3x with deionized and double distilled water (ddH₂O) for 5 min at a time. Next, gel slabs were placed in ddH₂O overnight (16-24 h) with constant agitation at room temperature. Subsequently, the supernatant was discarded and gel slabs were incubated in 0.02% (w/v) sodium thiosulfate for ~ 1 min with agitation. Gel slabs were then washed 3x with ddH₂O for 2 min each time and then were developed using 0.01% (v/v) formaldehyde and 2% (w/v) sodium carbonate for ~ 5-10 min. The success of the previous step is highly dependent on the amount of protein content loaded. Following exposure, gel slabs were placed in 1% (v/v) acetic acid, to stop the reaction. Gel slabs were then kept for as long as needed at room temperature or were processed immediately for in-gel separation and digestion for mass spectrometry analysis. Before proceeding to in-gel digestion, images of the gel slabs were taken using the Epson Perfection 2580 scanner and Epson photo software (Epson; Markham, ON).

In-gel Separation and Digestion:

Upon silver staining, protein bands of interest were identified and cut into small cubic pieces and placed in a clean 1.5 mL minitubes. Gel pieces were first washed with 100 µl of 50 mM

ammonium bicarbonate then centrifuged briefly; the supernatant was discarded and gel pieces kept. Gel pieces were then reduced in size using 50% (v/v) acetonitrile (ACN) and 25 mM ammonium bicarbonate for ~15 min. The supernatant was discarded and gel pieces were dried using a speed vacuum (Fisher Scientific; Waltham, MA). Next, the dried gel pieces were rehydrated using 50 mM ammonium bicarbonate, 10 mM DTT for 15 min at 56°C with constant agitation. Heated minitubes were removed and allowed to cool at room temperature for ~ 10 min. The supernatant was discarded and gel pieces were alkylated using 100 μ L of 100 mM iodoacetamide and 50 mM ammonium bicarbonate. Samples were then wrapped in tin foil and placed in the dark for 15min. Following alkylation, the supernatant was discarded and gel pieces were washed using 50 mM ammonium bicarbonate. Gel pieces were then reduced in size using 50% (v/v) ACN, and 25 mM ammonium bicarbonate for 15 min. Supernatant was discarded and gel pieces were dried using speed vacuum (Fischer Scientific; Waltham, MA). Gel pieces were then subjected to tryptic digestion using trypsin in 50 mM ammonium bicarbonate (enzyme to protein ratio 1:100) at 37°C overnight with constant agitation. Following tryptic digestion the supernatant was kept in a clean minitube, while the gel pieces are hydrated using 25 mM ammonium bicarbonate for 20 min. Next, the supernatant was removed and added to the previously kept supernatant, as these steps are sequential steps to elute digested peptides from gel pieces. Lastly, peptide samples were extracted from gel pieces by incubating for 20 min in 5% (v/v) formic acid and 50% (v/v) acetonitrile. Following extraction, the supernatant was removed and added to the previously eluted peptides and stored at -20°C for later analysis by mass spectrometry.

Phosphoproteomics and In-solution Protein Digestion:

1-3 mg/ml of mitochondrial lysate or 10 µg of purified His-tagged recombinant UCP1 was resuspended in denaturing buffer containing 200 µl of 8 M urea and 100 mM triethyl ammonium buffer (TEAB) in preparation for mass spectrometry analysis. Briefly, full length UCP1 (1-307aa) was produced in *E.coli*, His-tagged at the Nt-terminus, purified using a Ni-sepharose bead column and stored in 1M PBS containing 58mM disodium hydrogen phosphate, 68mM sodium chloride at pH 8 with 300mM imidazole, 0.7% Sarcosyl, and 15% glycerol. Briefly, samples were reduced with 10 µM DTT at 37°C for 2h followed by treatment with 20 µM iodoacetamide at room temperature in complete darkness for 40 min. Samples were then diluted in 1 M urea with 100 mM TEAB with a (w/v) 1/100 trypsin to protein ratio. The sample was then incubated overnight (16-24h) at 37°C and was frozen at -20°C for further analysis.

Enrichment of Phosphopeptides:

Following tryptic digestion, peptide samples are subjected to a phospho-enrichment protocol. Peptide mixtures were incubated with 50 µL of titanium microspheres (Ti⁴⁺-IMAC microspheres) with constant agitation for 30 min. Following incubation, samples were collected and centrifuged at 15,000 xg using a T900 centrifuge (Thermo Scientific; Burlington, ON) for 10 min and the supernatant was discarded. In order to remove nonspecific binding, Ti⁴⁺-IMAC microspheres were washed with a 100 µL solution containing 50% ACN, 6% trifluoroacetic acid (TFA), and 200 mM sodium chloride (NaCl) followed by a 100 µL solution containing 30% ACN, 0.1% TFA. In addition, to elute the phosphoenriched peptides from the Ti⁴⁺-IMAC microspheres a 100 µL of 12.5% ammonium hydroxide (NH₃•H₂O) was added with agitation for

10 min. Eluted samples were then centrifuged at 20,000 xg for 5 min. The supernatant was kept and lyophilized using a speed vacuum (Fisher Scientific; Waltham, MA) and stored at -20°C.

Nanoflow Reverse Phase Liquid Chromatography (RPLC) Separation and Mass Spectrometry Analysis:

In order to analysis purified and phosphorenrched peptide (His-tagged recombinant UCP1 and immunopurified UCP1 from BAT mitochondria), samples were subjected to high performance (pressure) liquid chromatograph, which was also equipped with a degasser and a quaternary surveyor MS pump (Agilent; Palo Alto, CA). Proper separation of peptide fragments was achieved using 0.1% formic acid (FA) in water (mobile phase A) and 100% acetonitrile (mobile phase B). For proper separation of peptides, a gradient solution was used, which consisted of 0-3% of mobile phase B for 2 min, 3-25% of mobile phase B for 90 min, 25-80% of mobile phase B for 8 min, 80% of mobile phase for 10 min, 80-100% of mobile phase B for 5 min, and 100% of mobile phase B for 30 min. Peptide fragments were analyzed using the LTQ-Orbitrap Mass Spectrometer (Thermo Scientific; San Jose, CA) that was set to detect a mass to charge (m/z) ranging between 400 to 2000. Ion fragmentation was applied such that less intense ions were excluded using a set protocol as follows: repeat count 2, repeat duration 30s and an exclusion duration of 60s. Data analysis involved the use of the matrix mascot search database (Matrix Science; Boston, MA) with a mass to charge cutoff score of 25.

Bioinformatics:

Bioinformatic analysis was conducted in order to locate the potential sites of phosphorylation within the UCP1 sequence. The online database phosphomouse (Harvard; Boston, MA) was used to predict the location of serine, threonine and tyrosine phosphorylation residue. Furthermore, candidate kinases were determined using a combination of NephosK1.0 (Lyngby, Denmark) and the group based prediction system (Anhui, China). Subsequently, covalent modifications were detected by isoelectric focusing and subsequent shifts in isoelectric points were verified using PhosphositePlus (<http://www.phosphosite.org/isoelectricCalcAction.do?id=14307&residues=13>).

MCK-UCP1 Transgenic Mice:

Transgenic male MCK-UCP1 mice (referred to hereafter as Tg) were kindly provided by Dr. Frédéric Bouillaud, Université Rene Descartes, Paris, France and through the laboratory of Dr. Daniel Kelly. Tg mice were previously developed using cDNA coding rat UCP1 associated with the SV40 polyadenylation sequence, which was inserted seven base pairs from the transcriptional start site of a muscle specific creatine kinase promoter construct (49). UCP1 Tg mice were of hemizygotic background backcrossed into C57Bl/6J mouse strain at least 8 times at the Sanford Burnham Medical Research Institute (Orlando, FL). Genotypes were confirmed by PCR amplification of a 442 base pair gene fragment using sense and anti-sense primers, gtgaaggtcagaatgcaagc and tgacgttcaggatccgagtcgca, respectively. Mice were housed singly at room temperature (22-23°C) with a normal light cycle (06:00-18:00 light; 18:00-06:00 dark). They were *ad libitum* fed a standard chow diet (44.2% carbohydrate, 6.2% fat and 18.6% crude protein; T.2018-Harlan). In addition, some mice were CE for indirect calorimetry analysis, as

described above. All experiments were approved by the Animal Care committee at the University of Ottawa in accordance with the guidelines of Canadian Council of Animal Care and were conducted in male mice aged 7-12 weeks.

Whole Body and Organ Weights:

Whole body, tissue and organ weights were collected from *ad libitum* fed Tg and Wt littermates post indirect calorimetry at room temperature and after CE. BAT, skeletal muscle, white adipose tissue (gonadal), liver, kidney and heart were all extracted blotted and weighed using the Mettler AE 50 balance (Mettler Toledo; Columbus, OH).

Food Intake:

Daily food intake and body weight changes were determined over a one month period with mice individually housed starting at 4 weeks of age. Briefly, body weight and food intake measurements were taken at the same hour (1-2pm) every 3-4 days. Mice were fed a standard chow diet as described above

Indirect Calorimetry:

Whole body oxygen consumption and respiratory exchange ratios were measured for mice housed in a 4-chamber Oxymax open circuit indirect calorimeter (Columbus Instruments; Columbus, OH). Mice were provided with water and their standard diet during measurements. Chambers were supplied with air at 0.5 L/min and maintained at room temperature (22-23°C)

with the normal light dark cycle (light 6:00-18:00; dark 18:00-6:00). Concentrations of O₂ and CO₂ were measured in each chamber for 60s every 4 min with a sample line-purge time of 2 min, as previously described (65). Data collection began after a 2h acclimatization period. For CE experiments, following initial measurements for 24h at ambient room temperature the temperature was decreased to 4°C to test the impact of ectopic UCP1 expression in muscle on adaptive thermogenesis. Measurements were taken for an additional 24h period and initiated 1h after the temperature was decreased.

In Vivo Assessments of Glucose Uptake using Positron Emission Tomography (PET):

Imaging was performed using an Inveon small animal scanner (Siemens, Knoxville, TN). Mice were weighed and saphenous vein blood glucose concentration was measured using an ACCU-CHEK Advantage glucometer (Roche; Laval, QC). Next, mice were anesthetized with 1.5-2.5% isoflurane and injected intravenously with ~ 1mCi [¹⁸F] fluoro-2-deoxyglucose ([¹⁸F] FDG) in a 100 µl volume, and subjected to an 80 min dynamic scan (constant rate of infusion 1.66 µl/min). An adrenergic agonist, norepinephrine (NE) (1 mg/kg of body weight) was administered intraperitoneally at 20 min into the 80 min scanning protocol, and the rate of [¹⁸F] FDG uptake was measured. Data analysis was performed using the Inveon Research Workplace software to determine the standardized uptake values (SUVs), which were defined as activity concentration/injected dose x body weight, based on regions of interest (ROI).

Immunoblotting:

Protein samples were diluted to 2mg/mL in Laemmli buffer boiled for 5 min and loaded into a gel that was precasted using the Mini-Protean II electrophoresis unit (Bio-Rad; Mississauga, ON). Resolving gels were prepared to final concentrations of 12% acrylamide/0.45% bis-acrylamide, 0.372M M Tris-base (pH 8.8), 0.1% sodium dodecyl sulfate (SDS), 0.05% ammonium persulfate (APS), and 50 μ l of N,N,N',N'-tetramethylethane-1,2-diamine (TEMED). The resolving gel mixture was poured into the gel cast and then overlaid with 100% isopropyl alcohol to prevent drying. Once polymerized the isopropyl alcohol was poured off and the top of the gel was dabbed dry. Next, the stacking gel was carefully poured with 1.5 mm combs inserted and was allowed to polymerize for 20 min at room temperature. The stacking gel was composed to a final concentration of 4.0% acrylamide/ 0.36%bis-acrylamide, 0.125M Tris base (pH 6.8), 0.1% SDS, 0.1% APS and 50 μ l TEMED). The gel assembly was carefully placed within the electrophoretic unit (Bio-Rad; Mississauga, ON) and gel wells were washed 2 x with electrophoresis buffer (192 mM glycine, 25 mM Tris-Base, and 0.1% SDS). Electrophoresis was conducted at a constant voltage of 100V which was increased to 150V once the running front entered the resolving gel. Upon completion gel-slabs were equilibrated in transfer buffer (50 mM Tris-base, 38 mM glycine, 20% methanol, and 0.1% (w/v) SDS) for ~10 min with constant agitation. Using the Mini-PROTEAN tetra cell (Bio-Rad; Mississauga, ON) a transfer sandwich consisting of two sponge fiber pads, four filter papers, a gel, and a nitrocellulose membrane was constructed and placed within the transfer apparatus with a block of ice. The assembled sandwich was electrophoresed at 100V for 1h at room temperature using the Bio-Rad power pac Basic 300 (Bio-Rad; Mississauga, ON). Membranes were then blocked with Tris-buffered saline containing 1% (v/v) Tween-20 (TBS-T) and 5% (w/v) non-fat powdered skim milk for 1h at

room temperature and then probed with a rabbit polyclonal anti-UCP1 antibody (1/5000; Sigma, St. Louis, MO). Rabbit polyclonal anti-succinate dehydrogenase (SDH) antibody (1/4000, Santa Cruz Biotechnology; Santa Cruz, CA) was used to establish the SDH loading control. Blots were incubated overnight at 4°C with constant agitation. Membranes were then washed 3x with TBS-T for 5 min and probed with secondary antibodies (goat anti-rabbit horseradish-peroxidase (conjugated) at 1/2000; Santa Cruz; Santa Cruz, CA) for 1h at room temperature. Membranes were then exposed using an enhanced chemiluminescent substrate for visualization (ECL Kit; Thermo Scientific, Waltham, MA) as previously described (136, 163)

Isolation of Mitochondria from Skeletal Muscle:

Mitochondria were isolated from skeletal muscle using differential centrifugation as described in (71, 136). All steps were performed on ice or at 4°C and all buffers were filtered using a 0.2micron filter (Sarstedt; Newton, NC). Briefly, hind limb, and forelimb muscle was excised and immediately placed in ice cold basic medium (BM;140 mM KCl, 20 mM HEPES, 5 mM MgCl₂, 1 mM EGTA, 10 mM pyruvate, and 2 mM malate; pH 7.0). Muscle was then cleaned of connective tissue and fat, blotted dry, weighed, and then minced on Teflon plates. The muscle was then placed in ~30 mL of homogenization media (BM+ 1 mM ATP, 1U subtilisin A and 1% (w/v) defatted BSA) and homogenized using an ice-cold glass/Teflon Potter-Elvehjem tissue grinder as follows; minimum 10 passes with a pestle that has higher clearance. Samples were then homogenized further (minimum 10 passes) using a pestle with lower clearance. This method ensures that muscle tissues are properly broken apart without disrupting mitochondrial

membrane integrity. Muscle homogenate was then centrifuged at 800 xg to pellet debris and connective tissue. The supernatant was then collected and centrifuged at 9500 xg for 9 min. The solution was decanted carefully and the inside of the tube was blotted dry to remove excess fat and HM without disturbing the pellet. The pellet was then resuspended in ~2 mL of BM and incubated for 5 min to induce myofibril repolymerization. The volume was then adjusted to ~30 mL with BM and centrifuged at 800 x g for 9 min to remove myofibers. The pellet was then discarded and the supernatant was centrifuged at 9500 xg for 9 min to pellet mitochondria resuspended in BM, and protein content was determined by the Bradford assay using 200ug/mL standard of BSA at a wavelength of 595 nm as previously described.

Mitochondrial ROS Emission:

Mitochondria isolated from BAT and skeletal muscles were tested for ROS emission using the fluorometric probe 2', 7'-dichlorodihydrofluorescein-diacetate (H₂-DCFDA) (Invitrogen; Burlington, ON). Mitochondrial preparations were diluted to 0.5mg/ml in reaction buffer C (20 mM Tris-Base, 125 mM sucrose, 2 mM MgCl₂, 4 mM KH₂PO₄, 1 mM EDTA) containing 20 μM H₂-DCFDA and warmed to 37°C for ~5 min. Baseline ROS measurements were taken for a period of 4 min with data points collected at 1 min intervals. Measurement of kinetics of mitochondrial ROS emission was initiated by the addition of substrate (5 mM pyruvate, 3 mM malate). Oligomycin (1.3 μg/mL; increases membrane potential by inhibiting ATP synthase and thus also inducing state 4o respiration) was added to test ROS emission under state 4o respiratory conditions in 1 min intervals for a period of 4 min. Next, 1 mM GDP was added to test ROS emission when UCP1 is inhibited. For all steps, ROS emission was measured for 4-6 min with 1 min measurement intervals at an excitation of 480 nm and emission of 528 nm.

Assays were carried out using BioTEK Synergy MX microplate reader and Gen5 software (BioTek; Winooski, VT). Data were normalized to background fluorescence and mitochondrial protein. Data were expressed as a fold change from substrate only.

Total Glutathione Measurements:

Total mitochondrial glutathione (reduced glutathione; GSH + oxidized glutathione disulfide; GSSG) was measured using the Agilent 1100 Series high performance liquid chromatography (HPLC) system (Agilent Technologies; Santa Clara, CA) (138). Briefly, isolated mitochondria from BAT or skeletal muscle were diluted to 0.5 mg/ml in 0.1% perchloric acid solution and incubated on ice for 10 min. Precipitate was then removed by centrifugation at 12,000 $\times g$ at 4°C for 10 min. The supernatant was carefully collected and then injected into an Agilent Binary Pump 1100 HPLC system equipped with a Pursuit C₁₈ column (150 $\times$ 4.6 mm, 5 μ m; Agilent Technologies; Santa Clara, CA) and operating at a flow rate of 1 mL/min. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid diluted in ddH₂O:HPLC-grade methanol (90:10). Mobile phase was filtered with 0.2micron filters prior to use (Sarstedt; Newton, NC). GSH and GSSG were detected using an Agilent variable wavelength detector set at 215 nm. Retention times of GSH and GSSG were determined by injecting standard GSH and GSSG solutions. Samples were quantified using the Agilent ChemStation software. Briefly, different volumes of 0.1mM and 1mM GSH and GSSG standard solutions were injected into the HPLC to generate standard curves with nmol GSH or GSSG as a function of x-axis and integration value for either peak a function of the y-axis. Area under GSH and GSSG peaks in each sample were then determined using Agilent Chemstation software. The resulting integration values were then

extrapolated from standard curves and the amount of total glutathione was calculated. All values were corrected to protein providing a quantitative value of nmol total glutathione/mg protein.

Measurement of Protein Carbonyls:

To determine if muscle specific expression of UCP1 induced oxidative stress, we measured the total amount of protein carbonyl adducts in *gastrocnemius* muscle using the Oxyblot assay kit (Millipore; Billerica, MA). *Gastrocnemius* muscles from Tg and Wt mice were placed in ice cold buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton-X 100, 50 mM, NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2% β -mercaptoethanol, 4 mg/L leupeptin, 50 mg/l trypsin inhibitor, and 0.1 mM benzamidine. The muscle was homogenized on ice for a minimum of 20 passes using the Tissue Tearor (BioSpec; Bartlesville, OK). Following homogenization the sample was centrifuged at 14,000 xg for 20 min at 4°C. The supernatant was kept and pellet was discarded. A Bradford assay was conducted following the addition of 2% β -mercaptoethanol to standards in order to correct for background reactions. Next, 15 μ g of protein aliquots of each sample were prepared either for derivatization or as negative controls. Proteins were denatured by addition of 12% SDS for a final concentration 6% to each sample. In addition, half of the samples were treated with 2, 4-dinitrophenyl hydrazine (DNPH) in order to derivative protein carbonyl adducts and the other half was treated with 1x derivatization-control solution (negative control) for 15 min at room temperature with periodic agitation. The reaction was stopped by addition of equal amount of neutralization solution to each sample as specified by the manufacturer's instructions. Samples were electrophoresed on a 12% isocratic gel at 150V for ~1h and transferred onto a nitrocellulose membrane at 100V for 1h using the Bio-Rad Power Pac Basic 300 (Bio-Rad; Mississauga, ON). Immunodetection proceeded by blocking in 1%

BSA/Tris-buffered saline solutions containing 1% Tween 20 for 1h followed by incubation overnight at 4°C in 1/150 primary antibody (rabbit Anti-DNP Ab, provided in the Oxyblot assay kit). Immunoblots were incubated with goat anti-rabbit IgG HP-conjugate at 1/300 for 1h and protein bands were detected by ECL substrate (Thermo Scientific; Waltham, MA).

Statistics:

Statistical analyses were done using a Student's t-test, or a two way ANOVA with a Fischer post-hoc test using Excel software (Microsoft, Redmond, WA) and Statsview 5.0 software (SAS Institute; Cary, NC), respectively. Data are presented as means $\pm$ SEM.

RESULTS:

INVESTIGATION OF UCP1 PHOSPHORYLATION AND ITS POTENTIAL ROLE IN THE CONTROL OF BROWN FAT THERMOGENESIS:

Proton leaks through UCP1 in brown fat mitochondria are well documented to be controlled by allosteric regulators. Proton conductance through UCP1 is inhibited by purine nucleotides (e.g., GDP and ADP), while cold exposure liberates FA (e.g., oleate) that bind to and activate UCP1 (56, 69). However, recent work by Carroll and colleagues has indicated that UCP1 is phosphorylated on, Ser³, Ser⁴, and Ser⁵¹ following cold exposure (29). Thus, it would appear that UCP1 is regulated by multiple mechanisms *e.g.*, allosteric inhibition and covalent modification. With this in mind, we decided to further explore the possibility that UCP1 is regulated by phosphorylation. Prior to testing if UCP1 can be phosphorylated, bioinformatics analysis was conducted. Using the phosphomouse database (<https://gygi.med.harvard.edu/phosphomouse/index.php>) it was predicted that UCP1 contained a phosphorylation residue in the second matrix loop region on serine 50 (Ser 50) (**Table 1.1**).

Table 1.1: Serine 50 identified as a phosphorylation residue in UCP1 by Phosphomouse analysis. Using the database Phosphomouse Serine 50 of the UCP1 mouse sequence was predicted as a potential phosphorylation residue. The ambiguity score (Ascore) calculated is a metric for determining the likelihood that the matched site was determined by chance based on the highest ion scores. An Ascore ≥ 19 indicates a 99% certainty of phosphorylation residue with a $p = 0.01$, an Ascore between 15-19 indicates a 90% certainty, an Ascore between 3-15 indicates a certainty of 80%, and an Ascore of < 3 indicates little to no proper information in order to predict phosphorylation residue.

Site Position	50
Peptide	LQIQGEGQA S STIR
A score	11.50

Next, using the group-based prediction system (GPS) kinase we determined which kinases may be required to phosphorylate this matrix loop region (238). Using cut-off and GPS scores, protein kinase A (PKA), protein kinase B (PKB) and mitogen activated protein kinase (MAPK) were identified as likely kinase candidates (**Table 1.2**). It is important to note that these kinases have been found to localize to the mitochondrial matrix (3, 30). Following the proteomic predictions of UCP1 phosphorylation, we next measured the energetics of mitochondria isolated from BAT of WA or CE mice. For this mitochondria were isolated from the brown fat of C57Bl/6J mice exposed to cold (CE, 4°C) for 24h. Mice exposed to ambient room temperature conditions (WA, 22-23°C) served as the control. Using a recently published technique by Rogers *et al.*(189) we assessed mitochondrial oxygen consumption using the Seahorse XF24 analyzer. We first measured coupled respiration in mitochondria. Unlike other tissues and organs, BAT mitochondria have low amounts of F_0F_1 ATP synthase, the molecular motor responsible for using the proton gradient in mitochondria for ATP production. BAT is specialized for heat production and thus uses PMF for thermogenesis rather than ATP synthesis. Measurements were carried out by first testing respiration under state 4 conditions (substrate only, 10mM pyruvate and 2mM malate) followed by assessment of ADP (0.1 mM) stimulated respiration (state 3) proton leak-dependent respiration (oligomycin 2.5 µg/mL; State 4o) and decoupled respiration (FCCP-4.5 µM). As expected, proton leak dependent respiration accounted for a substantial amount of O_2 consumption in BAT mitochondria from WA and CE mice (**Figure 1.6**). Indeed, ADP did not stimulate respiration. In fact, ADP treatment actually decreased O_2 consumption specifically in mitochondria from CE mice consistent with its inhibitory effect on UCP1 (decrease in ~38.5% relative to state 4). Most importantly, overall respiration was significantly higher in mitochondria from CE mice. These results confirm previous reports that 1) proton leaks

account for most of BAT mitochondrial respiration and 2) cold exposure drastically increases respiration in BAT (204). Next, we assessed the contribution of proton leaks through UCP1 to total O₂ consumption in BAT mitochondria from WA and CE mice. Sequential titration of GDP, a UCP1 inhibitor, up to 1 mM led a significant decrease in respiration only in mitochondrial preparations from CE mice. Calculation of the contribution of proton leaks through UCP1 to total respiration in CE mitochondria revealed that leaks through UCP1 account for 43% of respiration (**Figure 1.6**). No significant changes in respiration were observed in mitochondria from WA mice following GDP titration. CE is also well documented to increase UCP1 protein levels in mitochondria, a response required for enhanced thermogenesis. Immunoblot detection showed increased UCP1 protein expression following CE (**Figure 1.7**). Thus, our results confirm that CE increases proton leak through UCP1 which is in part due to 1) the activation of UCP1 by liberated FA and 2) an increase in UCP1 protein content. These results also confirm that our cold exposure model is appropriate for analyzing UCP1 regulation.

UCP1 Displays Covalent Modification Indicative of Phosphorylation:

We next sought to determine if cold exposure induces PTM of UCP1 as previously documented (49). We decided to use two-dimensional (2D) gel electrophoresis as a method to determine global covalent modification of UCP1 because of its general reproducibility. Briefly, protein separation is conducted using the principle of isoelectric point (pI) and an immobilized pH gradient (IPG) in the first dimension of electrophoresis. The native protein migrates towards either end (cathode or the anode) of the IPG until the reduced protein reaches its point of

neutrality. Subsequently, proteins in the IPG are then denatured and separated by mass for immunodetection using 12% isocratic SDS gel. Furthermore, protein migration from its native pI is correlated with the specific type of modification. Phosphorylation causes a protein to become acidic as indicated by the theoretical shifts of UCP1 (**Table 1.3**). Mitochondria were isolated from WA and CE mice and subjected to 2D-electrophoresis. Results indicate that CE causes a shift in the pI of UCP1 that was absent in WA samples (**Figure 1.8**). In order to confirm phosphorylation of UCP1 following CE, mitochondria were permeabilized with digitonin and treated with alkaline phosphatase. Note that for these determinations phosphatase inhibitors were excluded from the mitochondrial preparations to avoid inhibition of the phosphatase. Treatment of mitochondrial extracts from CE mice with shrimp alkaline phosphatase restored the pI of UCP1 (**Figure 1.8**). By contrast, alkaline phosphatase treatment had no effect on the pI of UCP1 in mitochondrial extracts from WA mice. These results indicate that UCP1 is covalently modified following cold acclimation as indicated by the shift in pI. Further, our findings indicate that the shift in pI is potentially due to UCP1 phosphorylation.

Table 1.2: Predicted Phosphorylation residue using the group-based phosphorylation scoring method. GPS scores are generated based on a predictive algorithm that matches the sequence motifs recognized by the respective kinases. A cut-off score is generated in order to reduce the likelihood of false positives.

Position	Peptide	Kinase	GPS score	Cut-off score
31	DIITFPL	PKB	10.000	5
36	PLDTAKV	IPL1(yeast)	7.000	6
76	KLYSGLP	PAK	12.000	3.5
87	RQISFAS	AMPK	6.429	4
87	RQISFAS	CaM-II	6.027	4
87	RQISFAS	ILK	12.000	12
87	RQISFAS	MAPKAPK2	4.778	4
87	RQISFAS	PAK	6.000	3.5
87	RQISFAS	PHK	4.765	4
87	RQISFAS	PKA	5.544	4
87	RQISFAS	PKC	3.043	3
109	GRETPAS	P34CDC2	5.000	5
118	NKISAGL	MAPKKK	13.000	12
169	TTESLST	DNA-PK	3.333	3
169	TTESLST	IKK	3.946	3.2
172	SLSTLWK	PKB	14.000	5
178	KGTTPNL	IPL1(yeast)	6.000	6
243	FINS LPG	RAF1	11.500	9
248	PGQYPSV	IGFR	6.696	6
253	SVPSCAM	S6K	9.000	9
260	SMYTKEG	S6K	12.000	9
31	DIITFPL	PKB	10.000	5

http://csbl.bmb.uga.edu/~ffzhou/gps_web/results.php

Figure 1.6: Cold exposure of mice leads to an overall increase in the rate of BAT mitochondrial oxygen consumption. BAT mitochondria were isolated from CE and WA mice and were diluted to 0.05 mg/ml for bioenergetic determinations. Briefly, mitochondria were attached to surface of a 24 well plate and respiration was analyzed using the XF24 Extracellular Flux Analyzer. **A)** Measurement of UCP1-dependent proton leaks. Following a measurement of non-phosphorylating respiration, mitochondria were treated with oligomycin (2.5µg/mL) to eliminate any respiration still associated with ATP synthase and then titrated with GDP to inhibit UCP1. **B)** Measurement of phosphorylating respiration Following measurement of state 4, mitochondria were treated with ADP (100µM), oligomycin (2.5ug/mL) and maximal respiration (FCCP 8µM) to measure state 3, state 4o (respiration not associated with ATP synthase), and maximal respiration. All values are corrected for extra mitochondrial respiration by antimycin A (4µM) treatment. Statistical significance was determined using a two way ANOVA with a Fisher post-hoc test N=4, n.s. - not significant; * and ** correspond to $p \leq 0.05$ and 0.01. CE (Cold exposure) and WA (Warm acclimated or ambient temperature (22-24°C))

Figure 1.6: Cold exposure of mice leads to an overall increase in the rate of BAT mitochondrial oxygen consumption.

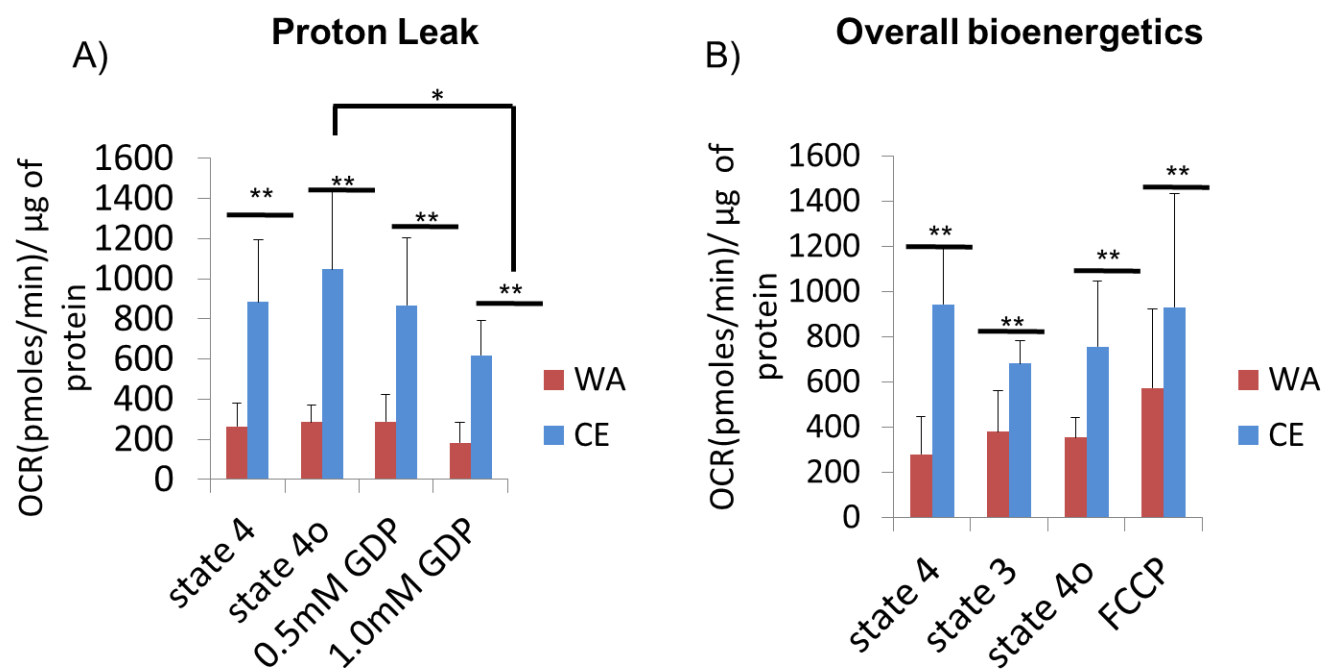
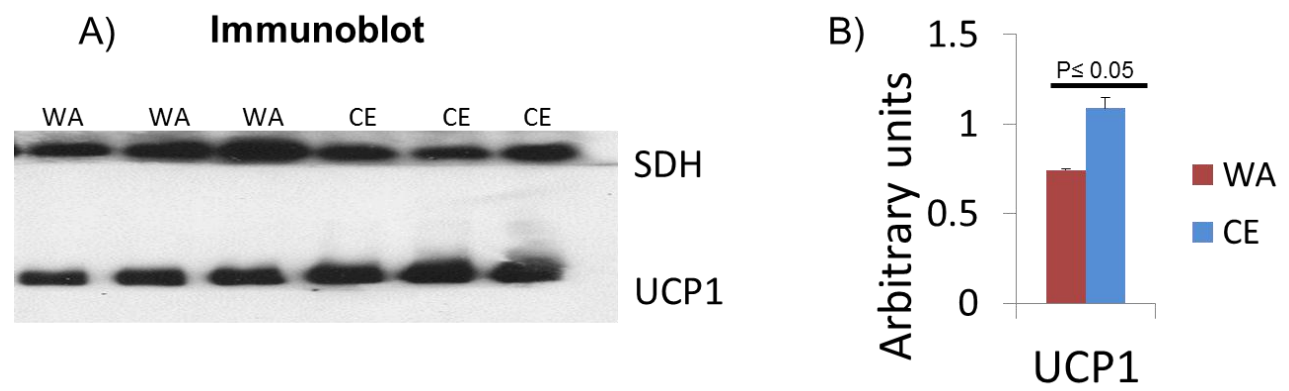


Figure 1.7: Cold exposure of mice increases UCP1 protein levels in BAT. **A)** Mitochondria were isolated from BAT of mice from which were CE or WA and subjected to Western blot analysis. 10ug of mitochondrial protein were loaded per well, and succinate dehydrogenase (SDH) was used an internal loading control. **B)** Densitometry using the software *Image J*. Student's *t*-test N=3, CE (Cold exposure) and WA (Warm acclimated (22-24°C))

Figure 1.7: Cold exposure of mice increases UCP1 protein levels in BAT.



We next conducted mass spectrometry analysis to determine the specific sites of phosphorylation. In comparison to the 2D gel electrophoresis, mass spectrometry provides increased sensitivity for covalent modification and requires less time to run and smaller quantities of protein samples (177). His-tagged recombinant and immunopurified UCP1 from isolated mitochondria (WA and CE mice) were used to confirm the presence of phosphorylation residue. Following mass spectrometry analysis we identified multiple phosphorylation residue in the recombinant His-tagged UCP1 (**Figure 1.9**). The locations of these modifications were shown to be in the first and second matrix loop, as well as in the second trans-membrane region (**Figure 1.10**). To validate our findings, isolated mitochondria from WA and CE mice were subjected to a cleanup protocol and an immunopurification (IP) using protein A beads. Briefly, we optimized our cleanup protocol by first adjusting the concentration of our anti-UCP1 antibody from 10µg to 5µg. Next, we increased the time from 10 to 30 min required for antibody coupling to protein A beads and eluted our coupled protein samples for 30 min at 4°C instead of 10 min at room temperature. We validated our IP protocol by immunoblotting for UCP1 as well as silver staining (**Figure 1.11A**). As a control, using BAT mitochondria not used for IP we were able to confirm that UCP1 protein can be detected in IP samples. UCP1 was not detected in liver mitochondrial extracts subjected to the same IP protocol (**Figure 1.11B**). Prior to mass spectrometry analysis, UCP1 IP samples were electrophoresed and silver stained. Prominent bands at ~32kDa, which corresponds to the mass of UCP1, were cut and analyzed for phosphorylation status. In addition, bands indicative of possible interacting partners were excised, digested and analyzed by mass spectrometry (**Figure 1.12**). Subsequently, a total of 25 different proteins were identified and analyzed from the excised bands using the HPLC-ESI-MS/MS generating ~ 100, 000 MS/MS spectra. Based on the bands excised and MS/MS spectra

generated, a list of interacting candidates was generated using the Mascot Analysis Software (Matrix Science; Boston, MA). Concisely, Mascot analysis is conducted using a simple probabilistic model to calculate the probability that the spectrum for each peptide detected could be produced by chance, and this calculation is converted into a relative score. Following the generation of the peptide score a pre-defined limit is imposed and all other peptide scores not above threshold is excluded. In addition, the analytic program, Prohits (Protein High-throughput solutions; Toronto, ON), was used to refine the list of candidate proteins generated that received a Mascot score of 25 and a p-value of 0.05 (128). An interactome was then generated using Ingenuity Pathway analysis software (Ingenuity Pathway; Redwood City, CA) based on UCP1 IP from WA and CE mice. Interestingly, the UCP1 IP from WA mice showed an indirect interaction with acyl-CoA dehydrogenase long chain (gene name: ACADL) and short-chain acyl-CoA dehydrogenase (gene name: ACADS) isoforms (**Figure 1.12A**). In contrast, UCP1 IP from CE mice mitochondria displayed indirect interaction with ACADL, ACADS, mediator complex subunit 1 (MEDS1; a peroxisome proliferator-activated receptor binding protein), Fatty acid translocase (CD 36 molecule), protein kinase A (PKA), and cAMP dependent protein kinase type II-beta regulatory subunit beta (PRKAR2B) (**Figure 1.12B**). However, despite these potential interactions with potential kinases, analysis of our results indicated that UCP1 IP for both samples isolated from WA and CE mice showed no potential phosphorylation residues (**Figure 1.13**).

Table 1.3: Hypothetical phosphorylation shifts based on predictions by Phosphosite Plus:

The isoelectric point (pI) is the region at which a protein is neutral on a standard titration curve. The pI of UCP1 based on amino acid sequence is predicted to be 9.29. Below is the theoretical shifts based on the addition of a negative charge by a phosphate group.

# of residues	pI
1	9.11
2	8.9
3	8.62
4	8.22
5	7.7
6	7.3
7	7.01
8	6.76
9	6.52
10	6.28
11	6.03
12	5.78

pI- (measured in pH)

Figure 1.8: Cold exposure induces an isoelectric shift in UCP1 indicative of phosphorylation. Isolated BAT mitochondria (200µg) from CE and WA mice were treated with 2% n-dodecyl-β-D Maltoside, 1% amidosulfbetaine, and 4-vinyl pyridine (1M) to rupture the mitochondrial membrane and solubilize the protein. Prior to isoelectric focusing solubilized proteins were treated with or without 8U of shrimp alkaline phosphatase. **A)** [-] indicates the absence of phosphatase, while **B)** [+] indicates the presence of phosphatase. Following isoelectric focusing of mitochondrial lysate, samples were electrophoresed to separate molecular weight and UCP1 was detected by immunoblot. Arrows indicate shifts in pI. N=3.

Figure 1.8: Cold exposure of mice induces an isoelectric shift in UCP1.

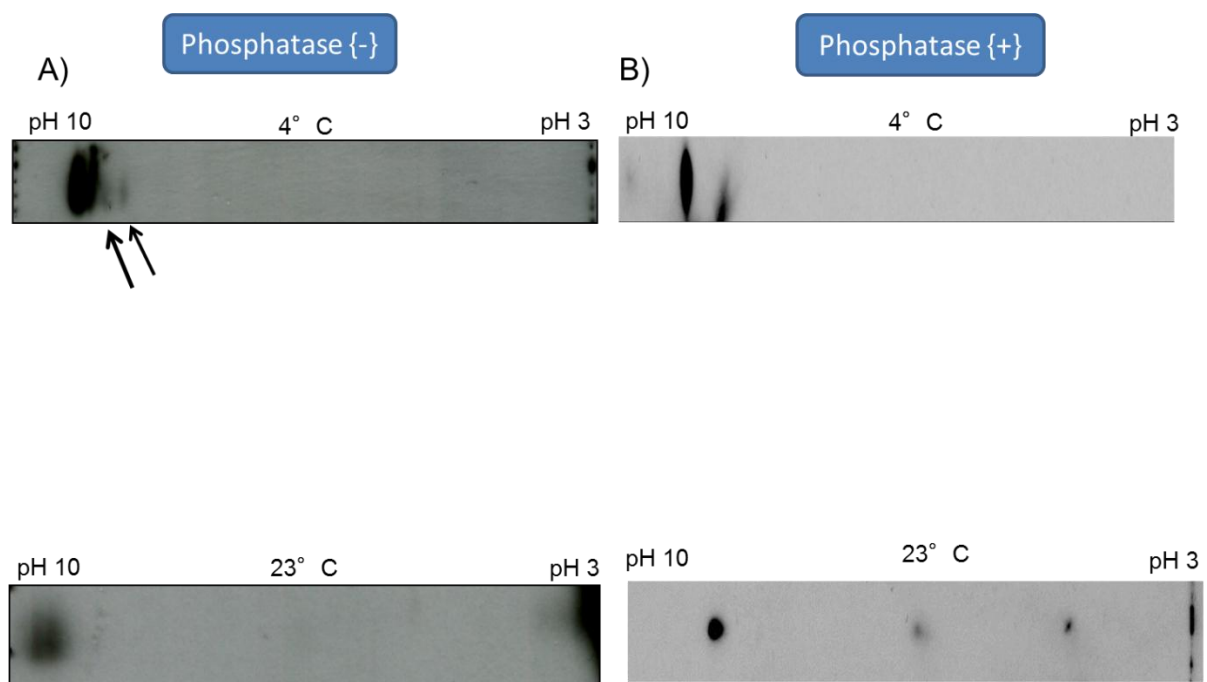


Figure 1.9: Phosphorylation residue identification of purified his-tagged UCP1. MS/MS spectrum of digested and purified his-tagged UCP1 was analyzed for potential phosphosites using the LTQ Orbitrap. Moreover, the fragment corresponding to the peptide sequence 1-16 of UCP1 was identified with a potential phosphorylation residue at residue 12 (threonine) based software analysis of raw spectrum by Mascot. N=2

Figure 1.9: Phosphorylation residue identification of purified his-tagged UCP1.

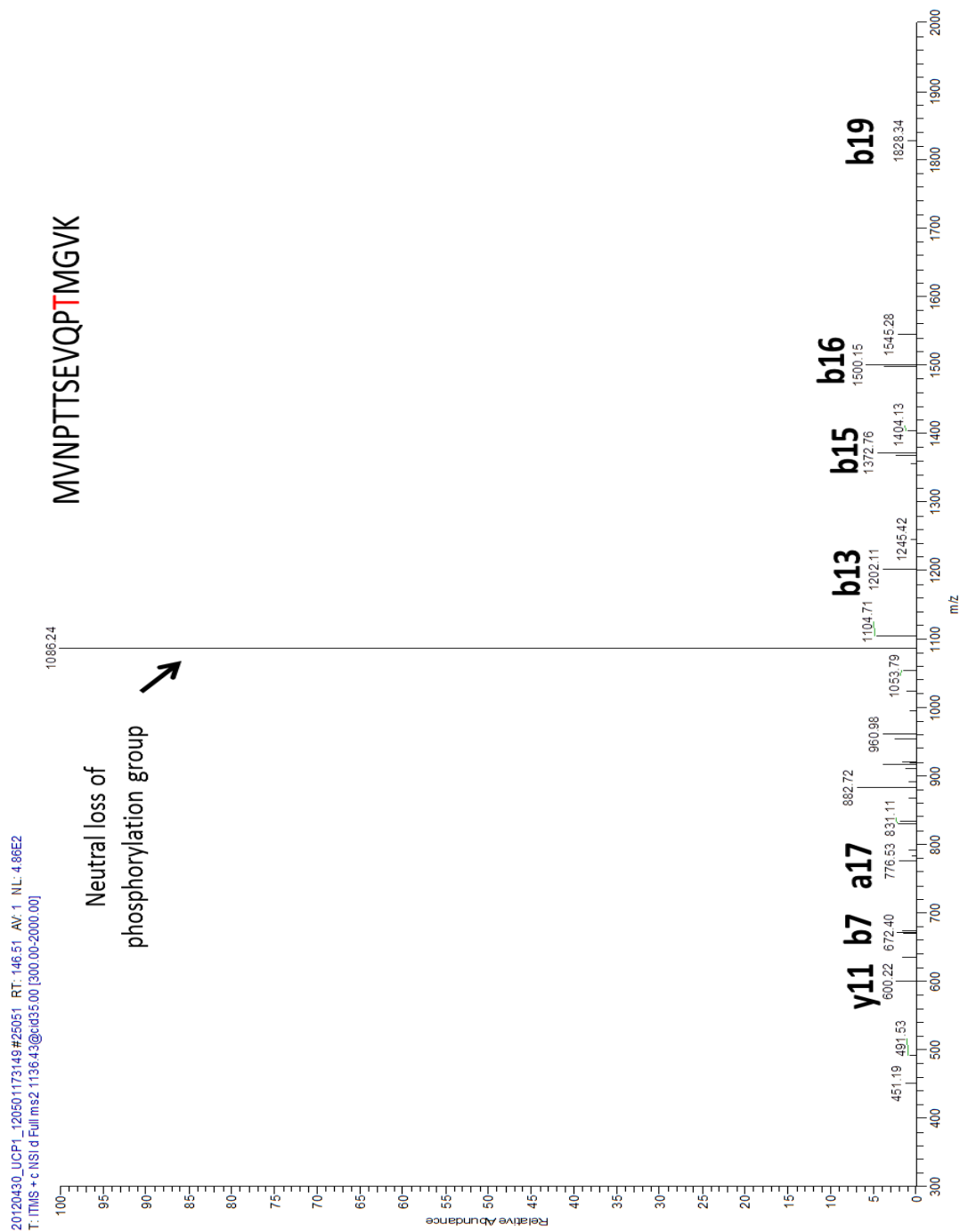


Figure 1.10: Two dimensional representation of RAT UCP1 amino acid sequence. Modified from (109). All shaded boxes indicate the regions of alpha helices with the orientation of the N to C terminal indicated left to right. Conserved residues among UCPs are indicated in green, while the UCP1 transmembrane regions are as follows (H1a, H2, H3a, H4, H5a, and H6). Black arrows refer to sites of phosphorylation shown in the literature by Carroll et al. (Ser³, Ser⁴, and Ser⁵⁰). Red arrow refers to phosphorylation residues identified by us (Thr¹²), while green arrow represents predicted phosphorylation residue based a predictive database phosphomouse (Ser⁵⁰).

Figure 1.10: Two dimensional representation of RAT UCP1 amino acid sequence. Modified from (109).

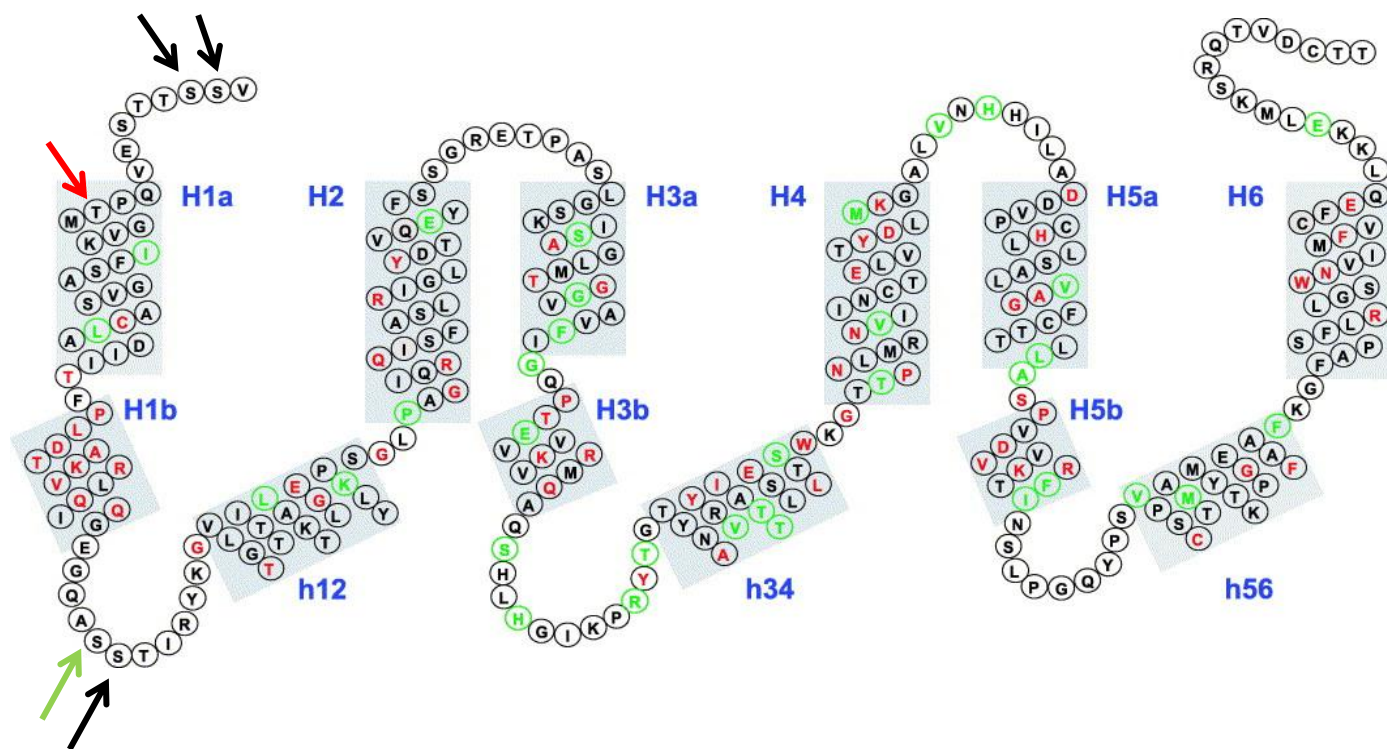


Figure 1.11: Purified UCP1 by immunoprecipitation and silver stained for interacting partners. **A)** 3 mg/ml of isolated mitochondria from WA and CE mice were sonicated and lysed using RIPA buffer. Protein extracts were subjected to an IP pull down using anti-UCP1 protein A beads. Eluted proteins were then electrophoresed, stained (sodium thiosulfate), and developed by 0.01% (v/v) formaldehyde and 2% (w/v) sodium carbonate. Note gel fragments 4 and 9 were shown to be highly abundant for UCP1 protein. **B)** UCP1 was immunopurified using anti-UCP1 antibody coupled to protein A beads. 5µg of isolated mitochondria from WA and CE mice were loaded as an input (INP) control. In addition, as a negative control IP liver mitochondria were loaded with a subsequent 5µg INP (warm acclimated WA; CE cold exposed (4°C) CE).

Figure 1.11: Purified UCP1 by immunoprecipitation and silver stained for interacting partners

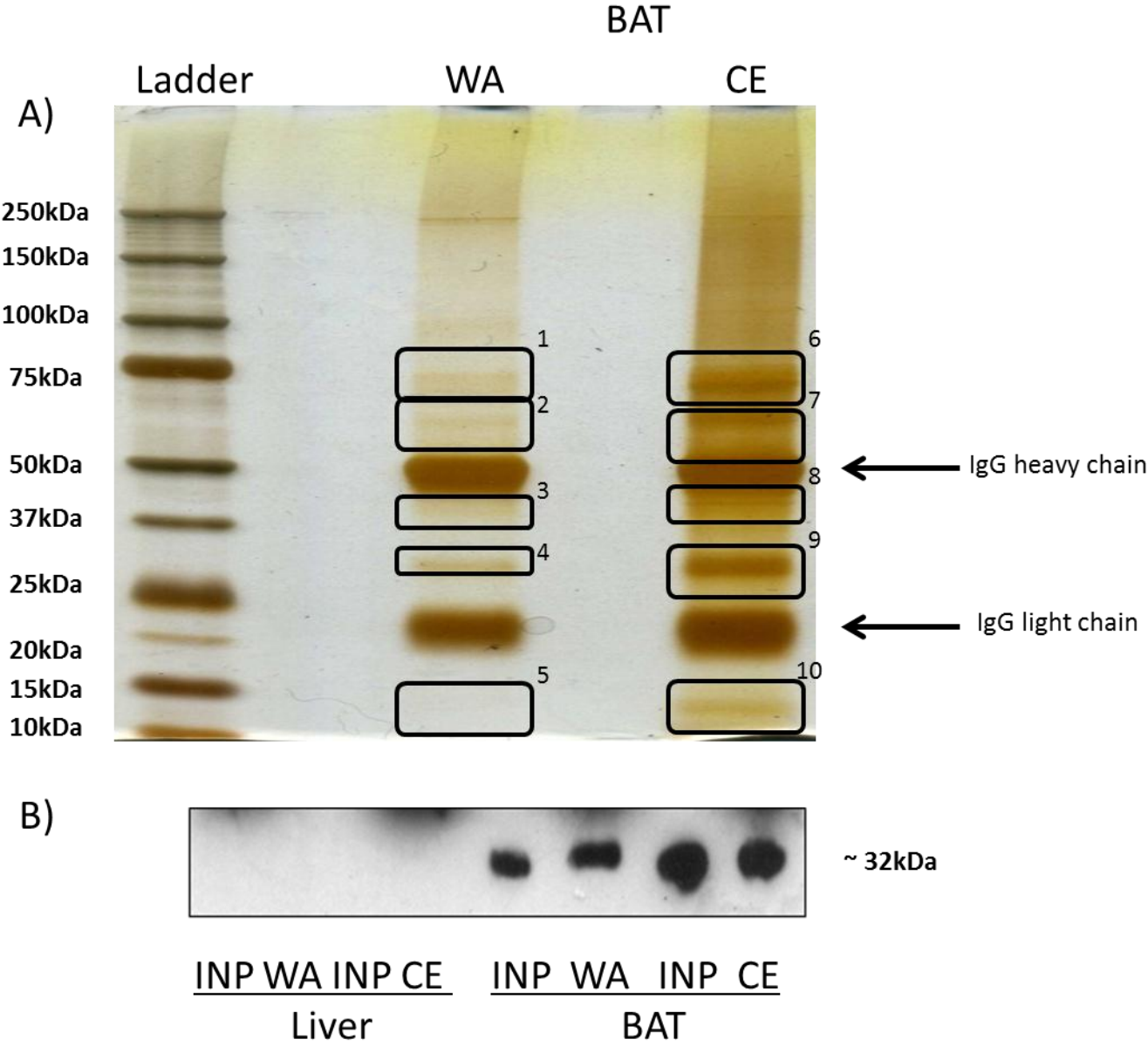


Figure 1.12: Immunopurified UCP1 from warm acclimated and cold exposed mice and its predicted binding partners. Following immunopurification of UCP1, samples were resolved by gel electrophoresis and silver stained. The banding pattern represents proteins that were enriched with UCP1 immunopurification. Protein bands were digested and analyzed using the LTQ Orbitrap mass spectrometer. Possible UCP1 binding partners were identified using Ingenuity Pathway Analysis software. Dashed lines indicate indirect interaction, while solid lines indicate direct interactors. **A)** Panel indicates IP UCP1 from WA mice. Isolated BAT mitochondria showed that UCP1 was a secondary hit in the ingenuity pathway with indirect interaction between ACADS- acyl-CoA dehydrogenase short chain and ACADL- acyl-CoA dehydrogenase long chain. Alternative proteins identified are RPL8- ribosomal protein R8, Cyp2b13/cyp269- cytochrome P450 family 2 subfamily B polypeptide 6 and 9, AGPAT9- 1-acylglycerol-3-phosphate o-acyltransferase 9, SERPINB1- serine peptidase inhibitor clade B member 1, acyl-coA thioesterase 2, KRT 23- keratin, CTBP2- c-terminal binding protein 2, GPD1-GPD2, glycerol-3-phosphate dehydrogenase 1 and 2, CDH1-cadherin 1, HNRNPA12B1-heterogeneous nuclear ribonucleoprotein A2/B1, SNRNPB2-small nuclear ribonucleoprotein polypeptide B, JUN-jun proto-oncogene, PPM1D- protein phosphatase Mg^{2+}/Mn^{2+} dependent, APEX multifunctional DNA repair enzyme 1/nuclease, ALB-albumin, HMGB1- high mobility group box 1, IL-12-interleukin-12, GOS2- GO/G1 switch protein 2, ACSL-acyl-CoA synthetase long-chain family member 6, SLC13A1-solute carrier family 13, MVP- major vault protein, AQP8- aquaporin 8, GPAM-glycerol-3- acyltransferase mitochondrial, HMGCS2- 3-hydroxy-3-methylglutaryl-coA synthase 2, PPARA- peroxisome proliferator activated receptor alpha, ELOVL3- fatty acid elongase 3, AKR1B7- aldo-keto reductase family 1 member 7, and C6,C8A and C9-complement component 6, 8 alpha, and 9 peptide.

B) UCP1-uncoupling protein 1 Isolated from CE mice was identified as the primary hit with direct and indirect interactions with the following candidates ACADS- acyl-CoA dehydrogenase short chain, ACADL- acyl-CoA dehydrogenase long chain, PRKAR2B- cAMP-dependent protein kinase type II-beta regulatory subunit, MED1- mediator complex subunit 1, CD 36- Fatty acid translocase. Alternative proteins identified are SLC25A20- solute carrier family 25 (carnitine/acylcarnitine translocase), ECH1-enoyl CoA hydratase 1, ACADM- acyl-CoA dehydrogenase medium chain, CPT2-carnitine palmitoyltransferase 2, HSDL2- hydroxysteroid dehydrogenase like 2, HADHB-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl CoA hydratase beta subunit, STARD4-STAR- related lipid transfer domain containing 4, NCOR- nuclear receptor corepressor, AMPK- AMP-activated protein-kinase, PDHA1, SUCLA12, MYH4, NF κ B, MAVS, PKA- protein kinase A, IL-12-interleukin-12, LYZ1/LYZ2- lysozyme 1 and 2, MGLL- monoglyceride lipase, TFAM- transcription factor A, PDK- pyruvate dehydrogenase kinase, POR- P450 (cytochrome) oxidoreductase, ATF58-ATP synthase (F1 complex beta polypeptide), ACSL3-acyl-CoA synthetase long-chain family member 3, ACACA- acetyl-CoA carboxylase alpha, LIPE-hormone sensitive lipase (testicular isoform), PRDX2- peroxiredoxin 2, HSD17B4-hydroxysteroid (17-beta) dehydrogenase 4, and CAMKK1- calcium/calmodulin-dependent protein kinase1.

Figure 1.12: A) Immunopurified UCP1 from warm acclimated mice and its predicted binding partners.

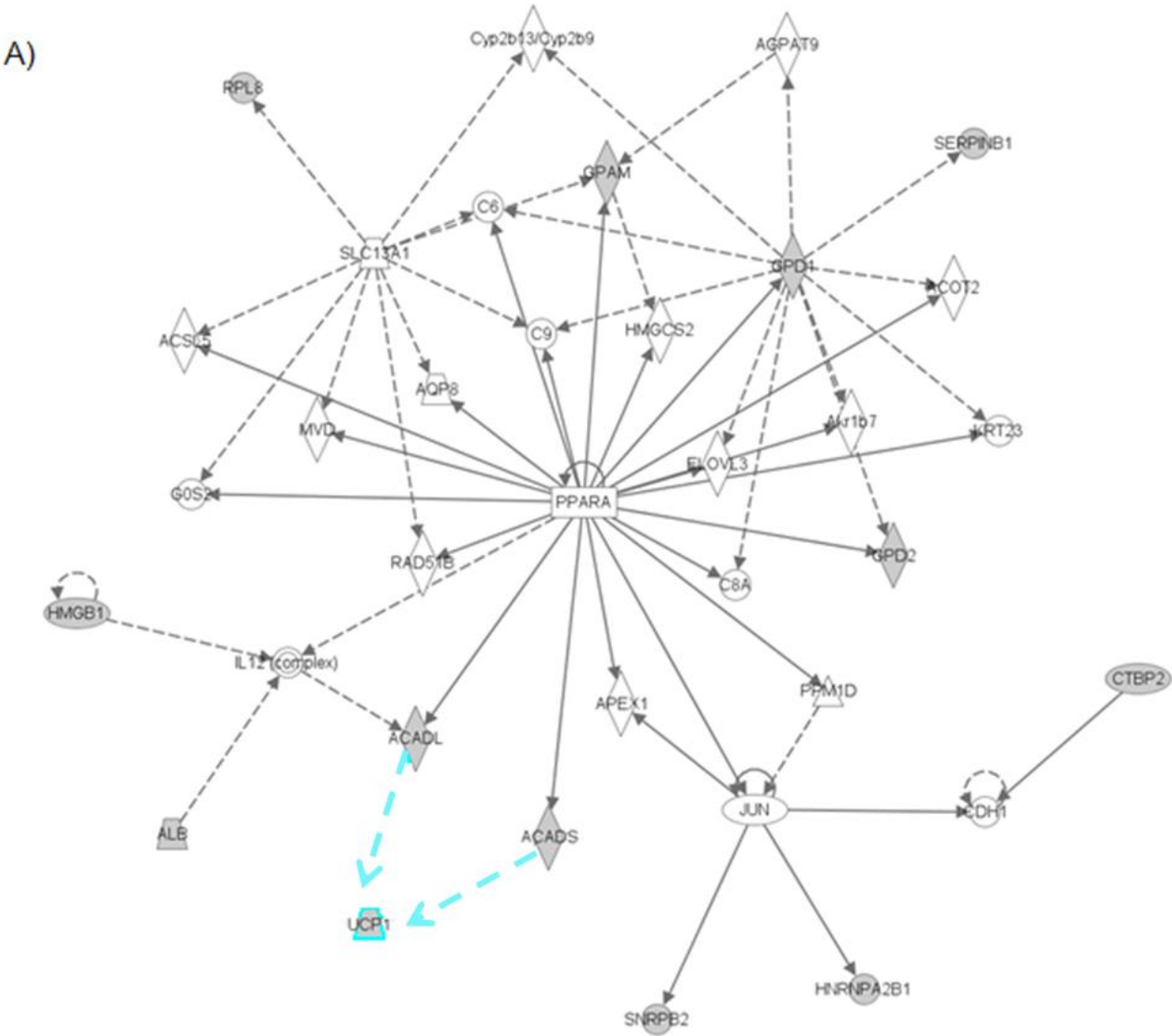


Figure 1.12: B) Immunopurified UCP1 from cold exposed mice and its predicted binding partners.

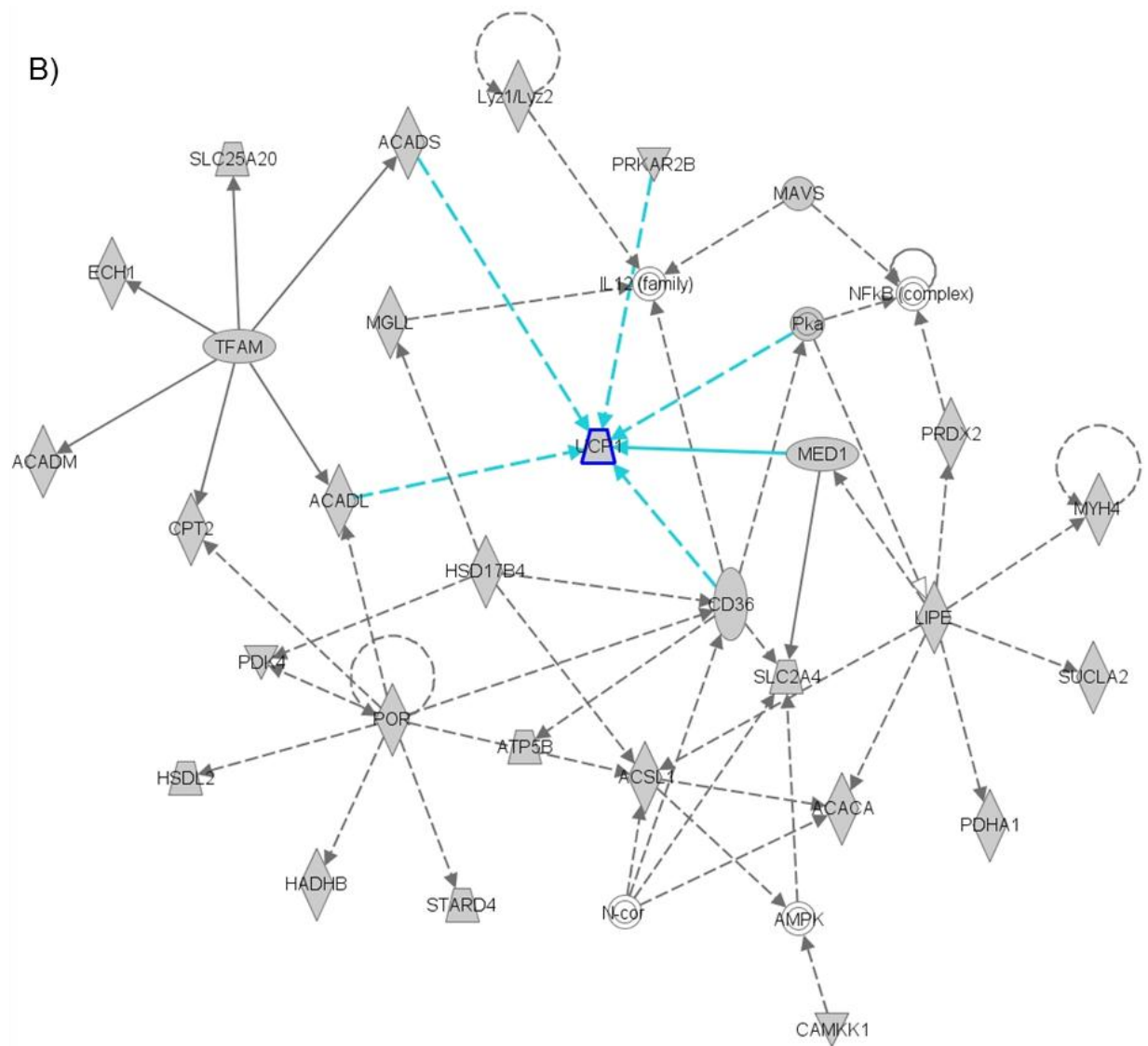


Figure 1.13: Immunoprecipitated UCP1 examined for potential phosphorylation residues. MS/MS spectrum using the LTQ Orbitrap was employed to analyze UCP1 following immunopurification using the Protein A dynabead kit. Moreover, the peptide fragment corresponding to the peptide sequence 41-54 was identified with no phosphorylation residues.

Figure 1.13: Immunoprecipitated UCP1 examined for potential phosphorylation residues

Collaboration_Rui_20130106_UCP1_IP_80% #11872 RT: 91.96 AV: 1 NL: 1.06E3
T: ITMS + c NSI d w Full ms2 758.41@cid35.00 [

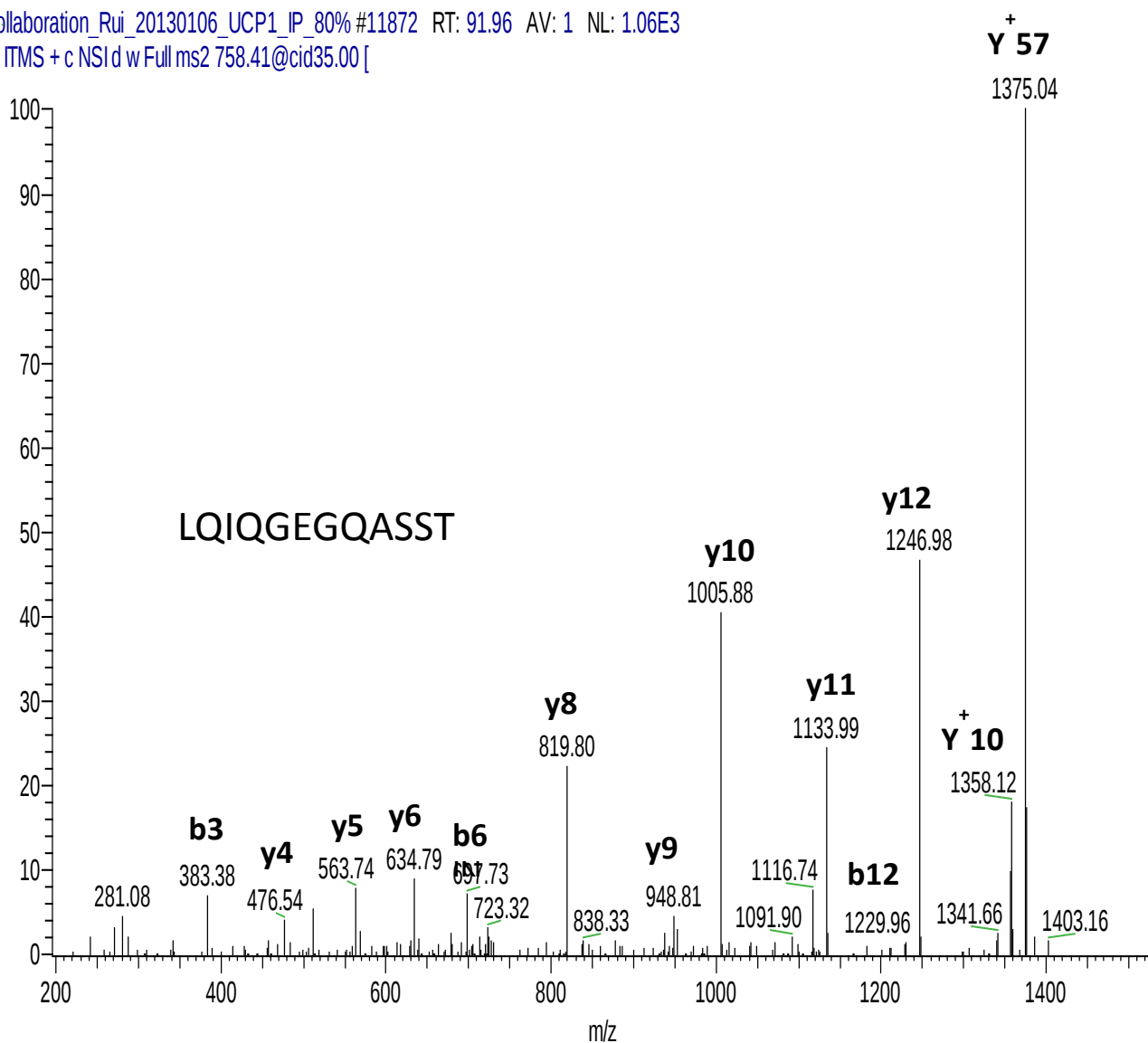
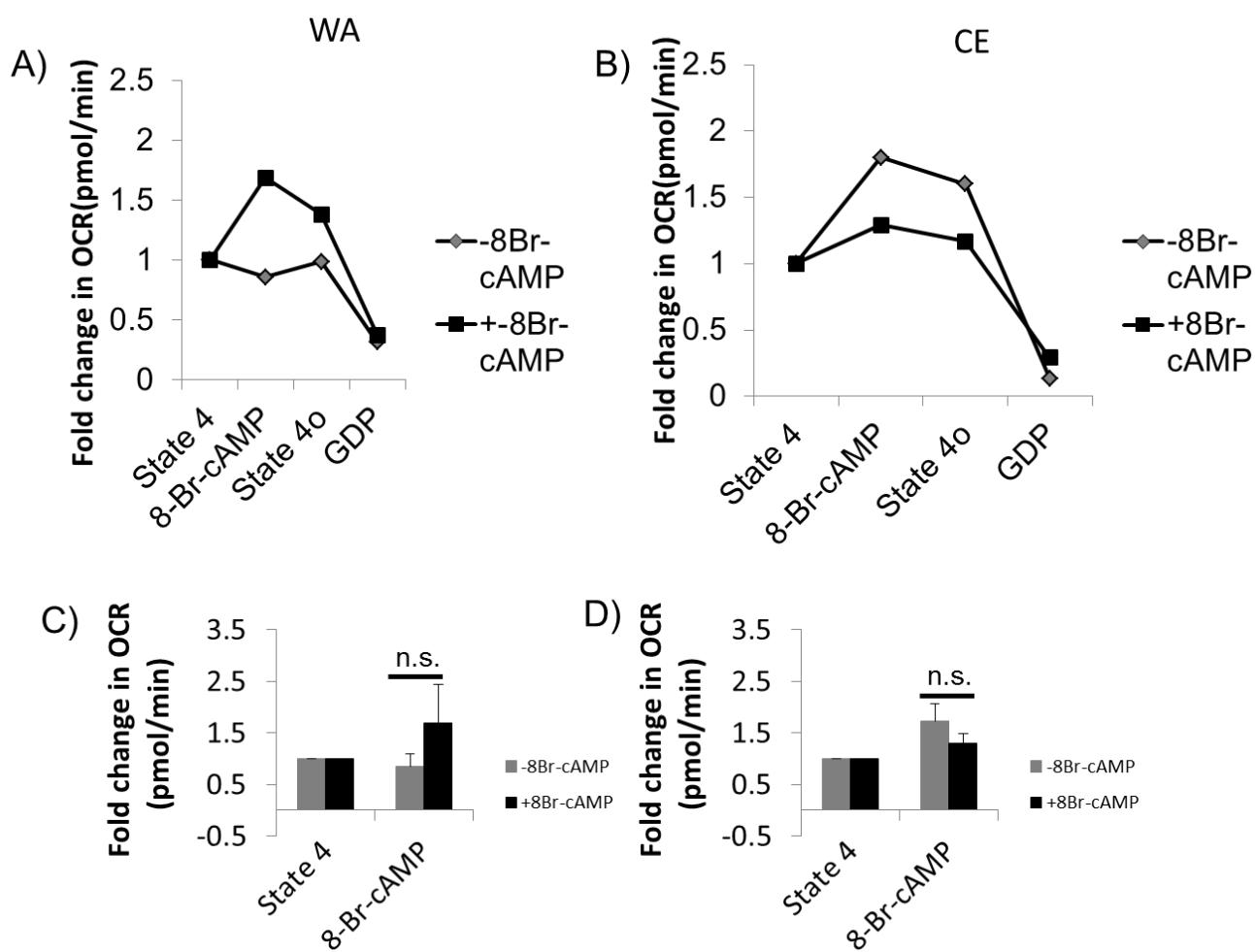


Figure 1.14: 8-Br-cAMP tends to increase respiration and GDP sensitivity in BAT mitochondria from WA, but not CE mice. Oxygen consumption rate was measured in isolated mitochondria (0.02mg/ml) from BAT of CE and WA mice. **A)** Mitochondria isolated from WA mice were either treated with or without 8Br-cAMP (+ 8Br-cAMP presence and -8Br-cAMP absence) (20 μ M) followed by sequential treatments with oligomycin (2.5 μ g/mL), GDP (1mM), and antimycin A (4 μ M) (note that values were normalized to antimycin A background). **B)** Mitochondria isolated from cold exposed mice were treated with or without 8Br-cAMP followed by sequential treatments as described above. **C)** Representative bar graph for panel A. **D)** Representative bar graph for panel B. PKA activation by 8-Br-cAMP leads to a trend for increased state 4 OCR in mitochondria from WA mice (**Panel A and D**). In contrast, PKA activation does not increase state 4 OCR in mitochondria from CE mice (**Panel B and D**). N=3, mean $\pm$ SEM. Student's *t*-test, and n.s. (not significant). CE (cold exposure) and WA (warm acclimated).

Following our inconclusive analysis of UCP1 phosphorylation, (His tagged UCP1 vs. immunopurified UCP1) we next assessed if we could modulate UCP1 phosphorylation *in vitro* using isolated mitochondria and a known PKA activator 8Br-cAMP. The rationale for this investigation was as follows; 1) it is well known that PKA activation plays a crucial role in uncoupled respiration in brown adipocytes (25, 202), 2) a recently discovered pool of soluble adenylyl cyclase has been linked to mitochondria respiration modulation through PKA activity (3), and 3) modulation of respiration using 8Br-cAMP under conditions of WA and CE would offer a potential link between UCP1 phosphorylation and function. Furthermore, our initial bioinformatic analyses also identified PKA as a potential candidate for the phosphorylation of UCP1 and mass spectroscopy of the UCP1 interactome revealed that PKA was a UCP1 binding partner. With these considerations we decided to test if PKA agonist 8Br-cAMP could stimulate proton conductance through UCP1. To test this, BAT mitochondria from WA and CE mice were treated with 8Br-cAMP and the impact on respiration was measured. Mitochondria were treated sequentially with 2.5µg/mL oligomycin and 1mM GDP to further examine the effect of 8Br-cAMP on proton leak through UCP1. Results showed a trend for 8Br-cAMP stimulation of respiration in mitochondria isolated from WA mice but not CE mice (**Figure 1.14A,B, C& D**).

Figure 1.14: 8-Br-cAMP tends to increase respiration and GDP sensitivity in BAT mitochondria from RT but not CE mice.



CHAPTER 2- ECTOPIC EXPRESSION OF UCP1 ALTERS MITOCHONDRIAL REDOX ENVIRONMENT AND BIOENERGETICS:

Mice Carrying Muscle Creatine Kinase-UCP1 Transgene Selectively Express UCP1 in Muscle: Ectopic Expression of UCP1:

Muscle creatine kinase-UCP1 mice (Transgenic; Tg) and wild-type (Wt) male mice aged 7-12 weeks were genotyped using the polymerase chain reaction to detect the rat UCP1 cDNA transgene. As shown in **Figure 2.1A**, Tg mice express UCP1 mRNA in muscle. Expression of UCP1 protein in muscle of Tg mice was confirmed by immunoblot analysis. UCP1 protein was not detected in muscle mitochondrial preparations from Wt mice. We also quantified the relative amount of UCP1 protein in muscle mitochondrial preparations from Tg mice. This was achieved by comparing the relative amounts of UCP1 protein in Tg muscle mitochondria to amounts found in BAT mitochondria. Based on our calculations, we found that UCP1 expression in muscle mitochondria of Tg was ~87% less when compared to BAT samples (**Figure 2.1A**).

Ectopic Expression of UCP1 Alters Body Composition and Increases Metabolic Rate:

Next, Tg and Wt mice were assessed for differences in whole body, tissue and organ weights. Tg mice displayed a ~ 29% decrease in whole body weight compared to Wt mice (**Figure 2.1B**). Skeletal muscle (hind limbs and forelimbs) and gonadal white adipose tissue (gWAT) were analyzed. Results showed that Tg mice display a ~ 62% decrease in skeletal muscle weight, and a ~ 51% decrease in gWAT when compared to Wt mice (**Figure 2.1C, & D**). Assessment of weights of liver, kidney, heart, and BAT were ~ 20%, 27%, 24%, and 46% lower, respectively, compared to Wt (**Figure 2.1F, G, H & E**). A significant decrease in body length was observed

for Tg mice, in agreement with the aforementioned results (**Figure 2.1I**). Hence, expression of UCP1 Tg in skeletal muscle leads to significant changes at the whole body level, i.e., beyond skeletal muscle.

Next, we assessed the impact of ectopic expression of UCP1 in skeletal muscle on metabolic rate and food intake. Food intake studies showed that there was a 24% increase in daily food intake in Tg compared to Wt mice (**Figure 2.2A**). Using indirect calorimetry, we assessed volume of oxygen consumed and carbon dioxide produced over a 24h period at ambient temperature (22-24°C). Interestingly, oxygen consumption at ambient temperature was 30% and 26% higher in Tg mice in the light (6:00-18:00) and dark cycles (18:00-6:00) respectively compared to Wt mice (**Figure 2.2B**). These significant increases were also present when absolute values were normalized to lean body mass of mice. In addition, we tested if Tg mice retained these differences in whole body energetics after a 24h exposure to cold temperatures. The decrease in temperature lead to an elevation in oxygen consumption in both Tg and Wt mice indicating ectopic expression of UCP1 did not compromise adaptive thermogenesis overall (**Figure 2.2C**). However, even when exposed to cold, Tg mice still displayed significantly higher whole body O₂ consumption rates compared to Wt mice. We also calculated the respiratory exchange ratio (RER) in light and dark cycles to ascertain if there were differences in carbon source (i.e., FA vs. carbohydrate) oxidation at the whole body level. RER values were similar during the light cycle between Tg and Wt mice. However, during the awake and feeding hours of the dark cycle Tg mice preferentially used carbohydrates as a fuel source as indicated by greater RER values in Tg vs. Wt mice (**Figure 2.3A**). Intriguingly these differences in RER between Tg and Wt mice were abolished once adaptive thermogenesis was invoked following exposure to 4°C (**Figure 2.3B**).

Overall these results indicate that ectopic expression of UCP1 in muscle increases food intake and energy expenditure in the Tg compared to the Wt mice.

UCP1 Expression in Muscle Increases Glucose Uptake:

Based on our previous findings of preferential carbohydrate oxidation by Tg mice, we sought to examine if there were differences in *in vivo* muscle glucose uptake. Glucose clearance from circulation was dynamically measured in real time using a radiotracer glucose analog [^{18}F]-fluoro-deoxy glucose ([^{18}F] FDG) and micro positron emission tomography (microPET). Briefly, basal glucose was assessed by an initial bolus dose (1/3 of total dose) given in the first minute of a 20 min dynamic scan. Following the initial bolus dose a constant rate of infusion (1.66 $\mu\text{l}/\text{min}$) over an 80 min scanning period was initiated. Basal glucose uptake was measured for a period of 20 min followed by an injection of norepinephrine (NE), an adrenergic agonist, and the rate of glucose clearance was subsequently measured for an additional 60 min. Prior to adrenergic stimulation basal glucose clearance was measured in skeletal muscle, heart and BAT. Pre-injection of NE showed that there were no significant differences in rate of uptake between cardiac and BAT tissues when comparing Tg and Wt mice (**Figure 2.4A**). However, the rate of glucose uptake in skeletal muscle of Tg mice was increased compared to Wt (**Figure 2.4A**). Also, we measured the rate of glucose uptake post NE injection. Both BAT and cardiac tissue displayed no differences in the rate of glucose uptake between Tg and Wt mice (**Figure 2.4A**). In contrast, the rate of glucose uptake post NE injection yielded an increase in skeletal muscle of Tg mice, compared to Wt (**Figure 2.4B**). Circulating blood glucose was also measured pre and post

NE injection and results showed no difference in both states (**Figure 2.4C**). Representative images of Tg and Wt are shown (**Figure 2.4D**).

Mitochondrial Bioenergetics:

We then measured mitochondrial bioenergetics to assess the contribution of UCP1 mediated skeletal muscle uncoupling to mitochondrial oxygen consumption. Using the Seahorse XF24 analyzer we measured basal oxygen consumption in BAT and skeletal muscle mitochondria. Leaks through UCP1 in isolated mitochondria from muscle were defined as GDP –sensitive respiration. We also measured GDP-sensitive leaks through UCP1 in BAT to afford a comparison with muscle mitochondria preparations. GDP was titrated from 0.1mM to 0.2mM for skeletal muscle and from 0.5mM to 1mM for BAT mitochondria. This concentration range was used because muscle of Tg mice express almost 10-fold less UCP1 when compared to BAT. Fold change analysis of proton leak assessment in skeletal muscle mitochondria displayed a decrease with 0.1mM GDP in Tg mitochondria, but little to no response in Wt mitochondria, while further titration with 0.2mM did not induce decreases in proton leak (**Figure 2.5A**). As a control, BAT mitochondria from Tg and Wt mice were also assessed with GDP titration using concentrations of 0.5mM and 1mM. BAT mitochondria from Tg and Wt mice displayed similar sensitivities to GDP titration (**Figure 2.5A**). We also measured phosphorylating respiration in muscle mitochondrial preparations from Tg and Wt to determine if UCP1 expression in muscle had a negative effect on coupled respiration. Our findings showed that UCP1 expression in muscle did not compromise phosphorylating respiration, as represented by the respiratory values obtained following ADP treatment (**Figure 2.5B**). Thus, the increases in energy expenditure in Tg mice

can be attributed to the increase in UCP1-specific proton leak in muscle mitochondria without compromising phosphorylating respiration.

Ectopic Expression of UCP1 Alters Mitochondrial Redox Status and may Protect Against Oxidative Stress:

Our laboratory and others have recently shown that mitochondrial redox environment plays a crucial role in mitochondrial function. Although skeletal muscle and BAT have been shown to share a common lineage, the mitochondrial redox environments are very different (136, 201). These differences may be associated with UCP1 or other proteins that are differentially expressed between tissues. As such we assessed the redox environment of skeletal muscle and BAT. Indeed, we evaluated whether ectopic expression of UCP1 in muscle affected ROS generation. Treatment of muscle mitochondria isolated from Tg mice with GDP lead to a significant increase in the rate of ROS production (**Figure 2.6A**). In contrast, GDP addition did not elevate ROS production in Wt mitochondria (**Figure 2.6A**). Additionally, GDP treatment did not elevate ROS production in BAT mitochondria from both Tg and Wt mice (**Figure 2.6A**). Next, we analyzed the total glutathione (GSH+ GSSG) in muscle and BAT mitochondria from Tg and Wt mice. The results showed that muscle mitochondria from Tg mice had a significant increase in total glutathione when compared to Wt (**Figure 2.6B**). In contrast, no changes in total glutathione levels were observed in BAT mitochondria. In addition, we assessed if muscle expression of UCP1 elevated levels of oxidative stress. Finally, levels of total protein carbonyl adducts, which are a result of lipid peroxidation, were measured in the gastrocnemius muscle, and no differences were detected between Tg and Wt mice (**Figure 2.6C**).

Figure 2.1: Transgene expression of UCP1 in muscle and its effect on body composition **A)** UCP1 mRNA and protein. The numbers 1, 2, 3 represents individual replicates- n=3. Succinate dehydrogenase (SDH) was used as loading control. **B)** Body weight. **C)** Gonadal white adipose tissue (gWAT) weight, **D)** Skeletal muscle (pooled limbs) weight, and **E)** Interscapular brown adipose tissue (BAT) weight. **F)** Kidney weight, **G)** Liver weight, **H)** Heart weight, and **I)** Body length (cm). Wild-type (Wt); MCK-UCP1 (Tg). n=9, mean±SEM, Student's *t*-test. n.s. = not significant.

Figure 2.1: Transgene expression of UCP1 in muscle and its effect on mouse body composition.

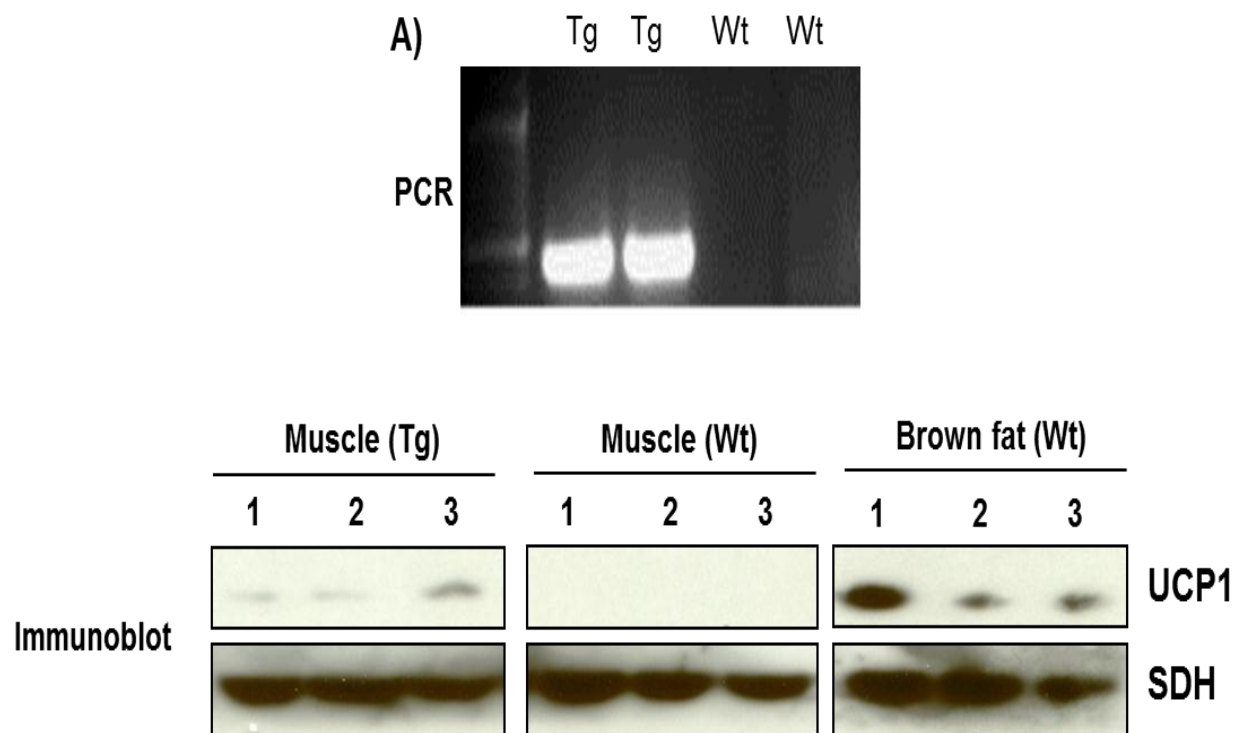


Figure 2.1: Transgene expression of UCP1 in muscle and its effect on mouse body composition.

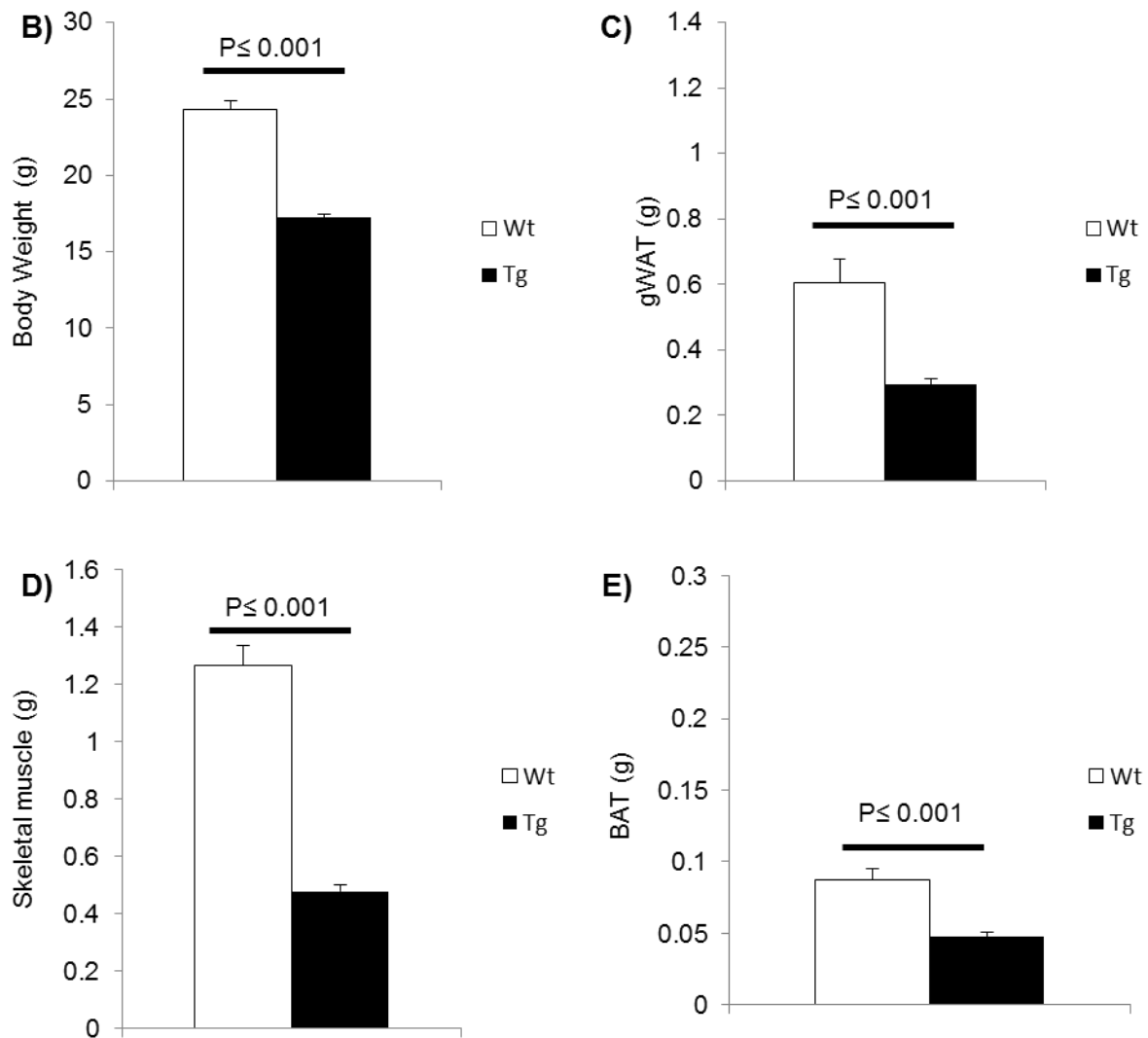


Figure 2.1: Transgene expression of UCP1 in muscle and its effect on mouse body composition.

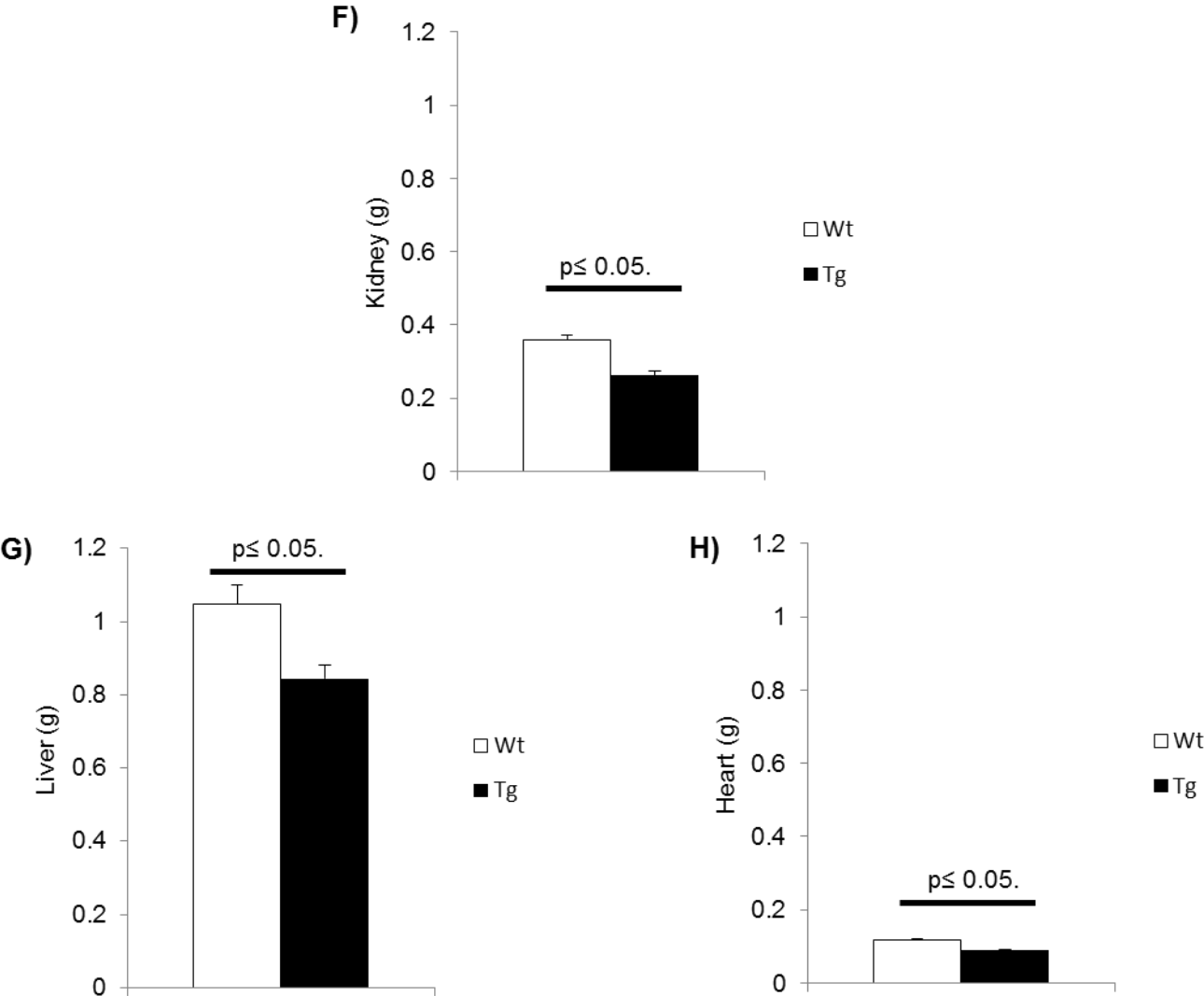


Figure 2.1: Transgene expression of UCP1 in muscle and its effect on mouse body composition.

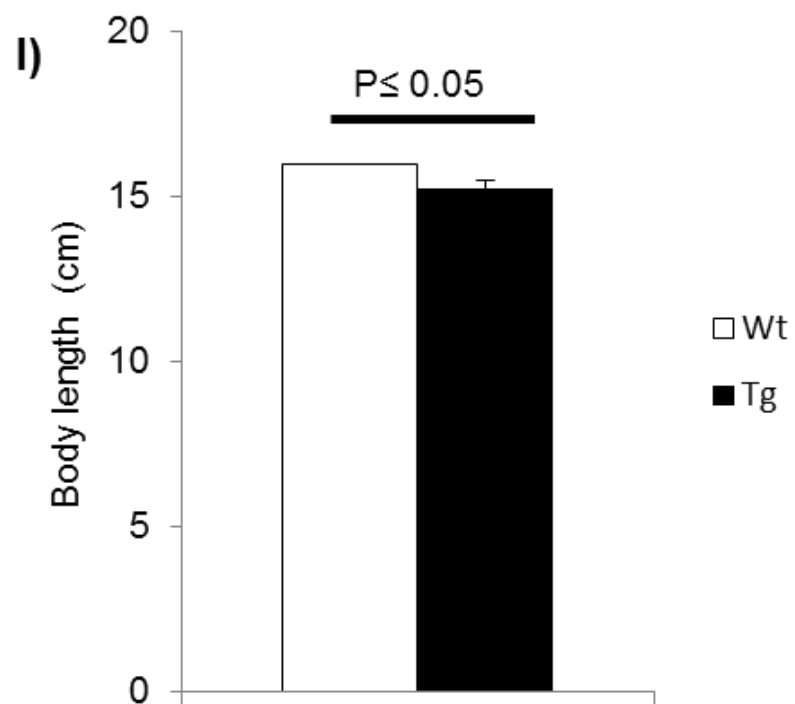


Figure 2.2: Ectopic UCP1 expression increases energy expenditure at ambient temperature and does not perturb adaptive thermogenesis. **A)** Food intake measured daily was normalized to whole body weight (g/day/body weight) over one month. $n=4$, mean $\pm$ sem, Student's t -test. **B)** Volume of oxygen consumed (VO_2 -ml/min) was assessed in Wt and Tg mice at room temperature (22-23°C) using indirect calorimetry. VO_2 was calculated for both light and dark cycles and was normalized to lean muscle mass. **C)** VO_2 was calculated in Wt and Tg mice exposed to cold (4°C) for 24h. Wild-type (Wt); MCK-UCP1 (Tg). $n=9$, mean $\pm$ sem, Student's t -test. n.s. = not significant

Figure 2.2: Ectopic UCP1 expression increases energy expenditure at ambient temperature and does not perturb adaptive thermogenesis.

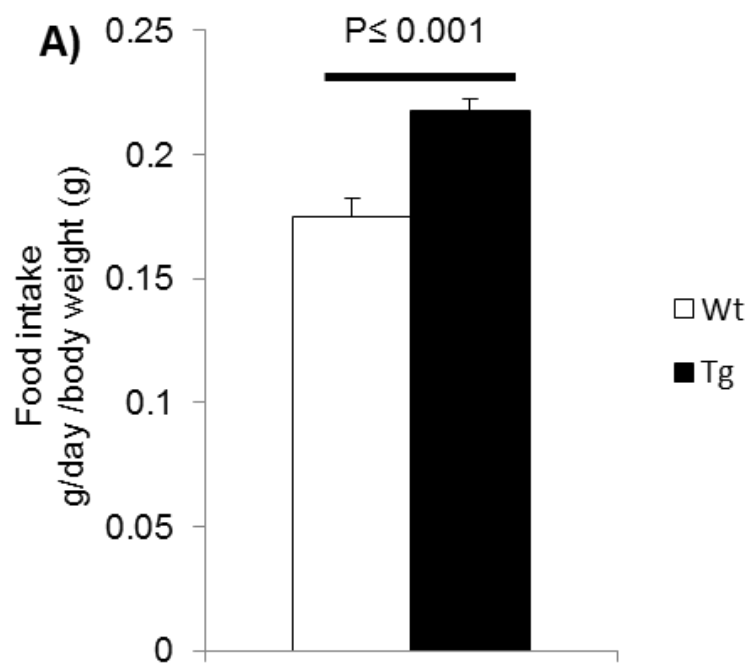


Figure 2.2: Ectopic UCP1 expression increases energy expenditure at ambient temperature and does not perturb adaptive thermogenesis.

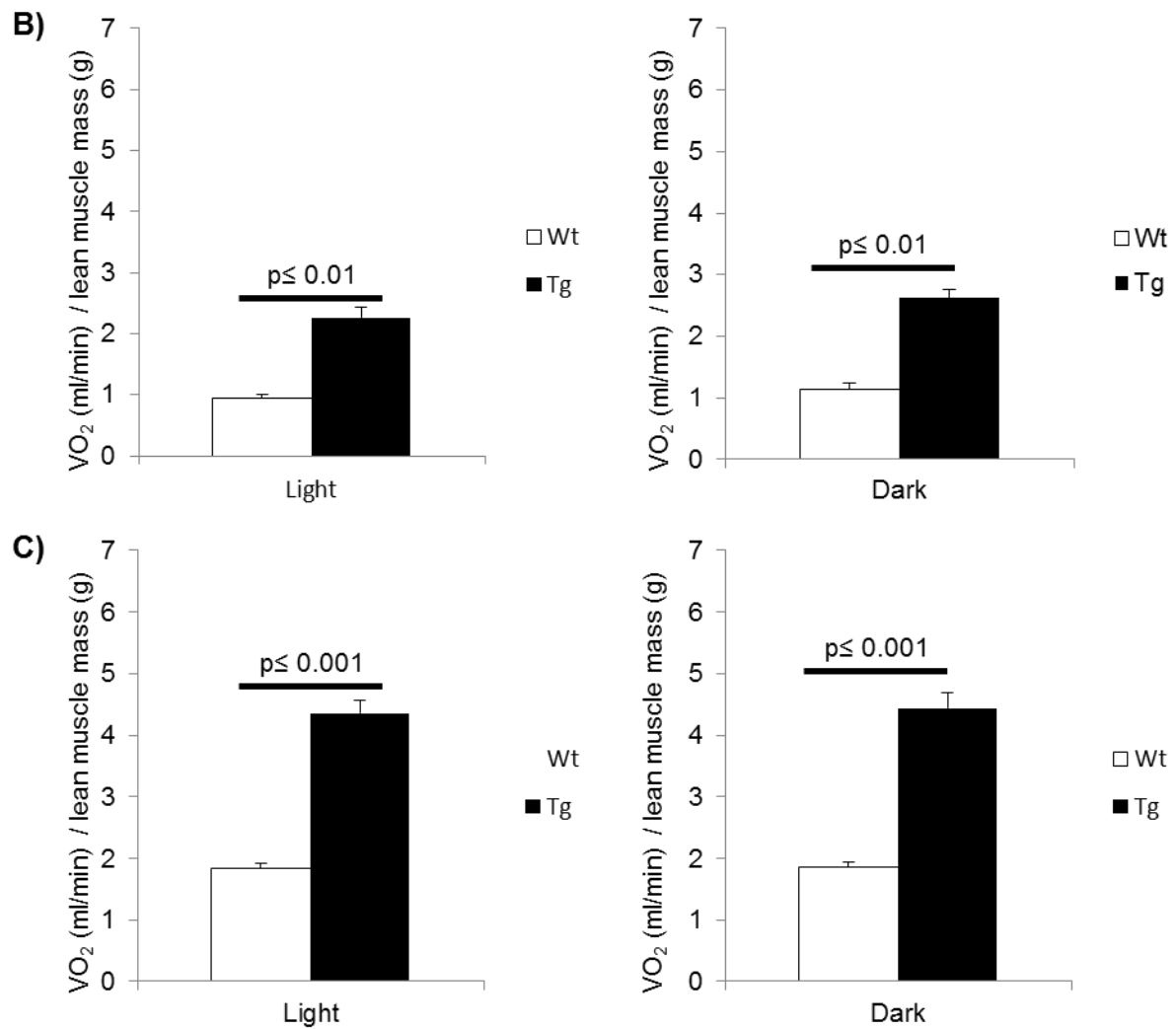


Figure 2.3: Ectopic expression of UCP1 in muscle increases whole body dependency on carbohydrates as fuel for metabolism: Respiratory Exchange Ratio (RER) was calculated from indirect calorimetry measurements over a 24 hour period. Calculated RER values for Wt and Tg mice exposed to **A)** room temperature (22-23°C) or **B)** cold (4°C) conditions. RER values are averages for both light and dark phases (12 hours). Wild-type (Wt); MCK-UCP1 (Tg). n=9, mean±sem, Student's *t*-test. n.s. = not significant

Figure 2.3: Ectopic expression of UCP1 in muscle increases the dependency on carbohydrates as fuel for metabolism:

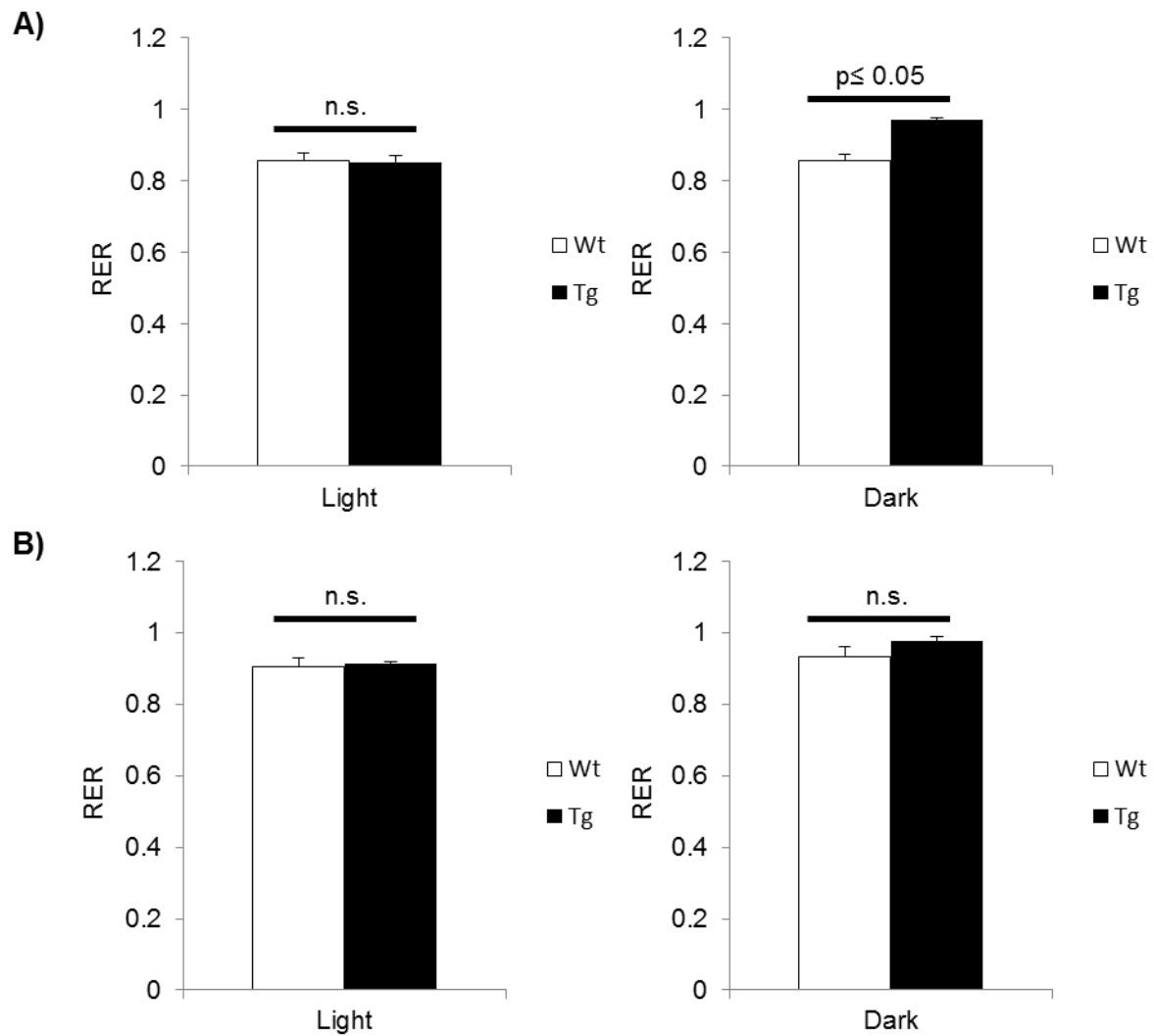


Figure 2.4: Rate of glucose uptake in muscle is increased as a consequence of UCP1 expression: Wt and Tg mice were anesthetized and injected with [^{18}F]FDG at a constant rate of infusion at (1.66 $\mu\text{l}/\text{min}$) for a period of 80 min. Initially, the scanning protocol assessed basal glucose uptake for 20min, and mice were then adrenergically stimulated with norepinephrine (NE). Mean standardized uptake values ($\text{SUV} = [\text{F}] \text{ FDG uptake}/(\text{injected activity}/\text{body weight}), \text{g}/\text{ml}$) as determined from regions of interest in the hind limb and forelimb muscle of Tg and Wt mice. Rate of glucose uptake into muscle, BAT, and heart was evaluated **A)** before NE treatment (pre-NE) and **B)** after NE treatment (post-NE) at a dosage of 1mg/kg of body weight. **C)** Plasma glucose levels were measured using the Accu-Check Advantage glucometer pre and post injection of NE. Statistical analysis was performed using a two way ANOVA with a Fischer post-hoc test. N=5, n.s.-Not significant. **D)** Coronal [^{18}F] FDG representative images were collected from Wt and Tg mice. n=5, mean $\pm$ SEM, Student's *t*-test. n.s.=not significant

Figure 2.4: Rate of glucose uptake in muscle is increased as consequence of UCP1 expression:

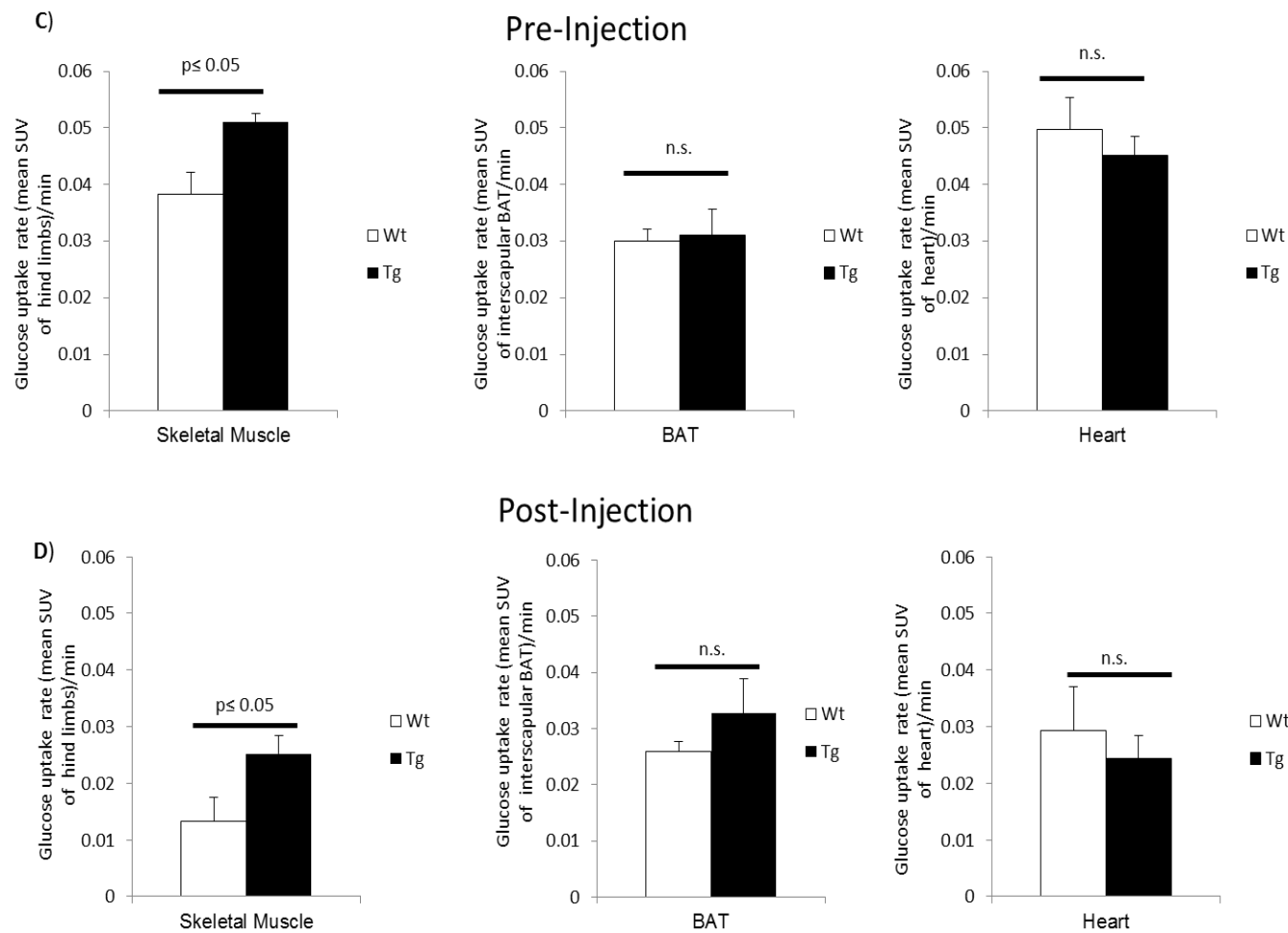


Figure 2.4: Rate of glucose uptake in muscle is increased as consequence of UCP1 expression:

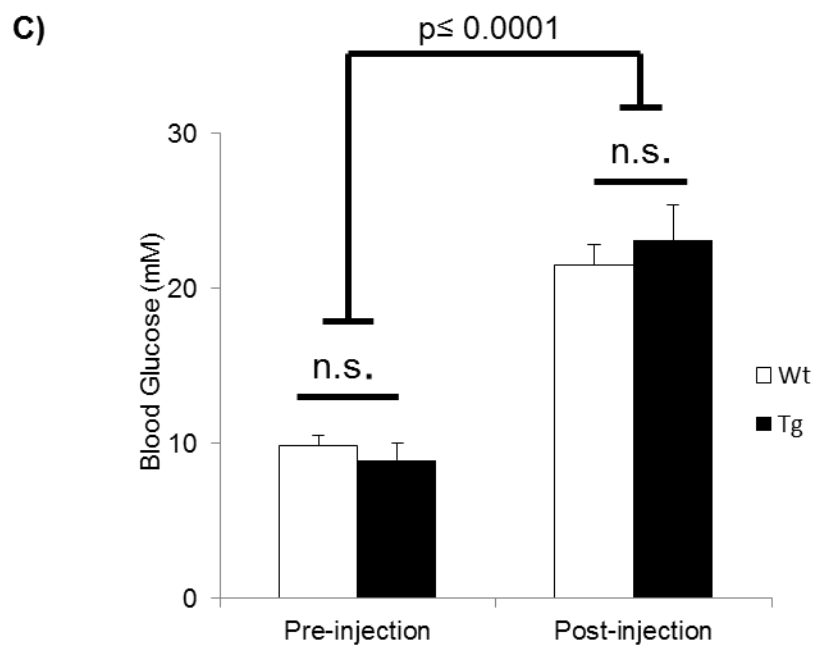


Figure 2.4: Rate of glucose uptake in muscle is increased as consequence of UCP1 expression:

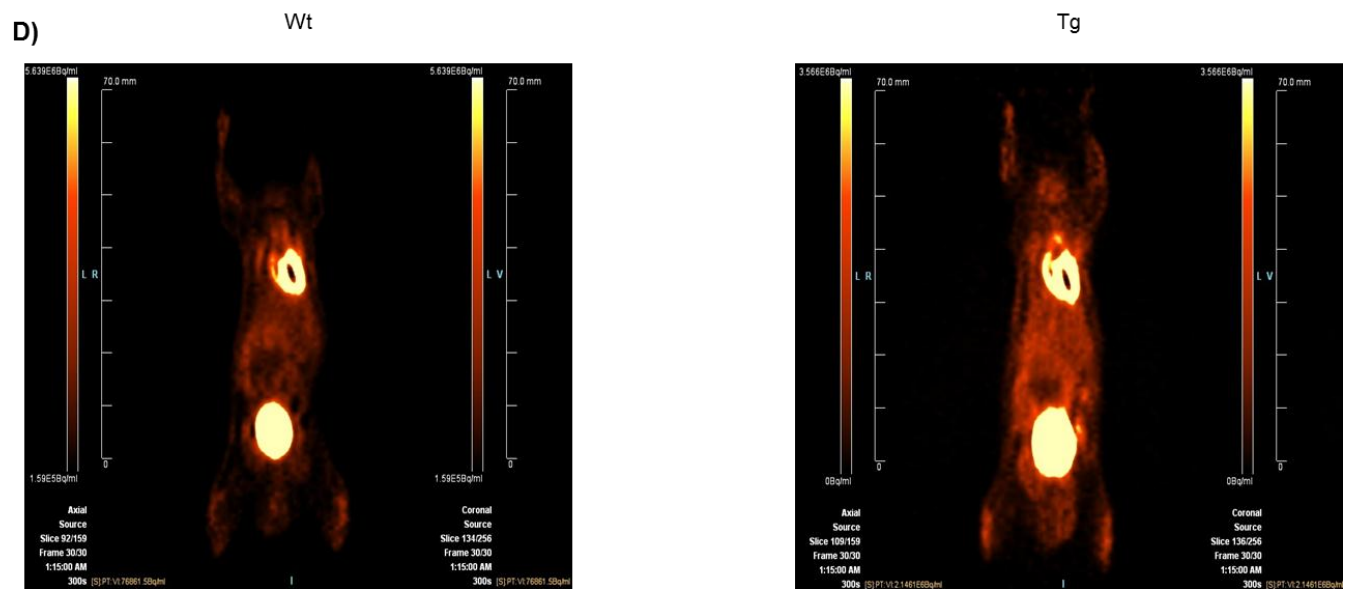
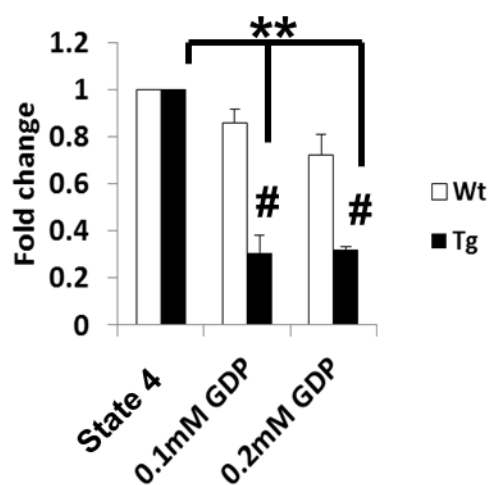


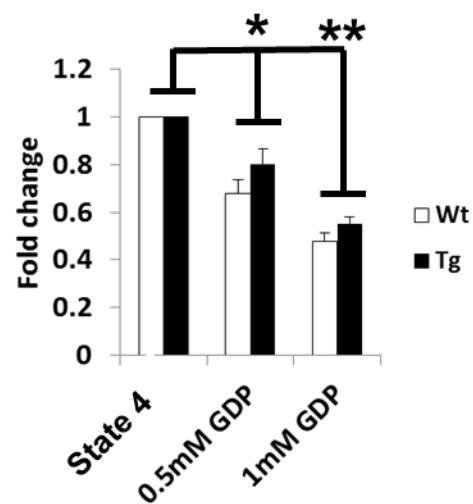
Figure 2.5: Proton leaks through a UCP1 mediated mechanism is increased in isolated mitochondria from muscle, without perturbation of oxidative phosphorylation: Oxygen consumption rates of isolated mitochondria from skeletal muscle and BAT of Wt and Tg mice were tested using the Seahorse XF24. **A)** Proton leak in muscle and BAT mitochondrial preparations from Wt and Tg mice were measured by GDP titration. Next, measurements of state 4 respiration (10mM pyruvate and 2mM malate in the absence of ADP) followed by oligomycin (2.5µg/mL; state 4 O) state 4o, and GDP titration (0.1-0.2mM for muscle and 0.5-1mM for BAT mitochondria) were conducted. **B)** Coupled respiration was measured by assessing sequentially state 4 respiration, ADP stimulated respiration (0.1mM; state 3), oligomycin, FCCP (8µM), and antimycin A (4µM). n=3, mean±SEM. Oxygen consumption rates are shown as fold changes relative to state 4 respiration rates. Two-way ANOVA with a Fischer post-hoc test. * and ** correspond to $p \leq 0.05$ and 0.01 for treatments compared to state 4. # corresponds to $p \leq 0.01$ for between genotypes.

Figure 2.5: Proton leaks through a UCP1 mediated mechanism is increased in isolated mitochondria from muscle, without perturbation of oxidative phosphorylation

A) Muscle Mitochondria



BAT Mitochondria



B)

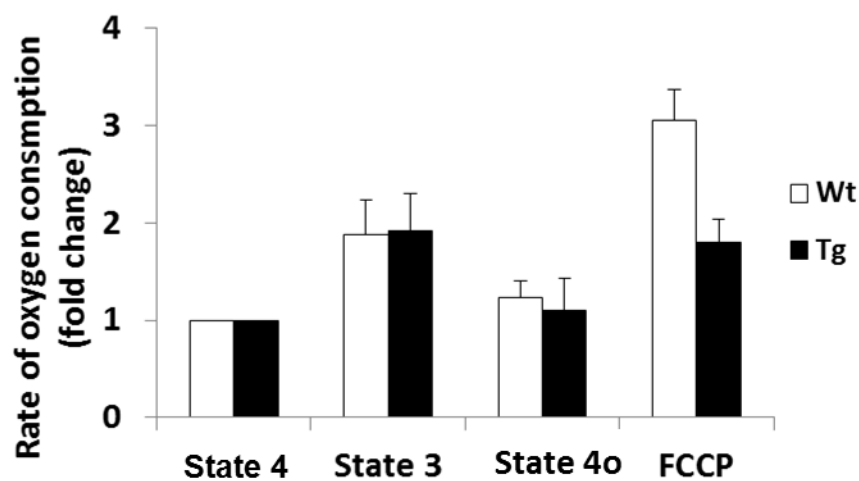


Figure 2.6: Inhibition of UCP1 alters rate of ROS production from muscle, but not in BAT mitochondria. **A)** ROS emission in skeletal muscle and BAT mitochondria of Wt and Tg mice were measured using the fluorometric probe H₂-DCFDA. ROS measurements were initiated with 5mM pyruvate and 3mM malate (Pyr+Mal) followed by the sequential addition of oligomycin (1.3µg/mL) and saturating amounts of GDP (1mM). ROS levels were measured for ~10min after each treatment. N=3, mean±SEM, two way ANOVA with a Fischer post hoc test. **B)** Total glutathione content in mitochondria isolated from skeletal muscle and BAT of Wt and Tg mice was measured using HPLC and quantified using the Agilent ChemStation software. **C)** Total protein carbonyl adducts were measured by immunoblot analysis in *gastrocnemius* muscle extracted from Wt and Tg mice. Lanes 1, 3, 5, and 7 correspond to Wt and a lane 2, 4, 6, and 8 correspond to Tg. Loading control was done by Ponceau S staining of membranes. Lane quantification was done using Image J software. n=4, mean±sem, A.u.= arbitrary units Student's *t*-test.

Figure 2.6: Inhibition of UCP1 alters rate of ROS production from muscle, but not in BAT mitochondria.

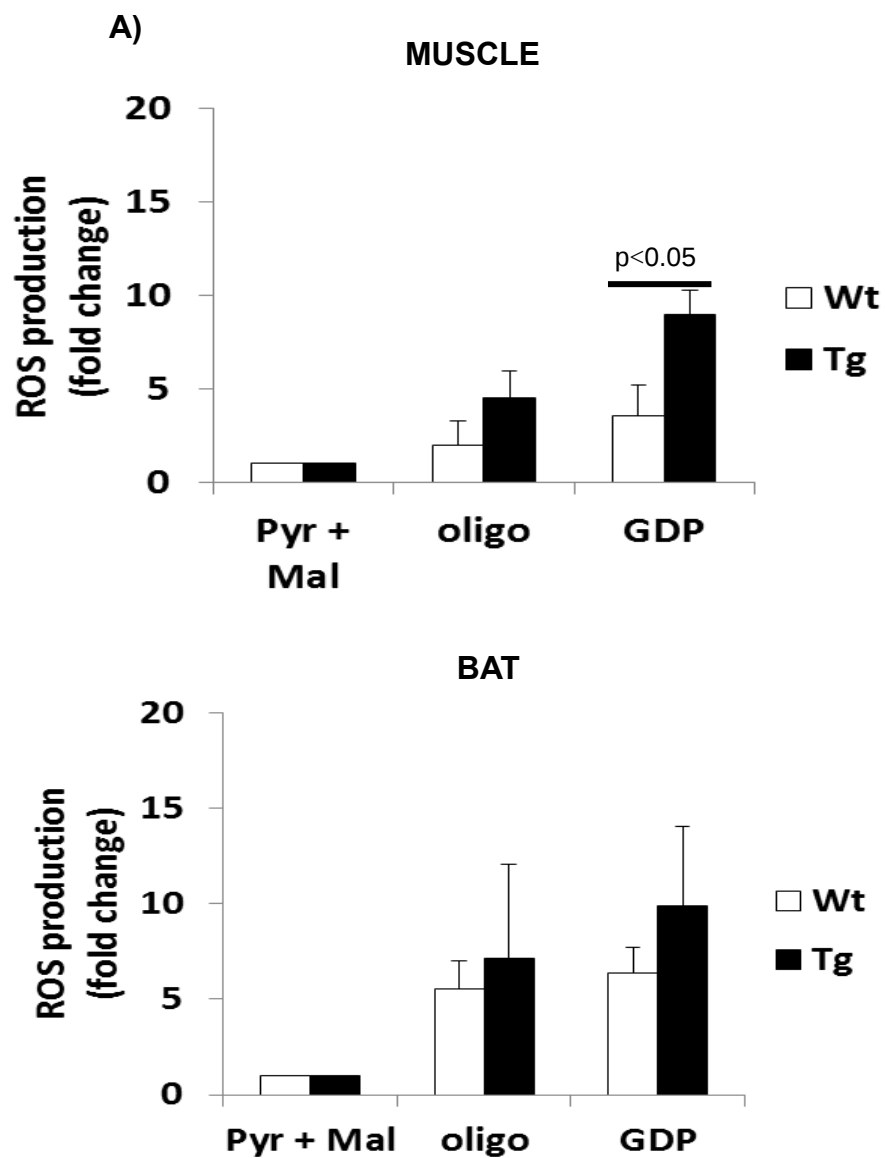


Figure 2.6: Inhibition of UCP1 alters rate of ROS production from muscle, but not in BAT mitochondria.

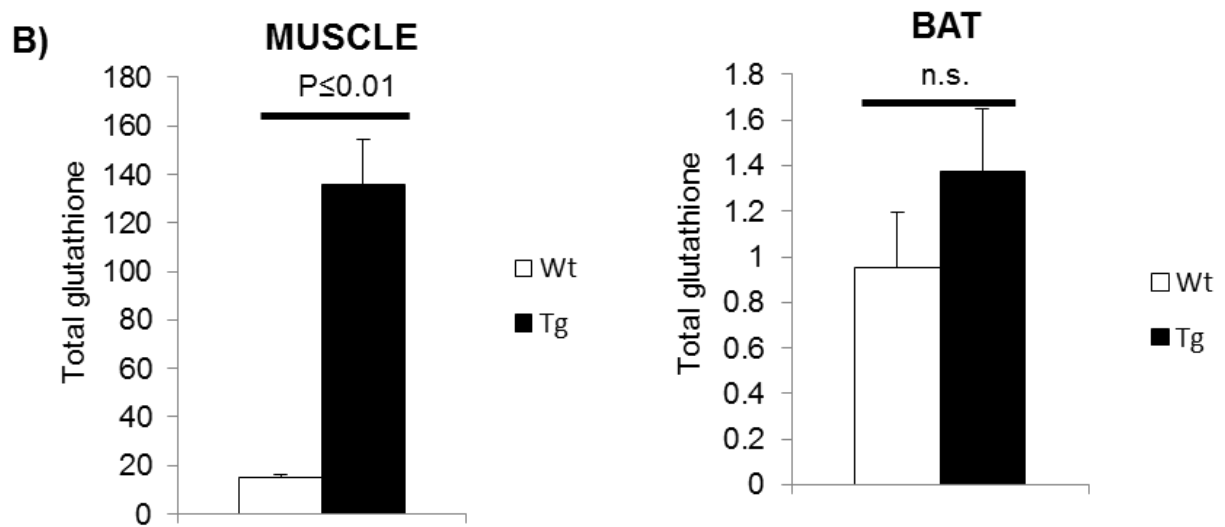
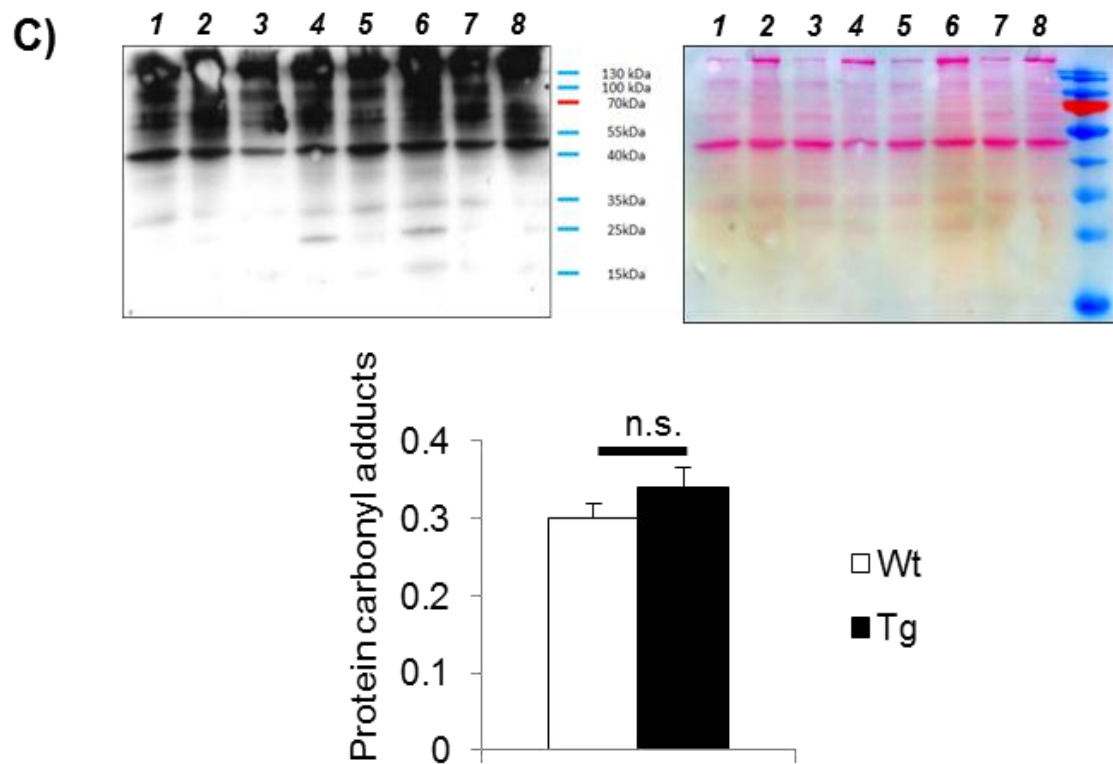


Figure 2.6: Inhibition of UCP1 alters rate of ROS production from muscle, but not in BAT mitochondria.



DISCUSSION:

The incidence of obesity worldwide is increasing at a tremendous rate and the fastest occurrence is now shown to be in developing countries (175). The development of obesity can be attributed to several causes such as genetic, environmental, and behavioral factors. These factors can result in the increase of EI (high caloric intake) and a decrease in EE (e.g., a sedentary lifestyle or defective EE wastage processes) leading to state of positive energy balance. Moreover, obesity can be associated with the development of other co-morbidities thereby furthering the disease state. In fact, excess fat accumulation in tissues and cells can lead to the onset of inflammation, disrupt insulin signaling, perturb hormonal release and energy expenditure, and induce oxidative stress. The disruption of the aforementioned pathways can lead to the development of heart disease, atherosclerosis, type 2 diabetes, hepatic steatosis, metabolic syndrome (combination of the former morbidities) and even cancer (22, 111, 118, 120, 219). Currently, treatment for obesity involves lifestyle modification (e.g., hypocaloric diets) and an increase in regular exercise. Also, there is a very limited repertoire of drug therapies. However, the pharmacological and non-pharmacological treatments have variable efficacy in obese persons. As such, given the need for additional pharmacological agents, it is important to identify new targets that can be exploited to restore energy balance in obese persons.

It has recently been shown that adult humans have BAT depots, and this discovery has renewed interest in using it as therapeutic target for obesity (227, 230, 236). It is evident that BAT activity in adult humans responds to decreases in ambient temperature, and the response in BAT activity are different in lean and obese individuals (51, 164, 227, 230). In fact, BAT appears to be inactive or atrophied in obese patients

(35, 51, 83, 227, 230). Rodent studies show that once activated, BAT consumes high amounts of fat for the purpose of thermogenesis, and this process is dependent on the expression of UCP1. However, the molecular details surrounding how UCP1 is controlled still require clarification. Currently, the only accepted method of UCP1 activation *in vivo* is through a fatty acid dependent mechanism. As such the first chapter of the current study was designed to explore the molecular mechanism of BAT regulation by PTM of UCP1, while the second part was to explore how the cellular redox environment impacts UCP1 function.

Control of UCP1 Activation by Phosphorylation:

While a previous study had documented that UCP1 was covalently modified by phosphorylation, the authors failed to either validate this modification using different methods or show the bioenergetic implications (29). Thus, the first part of our studies focused on validating UCP1 phosphorylation then exploring the physiological consequences. Hypothetically, phosphorylation of UCP1 in response to cold stimulation and adrenergic activation of BAT may cause conformational changes facilitating FA binding/or GDP release, and the subsequent activation of leaks. This sustained leak overtime drives up mitochondrial respiration and thereby leads to increases in EE. This mechanism of action could be a practical means of regulating UCP1 function as it would depend on the turnover of proteins and not on the availability of macro-nutrients such as fatty acids and energy molecules (GDP). Our main goals were to determine if 1) UCP1 was modified by phosphorylation and 2) if this modification was physiological relevant (i.e., is phosphorylation regulated by cold exposure and does this have implications for mitochondrial bioenergetics). In brief, we showed that CE of mice can augment mitochondrial

respiration in a UCP1 dependent manner. Moreover, using a protein kinase A agonist, 8Br-cAMP, we were able to stimulate (trends only) proton leak-dependent O₂ consumption in mitochondria from WA mice and not CE mice. IEF confirmed that CE of mice results in UCP1 modification by an acidic group, possibly phosphate. In contrast, mass spectrometry results were inconclusive for UCP1 phosphorylation.

Bioinformatics analysis was conducted to determine if UCP1 contains amino acid residues that may correspond to phosphorylation residues. We showed that based on sequence motif, Ser⁵⁰ of the *Mus musculus* UCP1 sequence was a likely candidate to be phosphorylated (Table 1-1). Interestingly, Ser⁵⁰ belongs to a peptide sequence (LQIQGEGQASSTIR) that is highly conserved among mammals and may offer a unique site that has not been documented in the literature. In fact, based on the online database Phosphosite Plus, Ser^{3, 4, and 51} in *Rattus norvegicus*, Thr²⁶⁵ in *Mus musculus*, and Tyr⁵⁵, Thr⁶¹, Ser²⁵⁰, and Thr²⁶⁵ in *Homo sapiens* were documented as possible phosphorylation residues in UCP1. However, biochemical analysis of these proposed sites has not been conducted. We followed our bioinformatic analysis with an examination of the potential kinases that could phosphorylate UCP1 using the group-based prediction software (GPS). The rationale for searching for potential kinases was to allow for targeted *in vitro* experiments to truly analyze the mechanism by which UCP1 was phosphorylated. However, we found that the LQIQGEGQASSTIR amino acid sequence located in the first matrix loop region of the UCP1 sequence was not recognized as a potential site for kinase phosphorylation. On the other hand, analysis of the full length UCP1 sequence showed that other sites were viable targets of kinases (table 2). These kinases included the P21 serine/threonine kinase, protein kinase B (PKB), protein kinase A (PKA), and protein kinase C (PKC). Intriguingly these identified

kinases are found in the intermembrane space and matrix environments of mitochondria (2, 3, 110). Taken together these results suggest that UCP1 may not be phosphorylated at the proposed sites by Carroll *et al*; instead different residues could be implicated. In fact, the concept of UCP phosphorylation was demonstrated in a recent publication in which mice and rats were treated with 3,4-methylenedioxymethamphetamine (MDMA). Treatment with MDMA lead to hyperthermia in these rodents and induced the phosphorylation of UCP3, a UCP1 paralog expressed in skeletal muscle (115). This would indicate that the UCPs in general can be modulated by PTM such as phosphorylation. However, it is important to note that UCP3 phosphorylation has not independently been confirmed and the actual phosphosite on UCP3 was never identified (115).

Nonetheless, mitochondria are now regarded as very important sites for regulation by phosphorylation, and various phosphorylation targets in mitochondria have been identified (53). Mitochondria also contain a distinct kinome that is responsive to signals from both the cytosolic and mitochondrial environments (104). In addition, there is an abundance of regulatory and non-regulatory phosphorylation residues in key enzymes of the ETC that have been linked to regulating mitochondrial respiration (3, 53). Also, ketogenesis and obesity development have been linked to fluctuations in the phosphoproteome status in mitochondria (87).

It is clear that phosphorylation of proteins plays a key role in regulating mitochondrial function. Whether or not this covalent modification is required to govern UCP1 function has yet to be determined. We sought to address this issue by measuring mitochondrial respiration under conditions we believed would induce phosphorylation of UCP1 such as CE. In fact, CE in BAT

stimulates adaptive thermogenesis through the cAMP dependent pathway, and could activate cold responsive kinases such as the p38 mitogen-activated protein kinase (MAPK) (28). The activation of cold responsive kinases and pathways may lead to the phosphorylation of proteins associated with oxidative phosphorylation.

We first decided to establish our model system for testing whether cold exposure leads to UCP1 phosphorylation. Mice were either WA or CE and mitochondria were isolated from BAT to test the characteristics of UCP1-dependent proton leaks. Using this method we were able to show that CE induces ~ 3-fold increase in mitochondrial respiration where ~ 41% was due to proton leak through UCP1. Thus, with our model established, we sought to confirm if UCP1 is phosphorylated using IEF and mass spectrometry analysis. We chose to evaluate covalent modification using the IEF technique because it offers a very efficient method of separating most proteins with accurate quantification of protein expression (17). Isolated mitochondria from WA and CE mice were subjected to IEF followed by immunodetection using anti-UCP1 antibodies. Immunoblotting showed acidic shifts in the pI of UCP1 following CE which is indicative of phosphorylation. Treatment with shrimp alkaline phosphatase resulted in restoration of UCP1 to its native pI, but with residual shifts remaining. The residual shifts detected under CE could indicate 1) a hyperphosphorylated state of UCP1, 2) improper solubilization and migration of UCP1, since membrane proteins are notoriously difficult to solubilize, and 3) the residual shifts are of a different type of covalent modification. In fact, modifications such as hydroxylation, palmitoylation, ubiquitination, and acetylation have all been documented to produce acidic shifts in pI (59, 84, 129). Indeed, acetylation could play a role in PTM of UCP1 since the histone deacetylase protein SIRT3, the mammalian homologue of the yeast *Sir2*, is regulated by cold

exposure in BAT. In fact, in a paper published by Tong and colleagues, they showed that SIRT3 localized to the mitochondrial inner membrane in BAT and that its overexpression in an immortalized brown adipocyte cell-line HIB-1B yielded an up regulation of UCP1 mRNA (209). Further, overexpression of SIRT3 increases mitochondrial respiration and UCP1-dependent proton leaks. On the other hand, agents associated with oxidative stress such as 4-hydroxy-2-nonenal (4-HNE) can modify proteins involved the ETC such as cytochrome c and have been documented to induce acidic shifts upon IEF analysis (105). Interestingly, the function of UCP1 ectopically expressed in *Saccharomyces cerevisiae* is regulated by 4-HNE and fatty acids (64). However cold exposure is not known to induce the overt production of oxidative damage end products such as 4-HNE, and, as such, the likelihood of this type of modification affecting our sample shifts is low. Although, residual shifts remain, it is still plausible that indeed the original shifts detected were phosphorylation and would fit well with the hypothesis of a hyper-phosphorylated UCP1. Thus, our results do point towards UCP1 phosphorylation but we cannot discount the possibility that other types of modification contribute to the observed pI shift in UCP1 following cold exposure.

Consequently, we turned to a more sensitive method of detecting protein modification. Using an LTQ Orbitrap MS/MS we analyzed a commercial recombinant UCP1 protein purified in *E.coli* vs. immunopurified UCP1 from BAT mitochondria isolated from WA and CE mice. We purchased recombinant UCP1 protein in order to maximize our ability to perform both proteomic analysis and functional assays. Likewise, immunopurified UCP1 was used as a mechanism to explore UCP1 regulation *in vivo* following CE of mice. Recombinant UCP1 subjected to MS/MS analysis indicated there was a potential phosphorylation residue at Thr¹² on the N-terminus of

UCP1. However, the mass to ion charge (m/z) was lower than expected at 23, while a typical m/z cutoff score is 25. In contrast, we were not able to detect protein phosphorylation of immunopurified UCP1 from WA and CE mice. To clarify, the discrepancies between our samples can be explained by the potential methods used for analysis as well as the source of our samples. Recombinant UCP1 produced in *E.coli* was analyzed with an in-solution digestion method which consisted of purifying our samples with titanium beads, while our immunopurified UCP1 from BAT was analyzed by an in-gel digestion protocol. In order to perform in-solution digestion we used concentrated amounts of a His-tagged recombinant UCP1 produced in *E.coli*. However, a major issue with the production of recombinant protein in *E.coli* is the addition of a molecular Tag in order to improve solubility and purification. However, with protein tags comes the concern of mis-folding and changes in protein activity which could be detrimental to the function of protein, and could exposes residues for modification, which otherwise would not be available (63). Hence, the sources of our UCP1 protein for analysis may play a role in the results discrepancy.

Next, immunopurified UCP1 isolated from BAT mitochondria of WA and CE mice was subjected to a preparatory clean-up under harsh solubilization and sonication conditions prior to an in-gel digestion protocol. Note we conducted an in-gel digestion protocol with immunopurified UCP1 because it offers the advantage of removing contaminants such as salts and detergents that may interfere with MS/MS analysis. It is plausible that through the process of solubilizing our proteins, the status of covalent modifications were disrupted. Next, we subjected our immunopurified UCP1 samples to SDS page and silver staining followed by the excision of our protein bands. We validated our in-gel digestion with immuno-detection of UCP1 in BAT using

liver mitochondria as a control. The presence of UCP1 was confirmed in BAT, while no UCP1 was detected in liver mitochondria. Hence, we are confident that our in-gel digested sample contained UCP1. However, phosphorylation of immunopurified UCP1 was not detected as discussed above. An inherent limitation of the in-gel digestion method is poor sequence coverage, as protein extraction from gel pieces rarely yields a full complement of digested peptides (88). We did conduct our studies in the presence of phosphatase inhibitors and on ice thus eliminating the possibility that endogenous enzymes were removing the phosphate groups. An alternative explanation as to why we weren't able to detect phosphorylation of immunopurified UCP1 could involve contaminating proteins. While antibodies are specific and as such are used in immunopurifications, it is possible that non-specific proteins bound to the antibody or to the coupled agarose beads. Furthermore, some of these contaminants may include endoplasmic reticulum proteins due to the use of differential centrifugation as an isolation method for mitochondria. To note, there is a large proportion of phosphorylated nuclear proteins, and as such if immunopurified could mask the less abundant phosphorylation levels of mitochondrial protein such as UCP1.

We next investigated whether or not PKA was required to phosphorylate UCP1. PKA plays an integral role in adrenergic signaling in BAT following cold acclimation. Specifically, PKA is implicated in 1) activating UCP1 gene expression and 2) inducing fatty acid mobilization for UCP1 protein activation. Further, PKA was recently identified in the mitochondrial matrix and was found to enhance electron transfer through the respiratory chain (1, 3). Our bioinformatics analysis also identified PKA as a potential kinase required to phosphorylate UCP1. Additionally, analysis of the UCP1 interactome revealed that PKA may interact with

UCP1 in CE mice. To determine if PKA was required to activate UCP1, mitochondria from WA and CE mice were treated with 8Br-cAMP, a PKA agonist that activates its kinase activity (1, 3). We hypothesized that CE may be a method of activating UCP1 dependent respiration and therefore treatment with the PKA agonist 8Br-cAMP should not alter mitochondrial respiration in BAT mitochondria from CE mice (since they might be already in a phosphorylated state). On the other hand, we predicted mitochondrial respiration from WA mice could be modified with 8Br-cAMP treatment. Addition of 8Br-cAMP tended to increase UCP1-dependent respiration only in BAT mitochondrial preparations from WA mice, but this increase was only at the level of a trend. No changes in respiration were observed in mitochondrial extracts from BAT of CE mice. Hence, our results could indicate that UCP1 dependent respiration is not controlled by phosphorylation. Our results also could indicate that PKA may not be the primary kinase responsible for phosphorylating UCP1. Other potential kinases include PKB and PAK which were also strongly predicted, based on GPS prediction site mapping, to act on UCP1. Taken as whole, these data indicate that UCP1 may be phosphorylated following cold exposure. However, it is clear that the nature of the modification is inherently complex and may also be mediated by other kinases. It is very well established that UCP1 is allosterically activated by FA (69, 157). Indeed, our results from pull down analysis and interactome mapping indicated that UCP1 has indirect interactions with enzymes related to fatty acid oxidation such as long and short chain acyl-coenzyme dehydrogenase ACADL and ACADS respectively. Additionally, interactome analysis showed that PKA indirectly interacts with UCP1. Thus, both fatty acids and phosphorylation may work in tandem to modulate proton leaks through UCP1. Thus, the modifications detected are indicative of phosphorylation *in vitro*, but we cannot rule out other types of modifications. It is thus unknown if UCP1 phosphorylation stimulates adaptive

thermogenesis; more kinases need to be tested. Future research should evaluate the direct interaction of a UCP1 modulator and its role in controlling adaptive thermogenesis.

Impact of Ectopic UCP1 Protein Expression on Proton Leaks in Muscle:

Activating proton leaks in mitochondria to increase energy expenditure has long been hypothesized as a novel mode to treat obesity. The original studies date back to the 1930's when it was found that proton ionophore 2,4-dinitrophenol (DNP) could be used as a weight loss drug. Specifically, DNP was found to increase energy expenditure by up to ~40%, which resulted in a decrease in fat mass (167, 218). DNP was used for a short period as a weight loss drug, but clinical use ceased after a series of deaths. Although the use of DNP had some dreadful consequences, it illustrated that the activation of proton leaks in a controlled fashion could potentially be used to treat obesity. As discussed above, it is now known that proton leaks are controlled by members of the anion carrier superfamily such as the uncoupling proteins. In fact, an improved understanding of the regulation of uncoupling proteins *in vivo* is of critical importance because of their diverse function and tissue locations. It's been shown that they can be modulated physiologically by allosteric activators or by changes in mitochondrial redox signaling. However, the relationship linking the cellular environment to UCP function still remains a subject to be explored. Recently, UCP1 was shown to curb ROS production when ectopically expressed in skeletal muscle and in BAT; however, other studies have refuted this claim (57, 114, 136). Although skeletal muscle and BAT share common lineages, their cellular redox environments are quite different and thus may regulate protein function differently as well. Indeed, Mailloux *et al* (136) showed that in skeletal muscle the ratio of reduced to oxidized glutathione (GSH/GSSG) is increased when compared to BAT. The importance of this ratio is

paramount in order to maintain a healthy cell environment since glutathione is the major non-protein anti-oxidant present within the cell (82). Given these ideas we explored how differences in cellular and tissue environments or redox biology may modulate UCP1 activity in different tissues. To explore this mechanism we chose a mouse model that ectopically expresses UCP1 in skeletal muscle using a muscle specific creatine kinase promoter. Briefly, we obtained results indicating that overexpression of UCP1 in muscle leads to changes in food intake, total body weight, tissue weights, energy expenditure, and RER. We also showed that there was an increase in muscle glucose uptake from circulation, an increase in proton leak, a decrease in ROS production, and an increase in glutathione content.

First, we analyzed physiological parameters such as whole body weight and the weights of selected tissues. It was imperative that we characterize the physiology of these mice to show that ectopic expression of UCP1 had no adverse effects on whole body health. We observed reductions in total body, skeletal muscle (fore and hind limb) and white adipose tissue weights (gonadal) in Tg compared to Wt mice. We measured forelimb and hind limb skeletal muscle and gonadal white fat as a proxy measurement of whole body skeletal muscle and adiposity, respectively. The reduction in whole body and adiposity weights could be a result of 1) a decrease in nutrient intake and/or 2) an increase in energy expenditure. Interestingly, our results showed an increase in food intake which agrees with the literature describing that UCP1 expression in muscle induces hyperphagia (49). Thus our findings are consistent with the idea that Tg mice had a significant increase in whole body energy expenditure. Thus, we further explored changes in energy expenditure by using indirect calorimetry (IC). We chose to use the IC method because it offers a very fast, easy and reliable method of assessing metabolic rate

(66). We measured total volume of oxygen (VO_2) consumed over a 24h period in Tg and Wt mice, and normalized data to total body weight or total lean mass. Furthermore, we normalized two different weights in order to account for the discrepancies in total body and skeletal muscle weights between Tg and Wt mice. Interestingly, even when VO_2 was normalized to total body or lean muscle weight, Tg mice displayed significant increases in VO_2 consumption rate. These results are in agreement with other studies that show ectopic UCP1 expression increases whole body EE (15). Based on these results and those in the literature, we would hypothesize that ectopic expression of UCP1 in muscle promoted negative energy balance by increasing mitochondrial proton leaks. This increase in muscle-dependent EE could account for the decrease in adiposity since muscle is reliant on triglycerides stored in WAT as an energy source. Furthermore, slight increases in the metabolic rate in muscle might have a significant impact at the whole body level because skeletal muscle accounts for a large proportion of total body weight and EE (96). Increases in UCP1-dependent leaks in muscle could also compromise cold adaptation and the induction of adaptive thermogenesis. However, we found that ectopic expression of UCP1 muscle did not compromise the ability of mice to increase their metabolic rate in response to cold temperatures. (15). In fact, UCP1 expression in skeletal muscle has been shown to have positive effects on metabolism such as decreasing blood pressure and improving insulin sensitivity in a genetic mouse model of obesity (Agouti mouse model) (15). Classically, the activation of leaks through UCP1 in BAT leads to increased glucose uptake and metabolism. Additionally, the ectopic expression of UCP1 in muscle has been shown to alter muscle fiber type by promoting a fast (glycolytic) to slow (oxidative) fiber type switch (49). In addition, Gaster et al (79) showed in oxidative fibers of non-diabetic patients that GLUT 4 (main glucose carrier in muscle) expression was increased. We then sought to determine if the increase in

energy expenditure in Tg mice was associated with an increase in the use of carbohydrates vs. fats to support fuel metabolism. Differences in glucose usage were measured using two methods; respiratory exchange ratio (RER) which provides an index for preferred fuel usage and microPET imaging, which provides *in vivo* measurements of muscle glucose uptake. Under ambient conditions Tg mice preferentially used more glucose than fatty acids as a substrates during awake and feeding time points (i.e., dark phase of the day) which is consistent with the increase in energy expenditure in these mice. However, following a decrease in the ambient temperature, there were no longer any substrates utilization differences between Tg and Wt mice. The increase in RER prompted measurements of glucose uptake by muscle using microPET. Basal glucose uptake into skeletal muscle, BAT, and cardiac tissues were simultaneously measured in Tg and Wt mice. UCP1 expression in muscle increased glucose uptake into skeletal muscle of Tg mice, corroborating the preferential glucose usage indicated by our RER values. Interestingly, adrenergic stimulation with NE did not further increase elevated the rate of glucose uptake in Tg mice even further. The NE effect was absent in Wt mice. Furthermore, measurements of circulating glucose pre and post adrenergic stimulation indicated there was no different in glucose levels between Tg and Wt mice. These results indicate the increased rate of glucose uptake in Tg mice is not dependent on differences in circulating glucose availability. Taken together, our results indicate that skeletal muscle uncoupling through UCP1 drives an increase EE and an increase in feeding as a compensatory mechanism and *in vivo* may lead to preferential glucose usage. Further, increase in EE could lead to the physiological differences seen in this study and by others (113, 156). Furthermore, we hypothesize that the preferential usage of glucose is needed to accommodate cellular and tissue ATP demands due to the increased EE. Indeed, if oxidative phosphorylation is compromised or proton leaks are increased,

then more substrates are needed to support the maintenance of a membrane potential in order to fulfill cellular ATP demands.

The increase in whole body energy expenditure and muscle glucose uptake prompted us to measure the energetics and redox biology of muscle mitochondria from Wt and Tg mice. It has been suggested that proton leaks produced by the ectopic expression of UCP1 in different systems is uncontrolled and detrimental (216). Moreover, some studies have shown that high expression of ectopic UCP1 in skeletal muscle can lead to mitochondrial dysfunction and intramyocellular lipid accumulation (92). However, this dysfunction was not seen in Tg mice that expressed UCP1 in low amounts. In fact, UCP1 expression in our model was ~13% less than the endogenous expression in BAT and these expression patterns are similar to other studies in which no pathologies were documented (156). Thus, we started by assessing coupled respiration in isolated mitochondria. Our results showed that UCP1 did not compromise the ability of mitochondria to respond to ADP stimulation as validated by previous literature (123). Overexpression of uncoupling proteins 1-3 in mitochondrial membranes can lead to uncontrolled proton leaks. This is due to improper folding which facilitates passive diffusion of protons back into the matrix and the depolarization of the mitochondrial inner membrane. The fact that UCP1 expression does not compromise coupled respiration indicates that UCP1 is not improperly folded and does not disrupt mitochondrial energetics in muscle. However, to determine if the increase in EE was due to the expression of UCP1 in muscle we measured GDP-sensitive proton leaks in muscle mitochondria. Indeed, oxygen consumption measured in mitochondria isolated from Tg mice showed increased sensitivity in presence of GDP. These results indicate that the increase in EE is associated with UCP1 proton leaks. Further, these data illustrate that UCP1 is properly folded and active. A similar experiment was performed in BAT mitochondria with both

Tg and Wt mitochondria showing similar response with GDP titration. These results are enticing because 1) they show that proton leaks through skeletal muscle mitochondria could account for the changes seen at the whole body level, 2) mitochondrial integrity was not compromised in skeletal muscle and 3) ectopic expression of UCP1 in muscle did not compromise the function of other tissues such as BAT.

The physiological role of UCP1 is to induce proton leaks for adaptive thermogenesis. However, there is currently a debate as to whether UCP1 like UCP2 and UCP3 plays a role in controlling mitochondrial ROS production. Previous work has shown that leaks through UCP1 do not curtail superoxide production by mitochondria (73). Similarly, Mailloux and colleagues generated results with BAT mitochondria showing that activation of leaks through UCP1 actually augments mitochondrial ROS production in BAT (136). Conversely, several other studies using various model systems in which UCP1 was expressed endogenously, ectopically, and or ablated, it was shown that UCP1 plays a part in ROS control (41, 57, 114). Hence, the true function of UCP1 in the control of mitochondrial ROS production is controversial (9, 166). However, it is important to note that Mailloux and colleagues revealed that UCP1 does not control mitochondrial ROS production due to fundamental differences in redox environment between BAT and other tissues. Indeed, their results indicated that BAT mitochondria had a decrease in reduced glutathione (GSH) and an increased in oxidized glutathione (GSSG). Further, BAT mitochondria contain low amounts of glutaredoxin-2 (Grx2), a matrix oxidoreductase that is involved in regulating mitochondrial protein function in response to fluctuations in redox (139). The glutathione ratio in BAT mitochondria displays an oxidized environment. In contrast, skeletal muscle mitochondria displayed a reduced environment as indicated by a higher GSH to GSSG ratio. Given these

exciting differences between tissues and the question of UCP1's role in ROS control we decided to measure ROS production in our mouse model which ectopically expressed UCP1 in muscle. Findings herein show that GDP inhibition of UCP1 in skeletal muscle mitochondria caused an increased in ROS emission. GDP had no effect on ROS in Wt skeletal muscle mitochondria. Conversely, BAT mitochondria from Tg or Wt mice displayed elevated ROS production with no changes in the rate of production upon addition of GDP. Our findings therefore suggest that in skeletal muscle UCP1 may play a role in ROS control in contrast to what has been observed in BAT. (114, 136). These differences in function could be a result of the differences in redox environment between skeletal muscle and BAT. We measured total glutathione in skeletal muscle and BAT mitochondria. We found that the total glutathione levels in skeletal muscle of Tg mice when compared to Wt mice were significantly increased (~14 fold), while these stark changes were not observed in BAT mitochondria. Hence, the significant increases in the total glutathione, which is involved in redox control in the mitochondria, maybe a result of UCP1 expression in skeletal muscle. Given our results showing that ROS was modulated by UCP1 expression in muscle and that total glutathione levels were increased, we explored the question of whether skeletal muscle of Tg mice was oxidatively stressed. Thus, we measured protein carbonyl adducts in muscle and found no significant changes between our two groups. Our results indicate that 1) UCP1 may be involved in the control of ROS production in skeletal muscle, which is in contrast to others (207), or 2) UCP1 may indirectly affect oxidative stress by modulating the redox environment (increased total glutathione in muscle). Given these results future studies could be proposed to explore the exact mechanism by which UCP1 controls ROS in muscle and its effect on the anti-oxidative defense systems *in vivo*.

CONCLUSION:

In summation, this thesis provides new information about the role of alternative mechanisms that may regulate UCP1 function. Specifically, we show here that UCP1 can undergo post-translational modification indicative of phosphorylation and that this covalent modification may be important for UCP1 function. However, more evidence is needed in order to confirm our initial phosphorylation results as well as determine the physiological implications. On the other hand, we show that mitochondrial redox environment in different tissues can play a significant role in regulating UCP1 function. Specifically, we show that ROS production in muscle of mice expressing UCP1 is sensitive to UCP1-mediated proton leak. In contrast, the control of ROS production in BAT shows no sensitivity towards proton leaks through UCP1. Furthermore, we show that the divergence of UCP1 function in muscle compared to BAT could be a result tissue differences in mitochondrial redox status. Indeed, total glutathione was ~ 14 fold higher in mice expressing UCP1 in Tg vs. Wt muscle, while no such difference were seen in BAT. In addition to these exciting findings, we also reinforced the role of UCP1 as a potential target for obesity. Assessments of body weight and adiposity showed significant decreases in mice ectopically expressing UCP1 compared to Wt controls. Additionally, expression of UCP1 in muscle lead to increased EE, increased mitochondrial proton leaks and did not compromise mitochondrial function. Taken together, these results indicate that alternative mechanisms regulating UCP1 are of importance and are in need of more research. Overall, these results indicate there are complex cellular mechanisms that control UCP1 function.

FUTURE WORK:

Given the described results, further research is needed to determine the roles of alternative PTM mechanisms that regulate UCP1. The current findings reveal that CE of mice increases acidic shifts in the pI of UCP1. These shifts were indicative of phosphorylation; however, we could not conclusively determine whether this modification could be regulated *in vivo* by CE. Hence, to determine if phosphorylation is indeed a mechanism that controls UCP1 function future research should explore the kinases responsible for phosphorylation. Also, we cannot ignore other post-translational modifications, such as acetylation, as a means to regulate UCP1. As such *in vitro* and *in vivo* acetylation studies are needed to either rule out or shed light on the role of this mechanism. Further work is needed to identify potential interacting protein partners of UCP1 as this would offer even more insight into UCP1 regulation. Finally, our results show that UCP1 regulates ROS production differently depending on the tissue in which it is expressed (i.e., muscle vs. BAT). As such more research is needed to elucidate tissue specific factors that are important in the control of mitochondrial uncoupling.

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235. **Yang X, Pratley RE, Tokraks S, Tataranni PA, and Permana PA.** UCP5/BMCP1 transcript isoforms in human skeletal muscle: relationship of the short-insert isoform with lipid oxidation and resting metabolic rates. *Molecular genetics and metabolism* 75: 369-373, 2002.
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237. **Yu XX, Mao W, Zhong A, Schow P, Brush J, Sherwood SW, Adams SH, and Pan G.** Characterization of novel UCP5/BMCP1 isoforms and differential regulation of UCP4 and UCP5 expression through dietary or temperature manipulation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 14: 1611-1618, 2000.
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APPENDIX:

CURRICULUM VITAE: Cyril Nii-Klu Adjeitey

Summary of Qualifications

- Bachelor of Science- Honours in Biochemistry with tentative M.Sc. defense date scheduled for Mid-May 2013.
- Served on five different councils involved in various policy creation and implementation
- Three years of teaching/tutoring/mentoring experience as well extensive experience in active leadership roles in different organization.
- Excellent technical skills in Microsoft Word, Excel, PowerPoint, Windows, and Internet search tools.
- Fluent in English and Ga with desire and willingness to improve French.

AWARDS, HONOURS, ACCOLADES

- | | |
|--|-------------|
| • Ontario Graduate Scholarship Program | 2011 & 2012 |
| • University of Ottawa Excellence Scholarship | 2011 & 2012 |
| • Graduate Poster day research award (Department of Biochemistry) | 2011 |
| • Undergraduate Research Excellence Award (Department of Biochemistry) | 2010 |
| • 2 nd Team All-Canadian-Football (Canadian Inter-University Sport) | 2010 & 2009 |
| • 1 st Team OUA All-star-Football (Ontario University Athletics) | 2010 & 2009 |

WORK EXPERIENCE

Academic Mentor University of Ottawa Sports Services

2010-present

- Coordinated with a group of mentors to build an academic survival guide for incoming first year student athletes (Football)
- Meet weekly, formulate goal setting strategies and assist student athletes in executing the survival plans
- Used various visual tools such as PowerPoint and Prezi to communicate academic material

Teaching Assistant- Biochemistry-BCH 2333

2011-present

- Hold discussion groups in order to teach course material to second year university students as well as host office hours to answer additional questions
- Mark quizzes, assignments, midterms and exams as required

Research in Mitochondrial Bioenergetics Lab

2009- 2010

- Worked with acute promyelocytic leukemia cells to discern the metabolic characteristics of drug resistant and drug sensitive cancer cells, and their relation with Uncoupling Protein (UCP) 2 expression
- Communicated my results through a written thesis, seminar and poster presentations

Administrative Assistant-University of Ottawa Men's Varsity Football team 2008-2010

- Worked with the men's varsity football coaching staff to coordinate banquet preparation for alumni events as well as host sports camps for prospective youths
- Worked on creating an up to date alumni and player contact information database for revenue generation purposes

EDUCATION & TRAINING

M.Sc. Candidate in Biochemistry (submitted) 2011-2013
University of Ottawa, Ontario

Honours B.Sc. with specialization in Biochemistry 2006-2010
University of Ottawa, Ontario

RESEARCH AND SCHOLARLY ACTIVITIES

Graduate studies M.Sc. Thesis Project (submitted) 2011-2013
University of Ottawa, Ontario.

- Using various techniques to assess the implication of Uncoupling Protein 1 phosphorylation on resting energy expenditure, glucose and lipid metabolism as well as brown adipose tissue bioenergetics

Undergraduate Honours Thesis Project 2009-2010
University of Ottawa, Ontario

- Used acute promyelocytic Leukemia cells, a cancer model to determine the link between cancer drug resistance and uncoupling protein 2 expression

RELEVANT POLICY AND ADVISORY COMMITTEE WORK

VSE committee-University of Ottawa Sports Services 2012

- Worked with the athletic director and various members of sports services as well as an independent consulting firm to develop and implement a varsity funding program
- See link for Final published report
- http://www.geegees.ca/sites/default/files/VSE_Final_Report2012.pdf

Biochemistry, Microbiology, and Immunology Graduate Association 2012-present

- Member of a student council that hosted social events in order to enhance the graduate student experience
- Coordinate the transition of policy implementation from the faculty to the departmental to students
- Facilitate and coordinate student invited seminar speakers as well as hosting career day events

Ad Hoc Committee- University of Ottawa 2011

- Worked with the vice-president of external relations, athletic director, and vice-president academic and provost to discuss the naming rights of the Sports complex at the university of Ottawa

University of Ottawa Football Alumni Committee 2012-present

- Responsibility includes evaluating and the selection criteria for the recently hired men's varsity head football manager
- Evaluate curriculum vitae of prospective coaches as well interview a total of 7 qualified candidates from a pool of approximately 50.
- Ensure a non-biased approach in the decision making process to hire the current and future football program managers.

PUBLICATIONS

- **C.Adjeitey**, R.Mailloux, R, deKemp and ME Harper. Mitochondrial uncoupling in skeletal muscle through UCP1 augments energy expenditure and glutathione content, while mitigating ROS production. INVITED FOR RESUBMISSION E-00057-2013.
- A. Pfefferle, R. Mailloux, **C.Adjeitey**, and ME Harper. Glutathionylation of UCP2 sensitizes drug resistant leukemia Cells to Chemotherapeutics. BIOCHIMA BIOPHYSICA ACTA. 2013 JAN;1833(1):80-9
- R. Mailloux, **C. Adjeitey**, J. Xuan, and ME Harper. Crucial yet divergent roles of mitochondrial redox state in skeletal muscle versus brown adipose tissue energetics. FASEB J. 2012 Jan;26(1):363-75
- R. Mailloux, **C. Adjeitey**, and ME Harper. Genipin-induced inhibition of uncoupling protein-2 sensitizes drug-resistant cancer cells to cytotoxic agents. PLoS One. 2010 Oct 13;5(10)

CONFERENCES

MOLECULAR FUNCTION & IMAGING SYMPOSIUM (Ottawa, Ontario) **2011**

- Invited guest speaker to present work on Uncoupling Protein 1 at the annual Ottawa Heart Institute Symposium

OTTAWA SYSTEMS BIOLOGY AND CHINA SYMPOSIUM AND THE 19TH METHODS IN PROTEIN STRUCTURE ANALYSIS MEETING (Ottawa, Ontario) **2012**

- Invited poster presentation to speak about the bioenergetics implication of post-translational modification of Uncoupling Protein 1

THE OBESITY SOCIETY MEETING (San Antonio, Texas) **2012**

- Invited poster presentation to share recent findings on Uncoupling Protein 1 function in brown adipose tissue (BAT) of *Mus musculus*

COMMUNITY INVOLVEMENT

Rogers House Palliative care **2011-present**

- Assist with the care of children/youth diagnosed with life limiting illnesses, residing at Roger's House for temporary respite care and/or end of life care
- Work with a multidisciplinary team, supervising, playing with and entertaining children/youth and communicating information with parents and family members

Ottawa General Hospital Rehabilitation Centre **2009**

- Hosted various social events for patients with brain trauma, neurological disorders, and chronic life-altering illnesses
- Provided psychological and physical assistance while developing supportive relationships with patients during their rehabilitation period

EXTRACURRICULAR ACTIVITIES

- Coaching youth sports (football and basketball, Gridiron Academy) **2008-present**
- Former member of the mens varsity football team (University of Ottawa) **2006-2010**
- Member of the Ontario University Athletics Champion for Life Program **2010**
- Academic Mentor for the varsity football team (University of Ottawa) **2011-present**

REFERENCES:

Mary-Ellen Harper, Ph.D.

Thesis Supervisor

Colin Timm

Assistant Director, Programs Sports Services (University of Ottawa)

Ryan Mailloux Ph.D.

Postdoctoral Fellow