

THE UPR IN ADIPOGENESIS AND ENERGY HOMEOSTASIS

CONTRIBUTION OF THE UNFOLDED PROTEIN RESPONSE (UPR) TO
ADIPOGENESIS AND WHOLE BODY ENERGY HOMEOSTASIS

By

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ABSTRACT

The endoplasmic reticulum (ER) is a specialized organelle that facilitates correct protein folding and maturation. Disruptions in ER homeostasis lead to ER stress and activation of a series of signal transduction cascades known as the unfolded protein response (UPR), which acts to restore ER homeostasis. In recent years, ER stress and UPR dysfunction have been linked to obesity, fatty liver and insulin resistance. Lipid-laden adipocytes, the main cellular component of white adipose tissue (WAT), play a critical role in whole body energy homeostasis as well as lipid and carbohydrate metabolism. Mature adipocytes, which are metabolically active endocrine cells, differentiate from precursor fibroblast-like preadipocytes, through a process called adipogenesis, leading to formation of cells capable of secreting numerous proteins, cytokines and hormones. ER homeostasis and UPR activation are essential to the function/differentiation of highly secretory cells, however, the role of ER stress/UPR activation in adipogenesis had previously not been examined. We hypothesized that *adipogenesis may rely on physiological UPR activation to accommodate the demand on the ER for increased folding and secretion of proteins.*

Initial experiments examining UPR activation during 3T3-L1 adipogenesis identified that expression of ER stress/UPR markers was modulated during adipocyte differentiation. Furthermore, inhibition of ER stress/UPR activation by the chemical chaperone, 4-phenyl butyric acid (4-PBA), inhibited adipogenesis and blunted high fat-diet induced weight gain in 4-PBA supplemented mice. These findings suggested that UPR activation modulates adipogenesis and adipose tissue metabolism.

Subsequently, we sought to identify novel candidate ER stress/UPR responsive genes that may be involved in adipogenesis and WAT metabolism. The expression of a recently recognized ER stress-responsive gene, T-cell death associated gene 51 (TDAG51) was identified to be differentially regulated during adipogenesis. However, the function of TDAG51 in adipogenesis or energy regulation was not known. Studies from this thesis showed that TDAG51 protein expression is attenuated by ER stress/UPR activation in preadipocytes and declines during adipogenesis. Based on these results, and given the importance of adipogenesis in WAT function and whole body energy metabolism, it was hypothesized that *TDAG51 may be a novel regulator of adipogenesis and energy homeostasis*. Indeed, as reported here, knock-down or absence of TDAG51 (*TDAG51^{-/-}*) in pre-adipocytes increased lipogenesis and lead to earlier and more potent expression of adipogenic markers.

Finally, we investigated whether absence of TDAG51 in mice affected adiposity and metabolic outcomes. Consistent with the *in vitro* results, we found that *TDAG51^{-/-}* mice fed a standard chow diet, exhibited an age-associated increase in WAT, developed fatty liver, and exhibited insulin resistance as compared to wild-type mice.

Taken together, the findings in this thesis indicate that physiological UPR activation and the UPR-responsive gene TDAG51 play important roles in regulating adipogenesis, lipogenesis and whole-body energy metabolism. Thus, therapeutic approaches aimed at modulating ER folding capacity, UPR activation and/or TDAG51 expression may have great potential in the treatment of obesity and its co-morbidities.

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PREFACE

This thesis is prepared in the “sandwich” format as outlined in the “Guide for the preparation of Master’s and Doctoral Theses” available through the School of Graduate Studies at McMaster University. Chapter 1 of this thesis contains parts of a book chapter and review article both of which were written by the candidate and serve as a general introduction. The body of this thesis consists of 3 chapters (Chapter 2-4), each one an independent study, one of which is published and the other two submitted for publication or under review at the time of the thesis submission. All submitted and published studies and manuscripts included in this thesis were written by the author of this thesis, who is also the first author on all included works. The preamble section preceding each chapter describes the contributions of other authors to the multi-authored work. Finally, the discussion section (Chapter 5) summarizes the conclusions of this thesis and draws out the overall implications. Parts of chapter 5 consist of sections written for a book chapter and review article which were modified to illustrate how the findings of this thesis project relate to recent advances in the field and the novel contributions of this thesis to expansion of knowledge in medical research.

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
4-PBA	4-phenyl butyric acid
ACC	acetyl CoA carboxylase
AdV-βGal	adenovirus containing β galactosidase
AdV-GRP78	adenovirus containing the 78 kDa glucose regulated protein gene
Akt	serine/threonine protein kinase Akt
ALT	alanine transaminase
aP2	adipocyte protein homologous to myelin P2
ATF6	activating transcription factor 6
ATGL	adipose triglyceride lipase
AUC	area under the curve
BAT	brown adipose tissue
BMI	body mass index
C/EBP	CCAAT/enhancer binding protein
CHOP	CCAAT/enhancer-binding protein homologous protein
CRT	calreticulin
CT	computed tomography
DAG	diacyl glycerol
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
eIF2α	eukaryotic translation initiation factor, alpha subunit
ELISA	enzyme-linked immunosorbent assay

EM	electron microscopy
ERK	extracellular signal-regulated kinase
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FA	fatty acid
FABP	fatty acid binding protein
FAS	fatty acid synthase
FBS	fetal bovine serum
FDG	fluoro-deoxyglucose
FFA	free fatty acids
GADD153	growth arrest and DNA damage inducible gene 153
GADD34	growth arrest and DNA damage inducible protein 34
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GK	glucokinase
GLUT	glucose transporter
GRP78	glucose regulated protein of 78 kDa
GSK3	glycogen synthase kinase 3
GTT	glucose tolerance test
H&E	hematoxylin and eosin
HF	high fat
HFD	high fat diet
HSL	hormone sensitive lipase
IBMX	3-Isobutyl-1-methylxanthine
IFN-γ	interferon gamma
IL-6	interleukin-6

INSIG	insulin induced gene
IR	insulin receptor
IRE 1	inositol requiring kinase 1
IRS-1	insulin receptor substrate-1
ITT	insulin tolerance test
JNK-1	c-Jun N-terminal kinase-1
kDa	kilo-dalton
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblast
NAFLD	non-alcoholic fatty liver disease
NEFA	non-esterified free fatty acid
ob/ob	obese (leptin)-deficient
ORO	oil red o
PA	phenyl acetate
p-eIF2α	phosphorylated eIF2 α
PERK	double-stranded RNA-activated protein kinase (PKR)-like ER kinase
PET	positron emission tomography
PH	pleckstrin homology
PHLDA1	pleckstrin homology-like domain family member A1
PI3K	phosphoinositol 3-kinase
PIKE	phosphoinositol 3-kinase enhancer
PIP2	phosphatidylinositol 4,5 diphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PPAR	peroxisome proliferator-activated receptor
RNA	ribonucleic acid

SCAP	SREBP cleavage-activating protein
SCD-1	stearoyl Co-enzyme desaturase 1
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
SREBP-1	sterol regulatory element binding protein-1
SVC	stromal-vascular cells
T2D	type 2 diabetes mellitus
TBARS	thiobarbituric acid reactive substances
TDAG51	T-cell death associated gene 51
Tg	thapsigargin
TG	triglycerides
Tm	tunicamycin
TNFα	tumour necrosis factor alpha
TUDCA	tauro-ursodeoxycholic acid
UCP-1	uncoupling protein-1
UPR	unfolded protein response
VO₂	oxygen consumption
WAT	white adipose tissue
WHO	world health organization
WT	wild-type
XBP1	X-box binding protein 1
XBP1s	spliced XBP-1

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

Preamble

Parts of this chapter have been adapted from a review article and a book chapter which were written by the author of this thesis. The details of these publications are listed here.

Publication 1 (Review Article)

Sana Basseri and Richard C. Austin. (2012) Endoplasmic Reticulum stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13. doi: 10.1155/2012/841362

Authors' Contribution: S. Basseri conducted the literature review, compiled and summarized the research in the field, and wrote the review article in its entirety. R.C. Austin edited the review article, provided intellectual input and acts as the corresponding author.

Publication 2 (Book Chapter)

Sana Basseri and Richard C. Austin. (2012) Chapter 10: Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In "Endoplasmic Reticulum Stress in Health and Disease" to be published by *Springer* in July 2012.

Authors' Contribution: S. Basseri conducted the literature review, compiled and summarized the research in the field, and wrote the book chapter in its entirety. R.C. Austin edited the chapter, provided intellectual input and acts as the corresponding author.

1.0. INTRODUCTION

1.1. Global prevalence of obesity and its complications

Overweight and obesity, which are defined as excessive fat accumulation with potential adverse health outcomes, are a growing public health concern. In adults, body mass index (BMI), a measure of weight to height ratio (kg/m^2), is often used to classify overweight and obesity, where a person with a $\text{BMI} \geq 25$ is overweight and a $\text{BMI} \geq 30$ is considered obese. According to the World Health Organization (WHO), in 2008 an alarming 1.5 billion adults over the age of 20 were overweight and of these, 500 million were obese (WHO, 2011). Today, overweight and obesity affect not only the developed world but are on the rise in low and middle-income countries (particularly in urban settings), and result in more deaths than underweight (WHO, 2011). Nearly 3 million adults die each year due to overweight and obesity complications. In Canada, during the 2007-2009 period, 61% of adults were overweight or obese, with 1 in every 4 adults considered obese (CHMS, 2010).

Although the cause of obesity is complex and multi-factorial, at the most fundamental level it is caused by an imbalance in energy intake and energy expenditure as the availability and consumption of energy-dense foods has increased while physical activity has decreased in many of today's societies. Obesity and its co-morbidities which include type 2 diabetes (T2D), hypertension, heart disease, osteoarthritis and certain types of cancers (Lau et al., 2007), are largely preventable but require modifications in lifestyle by the individual, as well as support and changes at the societal and food industry level.

The increasing prevalence of obesity in Canada and globally, is unlikely to reverse in the coming years as nearly 43 million children under 5 years of age are considered overweight and are at risk of being obese in adulthood according to 2010 global estimates (WHO, 2011). Therefore, there is a pressing need to better understand the biological and physiological components of obesity and weight gain in order to develop effective therapeutic strategies for treating and reversing obesity and its health complications. In response to the growing prevalence of obesity and its burden on the healthcare system, there has been a drastic expansion of knowledge and intense scientific investigations in the field of obesity and T2D in the last decade. While dietary intervention remains the cornerstone of obesity treatment, due to poor adherence and modest long-term favourable outcomes, surgical and pharmacological treatments are often required to treat obesity (Lau et al., 2007). However, effective and long-lasting pharmacological treatment modalities remain limited. A better understanding of the molecular and cellular processes that are involved in the regulation of energy homeostasis and metabolism will ultimately provide us with powerful tools to tackle this growing healthcare problem worldwide.

1.2. The Endoplasmic reticulum and its role in obesity and lipid metabolism: an overview¹

The obesity epidemic and its impact on human health have prompted researchers to examine fat (adipose) tissue function and metabolism more closely, which has led to

¹ This section has been adapted from the following book chapter with kind permission of Springer Science and Business Media: **Basseri S** and Austin RC. (2012) Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In “*Endoplasmic Reticulum Stress in Health and Disease*”. In press at *Springer*.

important scientific advances in the field. In recent years, it has become well-accepted that adipose tissue not only stores excess energy, but is the body's largest endocrine organ, secreting hormones, cytokines and other proteins, which can exert their effects on vital organs (Gesta et al., 2007). In adult humans, visceral and subcutaneous white adipose tissue (WAT) is the predominant type of fat, where excess calories are stored in adipocytes as triglyceride (TG) molecules. Obesity and excess WAT lead to alterations in glucose and lipid disposal as well as dysregulation of adipocytokine expression and secretion from WAT (Lefterova and Lazar, 2009). As a result, the body's energy homeostasis and metabolic regulation are affected leading to the development of obesity complications such as insulin resistance (IR), cardiovascular disease (CVD), and ectopic lipid accumulation resulting in fatty liver disease and pancreatic β cell dysfunction.

Inflammation and oxidative stress are present in obese adipose tissue and can cause IR and adipose tissue dysfunction (Gregor and Hotamisligil, 2007; Hotamisligil, 2006). Insulin-resistant adipocytes are burdened by mechanical stress caused by excessive lipid accumulation and lipid droplet expansion known as hypertrophy, as well as hypoxia and chronic inflammatory signalling (Gregor and Hotamisligil, 2007). In recent years it has become well-established that maladaptive responses in the insulin resistant adipocyte can impact organelles such as the endoplasmic reticulum (ER), the principal site of protein folding and maturation. Compromised ER function and ER stress can lead to metabolic disturbances by activating inflammatory and stress signalling pathways (Gregor and Hotamisligil, 2007). ER stress leads to impaired insulin signalling (Nakatani et al., 2005; Ozcan et al., 2004; Park et al., 2010; Su et al., 2009) and is

associated with insulin-resistant adipose tissue as well as fatty liver or hepatic steatosis, which are often present in obesity (Fu et al., 2011; Gentile et al., 2011; Ozcan et al., 2004; Puri et al., 2008; Su et al., 2009). In the liver, ER stress has been closely linked to lipid accumulation, and there is a growing body of evidence implicating specific ER stress response genes and pathways in the regulation of hepatic lipogenesis (Bobrovnikova-Marjon et al., 2008; Lee et al., 2008; Oyadomari et al., 2008; Rutkowski et al., 2008; Werstuck et al., 2001; Yamamoto et al., 2010a; Zeng et al., 2004; Zhang et al., 2011; Zheng et al., 2010). In addition to impacting adipocyte function, ER stress pathways also affect the differentiation of adipocytes from their precursor cells as demonstrated in **Chapter 2** and supported by recent publications (Basseri et al., 2009; Bobrovnikova-Marjon et al., 2008; Lowe et al., 2011; Sha et al., 2009). Adipocyte differentiation (adipogenesis) plays a critical role in adipose tissue metabolism, therefore, the adipogenic potential of multipotent mesenchymal² cells and preadipocyte commitment to the adipocyte lineage can affect whole body energy homeostasis.

1.3. The Endoplasmic reticulum and unfolded protein response³

The ER is a membranous organelle with several critical cellular functions. First, it is the site where nascent polypeptides fold into their proper conformation and any necessary

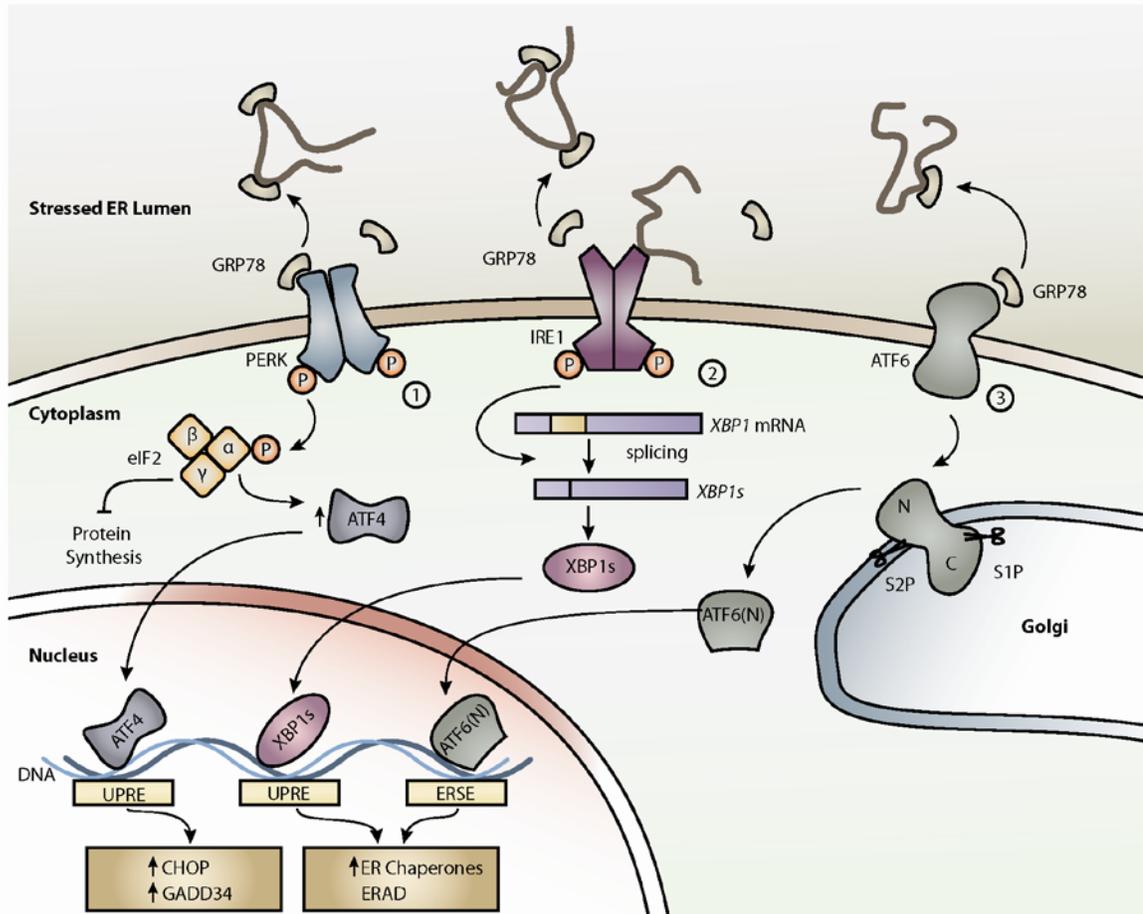
² Refers to cells that have the potential to become any of several mature cell types that develop into connective tissue (such as adipose), blood vessels and lymphatic tissue.

³ This section has been adapted from: **Basseri S** and Austin RC. (2012) Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13.

post-translational modifications such as glycosylation and disulphide bond formation take place. This task is accomplished by ER resident chaperones and foldases and protein disulphide isomerases (PDI) (Shen et al., 2004). Second, phospholipid synthesis takes place in the ER which allows for expansion of lipid bilayers in the cell (Lee and Glimcher, 2009). Third, the ER is a major storage site for calcium ions which are required for cellular signalling processes (Targos et al., 2005). Fourth, enzymes such as cytochrome p450 in the ER allow for efficient metabolism of drugs (Neve and Ingelman-Sundberg, 2010).

A number of physiological, pharmacological and pathological conditions are known to disrupt ER homeostasis and affect its protein folding capacity. The inability of the cell to efficiently fold and secrete proteins is defined as ER stress. Cells have evolved mechanisms to adapt to adverse conditions in order to maintain homeostasis and survive. One such coping mechanism is UPR activation in response to ER stress conditions (Kaufman, 1999; Welihinda et al., 1999). Activation of the UPR ultimately results in i) enhancement of ER protein folding capacity through expansion of the ER and increased expression of chaperones and foldases, ii) inhibition of protein translation, and iii) ER-associated protein degradation (ERAD) of misfolded proteins (Hosoi and Ozawa, 2010). If ER stress conditions are not resolved, ER stress-induced cell death may ensue. Generally, ER stress-associated cell death occurs through caspase activation (Hitomi et al., 2004; Nakagawa et al., 2000); however, caspase-independent necrosis and autophagy have also been observed (Ullman et al., 2008).

The UPR in mammalian cells is composed of three signalling branches which are initiated by three ER transmembrane sensors, inositol-requiring protein 1 (IRE1), double-stranded RNA-dependent protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Activation of these sensors is dependent on the dissociation of the ER-resident chaperone glucose-regulated protein of 78 kDa (GRP78), also known as BiP, from their luminal domains (Hendershot, 2004). This occurs during ER stress conditions when GRP78 is required for the folding of proteins in the ER and thus is recruited away from IRE1, PERK and ATF6, thereby activating the UPR. Activation of the UPR pathways is often used as an indicator of ER stress due to the technical difficulties in directly measuring compromised ER integrity or protein aggregates in the ER (Lee and Glimcher, 2009). **Figure 1.1.** depicts an overview of mammalian UPR signalling pathways.



(Figure is reproduced from Basseri S and Austin RC. (2012) Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13.)

Figure 1.1. ER stress and activation of the UPR signalling pathways.

Figure 1.1. ER stress and activation of the UPR signalling pathways. Accumulation of misfolded or unfolded protein aggregates in the ER lumen, a condition known as ER stress, leads to activation of three ER transmembrane proteins, PERK, IRE1 and ATF6. GRP78, a ubiquitous ER chaperone that is normally bound to these ER stress sensors and keeps them inactive, dissociates from them in order to assist with the folding of proteins in the ER lumen. However, this dissociation leads to activation of the three UPR signal transduction pathways. 1) PERK homodimerization and autophosphorylation results in the subsequent phosphorylation of the α subunit of eIF2 which by inhibiting global protein synthesis reduces the ER protein load. ATF4 expression however increases upon eIF2 α phosphorylation which translocates to the nucleus allowing for transcription of UPR target genes by binding to the UPR response element (UPRE). These genes include CHOP, a pro-apoptotic transcription factor that results in cell death if ER stress conditions persist, and GADD34, which acts as a negative regulator of the PERK pathway by dephosphorylating eIF2 α . 2) IRE1 is activated in a similar manner to PERK by homodimerization and autophosphorylation. Additionally, interaction of misfolded or unfolded proteins with the luminal domain of IRE1 can also further promote its activation. *XBP1* mRNA is an IRE1 substrate that undergoes splicing to produce *XBP1s*, encoding a transcription factor that can lead to up-regulation of ER chaperones and other UPR target genes. 3) ATF6 activation leads to its translocation to the Golgi where it is sequentially cleaved by site 1 and site 2 proteases. This leads to the release of the N-terminal ATF6 fragment which translocates to the nucleus, binds to the ER stress response element (ERSE) thereby activating UPR target genes.

1.3.1. PERK

Homo-dimerization and auto-phosphorylation of PERK following dissociation of GRP78 leads to its kinase activity. PERK phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2) resulting in translational attenuation (Harding et al., 2000b). Translation of certain mRNAs with short open reading frames in the 5'-UTR is enhanced by phosphorylation of eIF2 α . ATF4 is an example of such mRNA and its expression results in activation of C/EBP homologous protein (CHOP) which is a pro-apoptotic transcription factor (Harding et al., 2000a). GADD34 (growth-arrest and DNA-damage-inducible protein 34) is induced by CHOP, which acts to de-phosphorylate eIF2 α as a negative feedback loop and relieve the cell of the translational repression during prolonged ER stress (Marciniak et al., 2004).

1.3.2. IRE1

Similar to PERK, IRE1 is a type 1 transmembrane serine/threonine receptor protein kinase/endonuclease which upon dissociation of GRP78 homodimerizes leading to autophosphorylation and activation of its kinase and endoribonuclease functions (Hosoi and Ozawa, 2010). Unfolded proteins may also directly bind to IRE1 promoting its homodimerization and autophosphorylation (Credle et al., 2005; Gardner and Walter, 2011; Kimata et al., 2007). Activation of IRE1 results in splicing of *XBPI* mRNA, a process by which a 26-nucleotide sequence of *XBPI* mRNA is excised leading to a shift in its reading frame. Unlike the unspliced *XBPI* protein, which is rapidly degraded, spliced *XBPI* (*XBPIs*) encodes a bZIP transcription factor with a potent transactivation

domain (Todd et al., 2008). XBP1s translocates to the nucleus where it leads to expression of a number of UPR target genes including genes involved in protein folding and secretion, protein degradation and ER translocation (Lee and Glimcher, 2009; Lee et al., 2003). Consistent with these transcriptional targets, XBP1 is required for the secretory function of certain highly secretory cell types such as antibody producing plasma cells (Reimold et al., 2001).

1.3.3. ATF6

ATF6, the third arm of the UPR is comprised of two transmembrane bZIP transcription factors, ATF6 α and ATF6 β , which under normal conditions are held in the ER in a complex with GRP78 (Yoshida et al., 2000). ER stress and dissociation of GRP78 from ATF6 leads to its translocation to the Golgi where it is cleaved by site 1 and site 2 proteases. The sequential proteolysis by S1P and S2P leads to the release of the N-terminal cytosolic domain of ATF6 which then upon entry into the nucleus activates UPR target genes (Ye et al., 2000). Among these target genes are XBP1, CHOP, as well as ER chaperones such as GRP78 which allow the ER to cope with the increased protein folding demand (Haze et al., 1999; Yoshida et al., 2000). Interestingly, ATF6 and XBP1 possess very similar DNA binding specificity (Lee et al., 2003), and can heterodimerize suggesting that they may have common target genes (Yamamoto et al., 2007).

1.4. Adipocyte Biology and Obesity⁴

There is a well-documented rise in obesity over the last 40 years (Boardley and Pobocik, 2009; Mokdad et al., 2003), which develops when energy intake exceeds energy expenditure, culminating in expansion of adipose tissues. Obesity and increased adipose tissue mass are associated with inflammation, adipocyte IR, and dysregulation of TG lipolysis, leading to marked elevations in circulating non-esterified fatty acids (NEFAs) (Boden, 2008), the predominant cause of fatty liver in obesity (Donnelly et al., 2005). Elevated NEFA concentration is also linked to the development of IR and T2D, due to its impact on all major insulin sensitive organs (Boden, 2008). IR is characterized by increased fasting gluconeogenesis in the liver and decreased glucose uptake by peripheral tissues such as skeletal muscle, ultimately resulting in chronic hyperglycemia. Therefore, obesity, in particular excess abdominal adiposity, poses a great risk for development of T2D, dyslipidemia, ectopic fat accumulation and lipotoxicity, hypertension and CVD (Bays et al., 2008), a cluster of conditions referred to as the metabolic syndrome.

The adipose tissue is no longer just viewed as a TG storage organ, but rather its role in body energy balance, glucose homeostasis, and metabolic health are now the subject of intense scientific investigation (Rosen and Spiegelman, 2006). There are two types of adipose tissue, brown and white adipose tissue (WAT), which are functionally and developmentally distinct. While brown adipose tissue (BAT) is rich in mitochondria

⁴ This section and all its subheadings have been adapted from the following book chapter with kind permission of Springer Science and Business Media: **Basseri S** and Austin RC. (2012) Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In “*Endoplasmic Reticulum Stress in Health and Disease*”. In press at *Springer*.

and is therefore important for basal and inducible energy expenditure, WAT is the primary site of energy storage (Gesta et al., 2007). Given that WAT is the predominant type of fat in adult humans, and since obesity is associated with increased WAT, the focus of my thesis will be on WAT biology, from its cellular to tissue level, in order to gain a better understanding of obesity and its role in the development of metabolic complications.

1.4.1. Adipogenesis

Adipogenesis describes the process of conversion and commitment of a multipotent mesenchymal stem cell into a pre-adipocyte (determination phase), and its subsequent terminal differentiation into a mature adipocyte (Rosen and MacDougald, 2006). The mature adipocyte, in comparison to other cell types that accumulate esterified lipids, is unique in its ability to store a large quantity of lipid in unilocular lipid droplets, and efficiently metabolize them for use throughout the body in times of energy demand. Adipocytes also synthesize and secrete adipocytokines and proteins such as adiponectin, leptin, and resistin which can impact on the function of other organs (Rosen and Spiegelman, 2006). To carry out its functions, the mature adipocyte is equipped with the machinery required for lipid transport and TG synthesis, insulin responsiveness, and secretion of proteins unique to adipocytes (Rosen and MacDougald, 2006).

Preadipocyte cultures such as murine cell lines 3T3-L1 and 3T3-F442A, as well as primary preadipocytes, have been extensively studied to characterize the molecular and cellular regulation of adipogenesis. However, since these cells are already committed

to the adipose lineage, there has been greater focus on the terminal differentiation process, and much less on the determination stages (Rosen and MacDougald, 2006). Adipocyte differentiation occurs through a temporally regulated cascade of events involving elaborate transcriptional networks and changes in gene expression. Two principal transcription factors, peroxisome proliferator-activated receptor γ (PPAR γ), and CCAAT/enhancer binding protein (C/EBP) family member C/EBP α , control the entire terminal differentiation process, and are known as the master regulators of adipogenesis (Farmer, 2006). In addition, a number of early transcription factors in particular C/EBP β and C/EBP δ , are important in regulating the expression of C/EBP α (Yeh et al., 1995). During terminal differentiation, adipocytes gain sensitivity to insulin as the number of insulin receptors and glucose transporters increase. Furthermore, as *de novo* lipogenesis continues and lipid droplets form, expression of proteins such as adipocyte-specific fatty acid binding protein (FABP) known as aP2, the fatty acid transporter (FAT)/CD36, and the lipid droplet protein perilipin, also increase dramatically (Gregoire et al., 1998). As adipocytes expand with lipid and reach a critical cell size, other preadipocytes in the adipose tissue are recruited to differentiate in order to accommodate the increased demand for TG storage (Faust et al., 1978).

1.4.2. Adipose tissue biology

The adipose tissue is important for the release of fatty acids (FA) for use by other organs when glucose concentrations are low. Adipose mass is dependent on the rate of storage and removal of TG in adipocytes, as high TG storage, but low TG lipolysis and oxidation,

promotes WAT accumulation. At the cellular level, an increase in WAT mass results from adipocyte hypertrophy (increase in cell size), hyperplasia (increase in cell number) or both. WAT from individuals with adult-onset obesity typically have hypertrophic adipocytes, while adipocytes from individuals with early-onset obesity exhibit both hypertrophy and hyperplasia (Gesta et al., 2007). If during times of positive energy balance, lipogenesis and hypertrophy of existing adipocytes occurs without efficient recruitment and differentiation of preadipocytes into new adipocytes, it can result in dysfunctional and pathologic adipose tissue (Bays et al., 2008). Adipocyte hypertrophy is associated with IR (Craig et al., 1987; Ibrahim, 2010), hypoxia, and inflammation (Cinti et al., 2005; Trayhurn and Wood, 2004), which lead to WAT dysfunction. As the adipose tissue expands, the hypertrophied adipocytes undergo cell death and this leads to recruitment of macrophages to the WAT, and secretion of pro-inflammatory cytokines such as TNF α and IL-6 from the obese WAT (Trayhurn and Wood, 2004). WAT from obese and insulin-resistant individuals release more NEFA into circulation, which when taken up by non-adipose organs (ectopic lipid accumulation) results in lipotoxicity and organ dysfunction (Brookheart et al., 2009), ultimately contributing to the metabolic complications of obesity and diabetes.

In humans, WAT depots can be found in subcutaneous areas, as well as intra-abdominal areas, which are known as visceral fat. It is well-known that fat distribution influences the risk of metabolic complications with obesity (Gesta et al., 2007). Visceral adipocytes tend to be more hypertrophied, insulin resistant and hyperlipolytic (Ibrahim,

2010). As a result, increased visceral fat poses a greater risk for development of T2D and CVD than increased subcutaneous fat (Gesta et al., 2007).

A key aspect of WAT endocrine function is the synthesis and secretion of proteins and cytokines known as adipokines from mature adipocytes, which include leptin, adiponectin, and resistin among many others. Under physiologic conditions, these adipokines play an important role in whole body energy and glucose homeostasis. Leptin is primarily secreted from adipocytes and acts on the hypothalamus to decrease food intake and increase energy expenditure. In addition to its effects on energy balance, leptin also increases FA oxidation in muscle (Minokoshi et al., 2002), and reverses hyperglycemia in *ob/ob* mice (Pellemounter et al., 1995). In obese individuals, leptin levels are high, however, these individuals exhibit leptin resistance (Mark et al., 2002), leading to energy imbalance and metabolic complications. Unlike leptin, and for reasons that are currently under investigation, adiponectin levels are inversely correlated with WAT mass, and this adipokine has anti-hyperglycemic and anti-diabetic properties (Rosen and Spiegelman, 2006). Yet another adipokine that contributes to IR is resistin, a protein that is also positively linked to CVD (Burnett et al., 2006; Lefterova et al., 2009). Serum resistin levels are elevated in obesity, but their cellular source in humans has been debated. While in mice resistin is exclusively produced by adipocytes, in humans, adipose-resident macrophages may be the cells responsible for its production (Rosen and Spiegelman, 2006). It is evident that in obesity, expression of adipokines becomes dysregulated, leading to hyperglycemia and hyperlipidemia, which contribute to IR and cardiovascular complications seen in obese patients.

1.5. Lipid Metabolism and the Liver⁵

The liver plays a central role in whole body lipid homeostasis. Metabolic signals such as carbohydrates and dietary FAs regulate hepatic gene expression leading to glycolytic and lipogenic signalling pathways. In addition, the pancreatic hormones insulin and glucagon play a pivotal role in the transcriptional and post-translational regulation of lipogenesis and lipid oxidation (Lee and Glimcher, 2009). Lipogenesis, the process of *de novo* lipid biosynthesis, occurs when an excess of carbohydrates are consumed, or when circulating insulin levels are high. Carbohydrates undergo glycolysis to generate acetyl-CoA molecules which are the building blocks for FA synthesis. Following esterification, one glycerol molecule and three FA chains produce TG molecules which are transported in apoB containing very low density lipoprotein (VLDL) particles (Davidson and Shelness, 2000) to the adipose tissue for long term storage. Under fasting conditions when insulin levels are low and glucagon levels are high, FA oxidation or lipolysis occurs which allows for mobilization of FA and uptake by the liver (Canbay et al., 2007). However, disruption in these homeostatic mechanisms may lead to the development of dyslipidemia, IR, fatty liver and excess adipose mass.

1.5.1. Hepatic steatosis and liver disease⁶

⁵ Section 1.5. has been adapted from: **Basseri S** and Austin RC. (2012) Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13.

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Hepatic steatosis is defined as the accumulation of TG in the liver and represents the early manifestation of non-alcoholic fatty liver disease (NAFLD), a spectrum ranging from simple steatosis to advanced fibrosis and cirrhosis (Ferre and Foufelle, 2010). Steatosis itself is often seen as a benign condition, however 20-30% of cases of NAFLD have histological signs of steatohepatitis as indicated by fibrosis, inflammation and necrosis (Almeda-Valdes et al., 2009). Ultimately, these cases can result in end-stage liver disease and hepatocellular carcinomas (Almeda-Valdes et al., 2009). Currently, NAFLD is the most common form of chronic liver disease (Ferre and Foufelle, 2010). It is strongly associated with obesity and IR, and is thus known as the hepatic manifestation of the metabolic syndrome (Almeda-Valdes et al., 2009). The prevailing theory for the development of NAFLD is a “two-hit hypothesis” (Day and James, 1998), where the first hit, lipid accumulation, is followed by inflammation and oxidative stress, which represent the second hit, allowing for the initiation and progression of fatty liver disease into steatohepatitis and cirrhosis (Day and James, 1998). Currently, there is no effective treatment for steatosis and lifestyle modification is still the best therapeutic option (Ferre and Foufelle, 2010). Therefore, it is imperative that the cellular and molecular basis of hepatic steatosis is better understood in order to develop effective treatment options. There are four main mechanisms leading to the accumulation of TG and hepatic steatosis: increased FA delivery and uptake, *de novo* lipogenesis, defective TG transport as lipoprotein particles, and impaired FA oxidation (Anstee and Goldin, 2006). These mechanisms will be discussed in more detail below.

1.5.1.1. Fatty acid uptake

Excess dietary lipid and increased FA release from the adipose tissue due to lipolysis of intracellular TG, can both contribute to enhanced hepatic FA uptake. Non-esterified (free) fatty acids (NEFA) enter the hepatocytes through transporters or by diffusion (Nguyen et al., 2008). Members of the FA transport protein family are highly expressed in hepatocytes, and other transporters such as FAT/CD36, are also involved in FA uptake (Canbay et al., 2007; Nguyen et al., 2008). FABPs, which are intracellular lipid chaperones, reversibly bind saturated and unsaturated long-chain FAs and other lipids, thereby escorting them to their intracellular compartment to carry out their biological function (Furuhashi and Hotamisligil, 2008). The influx of FAs into the liver is proportional to the concentration of plasma NEFA, which may be a mechanism to prevent the potential toxicity of circulating FA to organs such as the pancreas (Lavoie and Gauthier, 2006; Miller, 2000). Excess uptake of circulating NEFA (mainly from adipose tissue lipolysis) is thought to be the main contributor to NAFLD in obesity (Donnelly et al., 2005; Lavoie and Gauthier, 2006).

1.5.1.2. SREBPs and insulin mediated de novo lipogenesis

Lipogenesis is a metabolic pathway that allows for the conversion of excess carbohydrates into FA and their esterification with glycerol 3-phosphate to form TG molecules. Since the activity of the lipogenic pathways is dependent on nutritional conditions, both insulin and glucose concentrations induce lipogenic gene expression (Ferre and Foufelle, 2010). Conversely, a high glucagon level during fasting, represses

lipogenesis (Tessari et al., 1981). Insulin and glucose exert their transcriptional effects through sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP), respectively. The synthesis of FA from acetyl-CoA is stimulated by insulin through increased expression of a SREBP-1c target gene, glucokinase, which allows glucose to enter the glycolytic pathway. Several lipogenic enzymes such as liver pyruvate kinase (L-PK), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearyl-CoA desaturase 1 (SCD1) are also SREBP-1c target genes (Tessari et al., 2009), and are important in hepatic lipid metabolism. L-PK leads to the production of pyruvate and its conversion to acetyl-CoA. The enzyme ACC carboxylates acetyl-CoA to form malonyl-CoA, a substrate for *de novo* lipogenesis and key regulator of FA partitioning between esterification and oxidation (Trumble et al., 1995). FAS, a key enzyme in palmitate synthesis converts malonyl-CoA to palmitoyl-CoA, which is then converted into palmitoleyl-CoA through the activity of SCD1. Finally, the enzyme glycerophosphate acyltransferase (GPAT), also a SREBP-1c target gene, esterifies FA chains to glycerol forming TG molecules (Tessari et al., 2009).

SREBP-1c is a member of the SREBP family of transcription factors which are synthesized as inactive precursors bound to the ER membrane. SREBPs are kept inactive through their interaction with SREBP cleavage-activating protein (SCAP), and insulin-induced gene (INSIG), an ER retention protein. SCAP interacts with INSIG and thereby retains the SREBP-SCAP complex in the ER (Yang et al., 2002). However, this interaction is tightly regulated by sterols which are sensed by SCAP through its sterol sensing domain (Nohturfft et al., 1998). Under sterol deplete conditions, a conformational

change in SCAP releases the SREBP-SCAP complex from INSIG, allowing its movement to the Golgi apparatus. In the Golgi, site 1 and site 2 proteases, the same proteases that cleave ATF6 during ER stress, sequentially cleave the nascent SREBP to produce a transcriptionally active N-terminal fragment, which can be released from the membrane to translocate to the nucleus (Colgan et al., 2011).

SREBP-2 and SREBP-1a are mainly involved in the regulation of cholesterol synthesis pathways, while SREBP-1c is important for hepatic TG biosynthesis, and is thus known as a master regulator of the lipogenic pathway (Ferre and Foufelle, 2010). Insulin is believed to be the most potent activator of SREBP-1c transcription, while glucagon is inhibitory (Shimomura et al., 1999). Furthermore, proteolytic cleavage of SREBP-1c is also stimulated by insulin and leads to a rapid increase in the nuclear mature form of SREBP-1c (Hegarty et al., 2005). Several studies have shown that phosphoinositide 3-kinase (PI3K) is important in mediating the effects of insulin on hepatic lipogenesis through a PI3K/PDK/Akt pathway resulting in enhanced SREBP-1c expression (Ferre and Foufelle, 2010; Leavens et al., 2009; Mora et al., 2005). Signalling through atypical protein kinase C (PKC) λ and β has also been shown to have an important role in insulin-mediated SREBP-1c transcription (Matsumoto et al., 2003; Yamamoto et al., 2010b). Furthermore, *in vivo* studies suggest that mammalian target of rapamycin complex 1 (mTORC1), which acts down-stream of Akt, is important in mediating SREBP-1c transcription in the liver (Li et al., 2010). A recent report, however, shows that pathways additional to mTORC1-SREBP-1c activation, mediate the effects of Akt2 on hepatic lipogenesis (Wan et al., 2011).

The mechanisms by which insulin signalling leads to SREBP-1c cleavage and activation are not entirely clear. Studies suggest that insulin causes SREBP activation via the down-regulation of mRNA encoding the INSIG2 protein, an ER protein which retains SREBP in the ER (Yabe et al., 2003; Yellaturu et al., 2009b). Insulin has also been shown to phosphorylate nascent SREBP-1c protein increasing its post-translational processing (Yellaturu et al., 2009a). In addition to insulin, it is been reported that liver X receptor α (LXR α), a nuclear receptor family member, is required for SREBP-1c transcription and activity, as mice lacking LXR α exhibit decreased expression of SREBP-1c and its target genes FAS, ACC and SCD-1 (Peet et al., 1998; Repa et al., 2000).

Another mechanism by which SREBP-1c and SREBP-2 are cleaved is through activation of ER stress pathways. ER stress-induced phosphorylation of eIF2 α leads to inhibition of general protein translation and down-regulation of INSIG1, which would allow for the SREBP-SCAP complex to translocate to the Golgi for proteolytic cleavage (Zeng et al., 2004). ER stress-mediated caspase activation has also been shown to cleave SREBP-1 and SREBP-2 (Pai et al., 1996; Wang et al., 1995). A recent study indicates that association of GRP78 with SREBP-1c or one of the proteins in the complex, such as SCAP or INSIG, may be responsible for sequestering SREBP-1c in this inactive form in the ER. Indeed, while there was a strong association between GRP78 and SREBP-1c proteins in livers from lean mice, in the livers from obese *ob/ob* mice that show signs of ER stress, this association was disrupted and reduced (Kammoun et al., 2009).

SREBP-1c deficiency in mice reduces FA synthesis by 50%, suggesting that SREBP-1c activity alone cannot fully account for the induction of lipogenic gene expression in response to carbohydrates (Ferre and Fofelle, 2010; Liang et al., 2002). The glucose-responsive transcription factor ChREBP has emerged as a key player in hepatic glucose metabolism and lipogenesis (Dentin et al., 2006; Dentin et al., 2005; Postic et al., 2007). Inhibition of ChREBP expression in the livers of obese mice lead to marked reduction in hepatic steatosis (Dentin et al., 2006). High glucose induces ChREBP expression, and activates a phosphatase which can dephosphorylate ChREBP thereby activating its DNA binding domain and allowing it to enter the nucleus (Kawaguchi et al., 2001).

1.5.1.3. Lipoprotein metabolism and export

Increased hepatic uptake of FA and lipogenesis are compensated by increased removal of lipids which are packaged as very low density lipoprotein (VLDL) particles and exported out of the liver. The packaging of TGs into VLDL takes place in the ER lumen and is dependent on the synthesis of apolipoprotein B100 (apoB100), the major protein component of VLDL particles (Canbay et al., 2007). Microsomal triglyceride transfer protein (MTP) plays a key role in regulating the incorporation of TG into apoB and thereby is required for the assembly and secretion of VLDL from the liver (Malaguarnera et al., 2009). Synthesis of apoB100 is enhanced by elevated NEFA, but it is inhibited by insulin (Julius, 2003; Tessari et al., 2009). Insulin has been shown to down-regulate the expression of MTP in liver cells (Malaguarnera et al., 2009), but hepatic IR leads to

increased MTP expression and apoB100 availability, leading to continued secretion of VLDL particles despite hyperinsulinemia (Chavez-Tapia et al., 2009; Choi and Ginsberg, 2011; Verges, 2010).

1.5.1.4. Fatty acid oxidation

During the post-absorptive state, FA oxidation which can occur in mitochondria, peroxisomes and microsomes, provides most of the body's energy supply. In the mitochondria and peroxisomes, oxidation of FA begins from the second carbon atom adjacent to the carboxy group and is therefore termed β -oxidation. In microsomes on the other hand, oxidation proceeds from the terminal carbon atom (ω -oxidation) (Tessari et al., 2009). Very long-chain FA are metabolized by peroxisomal β -oxidation and microsomal ω -oxidation systems, while mitochondrial β -oxidation is responsible for short, medium and long-chain FA oxidation.

Short and medium-chain FAs can freely enter the mitochondria, however long-chain FAs (14 carbons or more), require the enzyme carnitine palmitoyltransferase I (CPT-1), which is present in the outer mitochondrial membrane (Canbay et al., 2007; Nguyen et al., 2008). The activity of CPT-1 is inhibited by malonyl-CoA, the substrate for FA synthesis that forms as a result of ACC-mediated acetyl-CoA carboxylation. Therefore, CPT-1 is the rate-limiting enzyme in FA oxidation, and high malonyl-CoA levels decreases FA oxidation (Canbay et al., 2007; Nguyen et al., 2008). An enzyme up-regulated by energy deprivation, AMP-activated protein kinase (AMPK) can directly phosphorylate and inactivate ACC, leading to reduced malonyl-CoA formation and

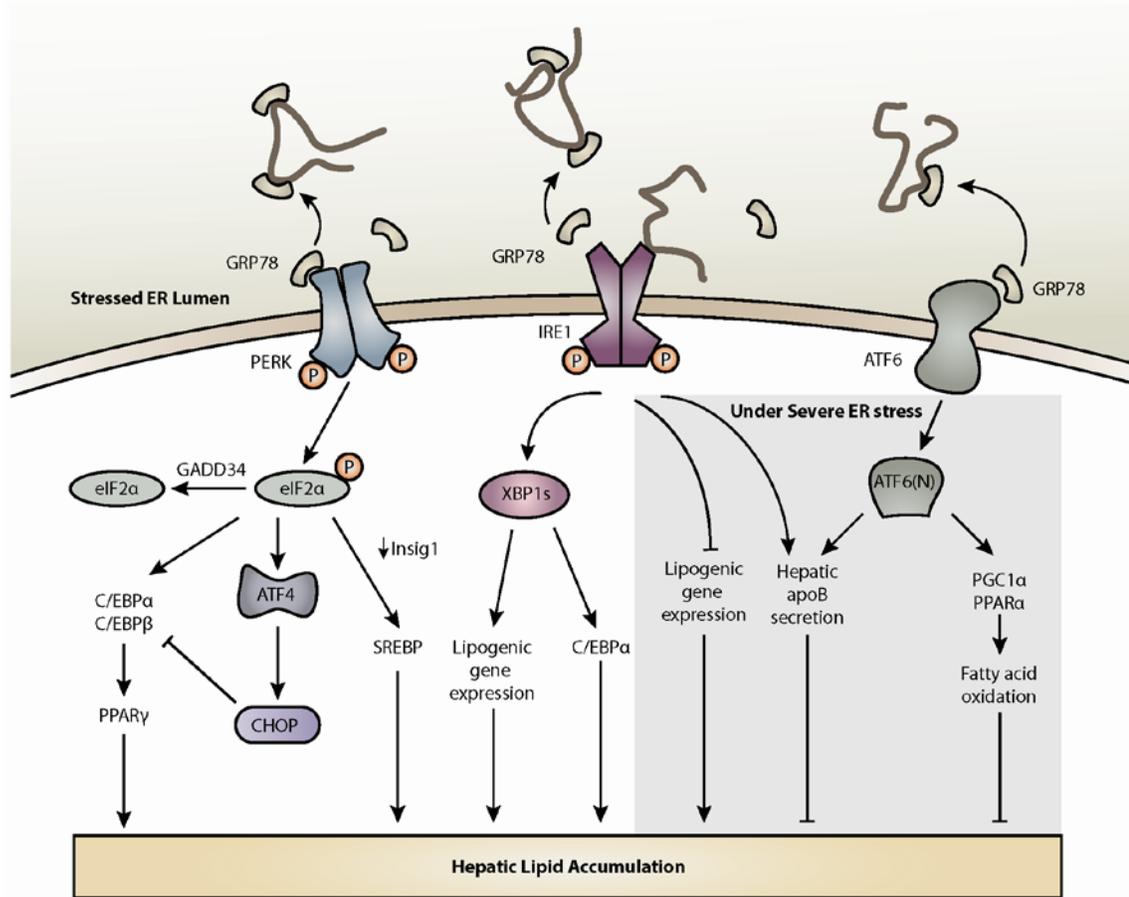
increasing FA transport into the mitochondria for oxidation (Tessari et al., 2009). Therefore, while a negative energy balance promotes FA oxidation, insulin, which induces ACC activity, inhibits it. Key enzymes involved in FA oxidation are regulated by PPAR α , a FA sensor, which is highly expressed in tissues such as liver (Nguyen et al., 2008). In addition to inducing expression of genes encoding CPT-1, FABP, and FAT, PPAR α also regulates mitochondrial and peroxisomal β -oxidation, and microsomal ω -oxidation systems (Hashimoto et al., 1999). Thus, as expected, PPAR α -null mice display massive hepatic and cardiac lipid accumulation (Djouadi et al., 1998).

Hepatic PPAR α activity and excessive mitochondrial uptake of FA can result in ketogenesis, a process by which acetyl-CoA is converted into ketone bodies, acetoacetate and β -hydroxybutyrate due to incomplete FA oxidation (Badman et al., 2007; Nguyen et al., 2008). Ketogenesis produces less ATP per mole of FA oxidized, but also increases the rate of FA utilization, and thus may serve as an important short-term fuel redistribution strategy during the post-absorptive state (Nguyen et al., 2008). This process allows for glucose to be spared for use by the brain and nervous system under prolonged fasting conditions.

1.6. ER stress and lipid metabolism⁷

It has been known for about a decade that ER stress can lead to altered lipid metabolism and hepatic steatosis. A study performed in our laboratory demonstrated that homocysteine-induced ER stress can lead to hepatic steatosis and altered cholesterol and TG biosynthetic pathways, both in cultured cells and in livers of hyperhomocysteinemic mice (Werstuck et al., 2001). Overexpression of GRP78, which attenuates ER stress and UPR activation, has been shown to decrease hepatic steatosis by reducing SREBP-1c activity (Kammoun et al., 2009). More recently, specific arms of the UPR and their downstream signalling molecules have been examined in cell culture and animal models to decipher their function and role in lipid metabolism. It is now well-established that various components of the UPR signalling network play a role in the regulation of lipid metabolism (Bobrovnikova-Marjon et al., 2008; Kammoun et al., 2009; Lee et al., 2008; Oyadomari et al., 2008; Rutkowski et al., 2008; Sriburi et al., 2004; Zhang et al., 2011). **Figure 1.2.** summarizes our current knowledge about the interactions between various components of UPR signalling and lipid metabolism. **Chapter 5** (Discussion) of this thesis presents in depth analysis of recent studies linking UPR pathways to lipogenesis, adipogenesis, obesity and metabolic homeostasis, with the goal of placing the results of this thesis project in the context of recent advances in the field.

⁷ This section has been adapted from: Basseri S and Austin RC. (2012) Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13.



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Figure 1.2. Crosstalk between UPR signalling pathways and lipogenesis.

Figure 1.2. Crosstalk between UPR signalling pathways and lipogenesis.

Phosphorylation of eIF2 α and activation of the PERK pathway under high fat diet-induced ER stress conditions allows for enhanced lipogenesis by inducing C/EBP α , and decreasing Insig1 protein translation which increases activation of SREBP. However, under severe or prolonged ER stress conditions, CHOP expression may lead to dysregulation of the C/EBPs. Similarly, high carbohydrate diet induced ER stress conditions depend on XBP1 for expression of lipogenic genes and to increase C/EBP α activity, both of which promote lipogenesis. However, severe ER stress conditions imposed by tunicamycin, lead to XBP1-mediated inhibition of lipogenic gene expression. Furthermore, both XBP1 and ATF6 are important for apolipoprotein B secretion from hepatocytes and activation of fatty acid oxidation pathways (PPAR α , PGC1 α) under such conditions. These pathways culminate in attenuation of lipogenesis and prevention of fatty liver disease under severe ER stress.

1.7 T-cell death associated gene 51 (TDAG51)

1.7.1 TDAG51 structure and function

TDAG51 was first identified in T cell hybridoma mutants that were resistant to anti-T cell receptor induced apoptosis (Park et al., 1996). Functional TDAG51 was required for Fas-mediated apoptosis in T cells. In other cell lines such as neuronal, melanoma and vascular endothelial cells, TDAG51 promoted Fas-independent apoptosis (Gomes et al., 1999; Hossain et al., 2003; Neef et al., 2002). While most studies suggest that TDAG51 is a pro-apoptotic protein, in NIH 3T3 cells over-expressing the IGF1 receptor, TDAG51 was a crucial mediator of the pro-survival effects of IGF1 (Toyoshima et al., 2004).

The human homolog of TDAG51, pleckstrin homology-like domain family A, member 1 (PHLDA1), is down-regulated in metastatic melanoma cells (Neef et al., 2002) and its decreased expression has also been linked to breast cancer progression (Nagai et al., 2007). Indeed, a recent study by Johnson *et al* illustrated that Aurora A, a serine/threonine protein kinase, phosphorylates PHLDA1 leading to its degradation in breast cancer cells (Johnson et al., 2011). More recently TDAG51 has been characterized and used as a follicular stem cell marker in tissue biopsy analysis (Sellheyer and Krahl, 2011; Sellheyer and Nelson, 2011). In a study using the yeast two hybrid method, TDAG51 was shown to interact with proteins involved in protein translation and biosynthesis (Hinz et al., 2001), suggesting that it may be involved in the regulation of protein translation.

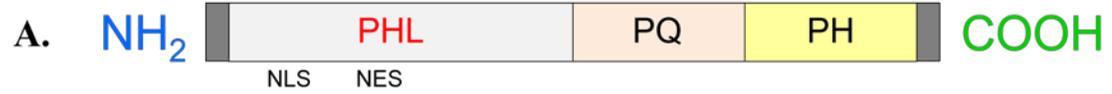
TDAG51 protein structural analysis indicates that it possesses several distinct motifs consisting of proline-histidine (PH), proline-glutamine (PQ) and polyglutamine (QQ) repeats (**Figure 1.3.**). Despite its predicted molecular mass of 29 kDa, TDAG51 is detected as a 40 kDa protein in SDS-PAGE immunoblot analysis with anti-TDAG51 antibody, likely due to the secondary structure imparted by the proline, glutamine, and histidine-rich content. Near the N-terminal region, TDAG51 contains a pleckstrin-homology like domain (**Figure 1.3.**). Such domains mediate associations with membranes, and are involved in cell signalling and cytoskeletal organization in cells. Based on sequence homology, TDAG51 belongs to a pleckstrin homology-related gene family that also consists of *Ipl/Tssc3* and *Tih1* (Frank et al., 1999).

The precise regulation of TDAG51 gene expression, or potential signalling pathways and cellular processes it may modulate, are not well-understood. TDAG51 was demonstrated to be specifically induced by IGF-1 stimulation in NIH-3T3 cells, mediating its effects on cell survival (Toyoshima et al., 2004). IGF-1 is known to mediate proliferation, protein synthesis and pro-survival signals (Butts et al., 2003; Toyoshima et al., 2004), therefore identification of TDAG51 as an IGF-1 responsive gene suggests that TDAG51 is a multi-functional protein and may play a role in various aspects of cell biology. In the presence of IGF-1, chemical inhibition of the mitogen-activated protein kinase (MAPK), p38, decreased TDAG51 expression suggesting that TDAG51 may be a p38 target gene (Oberge et al., 2004; Toyoshima et al., 2004). Another study suggests that MAPK/extracellular signal-regulated kinase (ERK) signalling results in TDAG51 induction, and that loss of TDAG51 increases ERK activation in mammary epithelial

cells (Oberst et al., 2008). ERK activation was also important for farnesol-induced ER stress/UPR activation in human lung carcinoma cells, where TDAG51 expression was found to be induced (Joo et al., 2007).

TDAG51 is also a target of heat shock transcription factor 1 (HSF1), an important regulator of the heat shock response (Hayashida et al., 2006). Absence of TDAG51 protected spermatocytes from apoptotic cell death suggesting that TDAG51 may promote heat-induced germ cell apoptosis *in vivo* (Hayashida et al., 2006). In T-cell hybridomas, TDAG51 mediates Fas up-regulation and T-cell apoptosis through protein kinase C (PKC) activity (Park et al., 1996; Wang et al., 1998). However, generation of the TDAG51-deficient (*TDAG51^{-/-}*) mouse revealed no differences in lymphoid organs when compared to wild-type littermates, nor any differences in T cell number, function, or proliferative capability (Rho et al., 2001), indicating that TDAG51 is not required for Fas expression and T cell apoptosis *in vivo*.

TDAG51 Protein – 261 aa



B. **NH₂**MLENSGCKALKEGVLEKRSDGLLQLWKKKCCILTEGLLLI
 PPKQLQQQQQQQQPGQGTAEPSQPSGPTVASLEPPVKLKEL
 HFSNMKTVDCVERKGYMYFVVMTEGKEIDFRCPQDQGWNA
 EITLQMVQYKNRQAILAVKSTRQKQQLVQQQPPQTQQIQPQ
 PQPQIQPQPQPQIQPQPQPQPQPQPQPQPQPQPQQLHSYPH
 PHPHPYSHPHQHPHPHPHPHPHPHPYQLQHAHQPLHSQP
 QGHRLLRSTSNSA-**COOH**

C.

Figure 1.3. Analysis of the mouse T-cell death associated gene 51 protein structure.

Figure 1.3. Analysis of the mouse T-cell death associated gene 51 protein structure.

A) The mouse TDAG51 gene encodes a 261 amino acid protein with several distinct regions.

B) TDAG51 amino acid sequence and functional domains. A pleckstrin homology-like (PHL) domain, indicated in red, is located near the N-terminus containing a putative nuclear localization signal (NLS) motif *KRSDGLLQLWKKK* indicated by a solid underline, as well as a putative nuclear export signal (NES) motif *VASLEPPVKL* indicated by a dashed underline. The PHL domain also contains a long stretch of glutamine (Q) residues. Near the C-terminus, there are proline-glutamine (PQ) and proline-histidine (PH) repeats.

C) Model of the predicted mouse TDAG51 protein structure based on its amino acid sequence. 57% of residues were modelled at >90% confidence using the Phyre2 software (Kelley and Sternberg, 2009).

1.7.2. The UPR and TDAG51

Others in the Austin laboratory have previously shown that TDAG51 is expressed in atherosclerotic lesions of *ApoE*^{-/-} (atherosclerosis-prone) mice with hyperhomocysteinemia. Homocysteine is an ER stress-inducing agent and TDAG51 was identified as a homocysteine-inducible transcript in human umbilical vein endothelial cells (HUVECs). Other ER-stress inducing agents (thapsigargin, dithiothreitol, peroxynitrite/Sin-1 treatment) can also induce TDAG51 expression in endothelial cells (Dickhout et al., 2005; Hossain et al., 2003). Furthermore, TDAG51 was also up-regulated under farnesol-induced ER stress conditions in human lung carcinoma cells (Joo et al., 2007). Given that UPR activation occurs during all stages of atherosclerotic lesion development (Zhou et al., 2005) and the presence of TDAG51 in early and late atherosclerotic lesions, it was hypothesized that TDAG51 may contribute to ER stress-mediated atherosclerotic lesion development.

Data from our laboratory that are currently under review for publication (Hossain GS *et al.* 2012), demonstrated that *ApoE*^{-/-}/*TDAG51*^{-/-} double knock-out mice have decreased atherosclerotic lesion size as compared to the *ApoE*^{-/-} mice. However, upon histological surveillance of organs relevant to atherosclerosis, a significant increase in hepatic steatosis, specifically hepatic triglycerides, was noted in the double knock-out mice as compared to the *ApoE*^{-/-} mouse. Cultured macrophages, endothelial and smooth muscle cells are currently being utilized to elucidate potential mechanisms mediating the effects of TDAG51 deficiency on atherogenesis. Importantly, the observation that

ApoE^{-/-}/*TDAG51*^{-/-} mice exhibit marked fatty liver suggests that TDAG51 may have differential effects in various tissues/cell types, such that lipid accumulation and necrotic core formation was reduced in the aortic root of *ApoE*^{-/-}/*TDAG51*^{-/-} mice, while hepatic lipid accumulation was concomitantly increased leading to fatty liver (hepatic steatosis). Though it seems contradictory, fatty liver in the presence of reduced atherosclerotic lesion development or cardiovascular risk has been previously observed. For example, individuals with hypobetalipoproteinemia are prone to fatty liver disease, but protected from cardiovascular disease due a profound reduction in LDL cholesterol (Sankatsing et al., 2005). Furthermore, treatment of *ApoE*^{-/-} or *LDLR*^{-/-} mice with an LXR ligand increases hepatic lipogenesis, while reducing serum cholesterol and reducing atherosclerotic lesion development (Schultz et al., 2000; Terasaka et al., 2003). The role of TDAG51 in lipid metabolism and energy homeostasis has not been previously examined. Therefore, **chapter 4** of this thesis aims to address the role of TDAG51 in lipogenesis, IR and obesity using *TDAG51*^{-/-} mice.

1.7.3. Link between TDAG51 and adipogenesis

It is well established that IGF1/insulin signalling are essential for the initiation of adipocyte differentiation (Smith et al., 1988; Zizola et al., 2002). The previous finding that TDAG51 expression is induced in response to IGF1 and its downstream p38 MAPK signalling (Oberge et al., 2004; Toyoshima et al., 2004), suggests that TDAG51 may be an important mediator of growth factor signalling during adipogenesis. Interestingly, p38 MAPK activity declines over the course of adipogenesis, but it remains somewhat

inconclusive whether p38 MAPK signalling accelerates or inhibits adipogenesis (Aouadi et al., 2007; Aouadi et al., 2006; Bost et al., 2005; Engelman et al., 1998). Furthermore, given the finding that TDAG51 is an ER stress-inducible gene (Hossain et al., 2003) and the importance of ER stress/UPR genes in adipogenesis (Basseri et al., 2009; Bobrovnikova-Marjon et al., 2008; Lowe et al., 2011; Sha et al., 2009), it was hypothesized that TDAG51 may be involved in adipogenic gene expression or post-translational events.

A comparison of mouse embryonic fibroblasts from WT and *TDAG51*^{-/-} mice using microarray analysis, identified a 29-fold induction in PPAR γ mRNA in *TDAG51*^{-/-} MEFs as compared to WT MEFs. This finding raised the possibility that TDAG51 may regulate adipogenesis, a process that requires and relies upon activation of PPAR γ for transcription of downstream genes required for differentiation and maintenance of adipocytes.

A recent microarray analysis of differentiating 3T3-L1 cells found TDAG51 mRNA to be induced early in adipogenesis, the levels of which peak between 0-8 hrs post-stimulation and drop drastically after 8 hours (Burton et al., 2004). Other genes within this cluster included C/EBP β , C/EBP δ and insulin substrate receptor-2, which are known to be transcribed very early during differentiation of 3T3-L1 cells. Interestingly, the TDAG51 gene was also found in the cluster of genes that are repressed 8 hours post-stimulation. This finding implies that upon stimulation to differentiate, 3T3-L1 cells increase the transcription of TDAG51, a gene with an unknown function in the area of

adipocyte differentiation, and glucose or lipid metabolism. Furthermore, the drastic drop in TDAG51 expression after 8 hours suggests that 3T3-L1 cells have a mechanism in place to repress the transcription of TDAG51, possibly in order to facilitate the adipogenic cascade of events. Other genes found within this cluster that were repressed after 8 hours included C/EBP δ , and macrophage colony-stimulating factor. Genes such as PPAR γ , C/EBP α , adipoQ, Adipsin, FAS, and LPL were induced 24-48 hours post-stimulation, which is consistent with previous studies and confirms the validity of the microarray study.

These results combined with our preliminary data and the inverse relationship between PPAR γ and TDAG51 in MEFs, led us to hypothesize that TDAG51 plays a critical role in the induction of adipogenesis and in the regulation of energy balance *in vivo*. Hence, **chapter 3** and **4** of this PhD thesis address this hypothesis and present the research findings examining the role of TDAG51 in adipogenesis and energy metabolism.

1.8. Hypothesis and Thesis Objectives

Given the importance of ER stress/UPR pathways in differentiation and/or function of secretory cells, it was *hypothesized* that adipocyte differentiation and adipose tissue metabolism rely on efficient ER function and the UPR signal transduction pathways. The *overall objective* of this PhD thesis was to determine the role of ER stress/UPR activation in adipogenesis, regulation of adiposity and whole body energy metabolism. Specifically, the *thesis objectives* were

- i) To examine the role of the UPR and ER stress in the process of adipogenesis *in vitro* and evaluation of the use of the chemical chaperone and inhibitor of ER stress, 4-phenyl butyric acid (4-PBA) on adipocyte differentiation and weight gain in high fat diet-fed mice;
- ii) To determine the role of an ER stress-regulated gene, T-cell death associated gene 51 (TDAG51), as a novel regulator of adipogenesis;
- iii) To elucidate the effects of TDAG51 deficiency on metabolic parameters: adipose tissue mass, hepatic lipid content, plasma metabolic markers, insulin sensitivity and energy homeostasis in mice.

In brief, the experimental results of this thesis show that UPR activation is an important regulator of adipogenesis, and demonstrate for the first time that TDAG51 is a novel modulator of adipogenesis, glucose homeostasis, and whole body energy metabolism. These findings have shed light on the importance of UPR activation and the gene

TDAG51 in adipose tissue biology and whole body energy homeostasis, thereby opening avenues for development of better pharmacological interventions for the treatment of obesity and its co-morbidities.

1.9. References

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CHAPTER 2

The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By Modulating The Unfolded Protein Response

Preamble

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Authors' Contribution

S. Basseri and R.C. Austin designed the study. S. Basseri performed all the cell culture experiments and related analyses, performed Western blotting, conducted the animal study, and assisted in tissue collection and analysis. S. Lhotak assisted in tissue collection, and provided technical assistance in histology and immunohistochemistry. S. Basseri wrote and prepared the manuscript. A.M. Sharma and R.C. Austin provided intellectual input. S. Basseri, S. Lhotak, A.M. Sharma and R.C. Austin reviewed and edited the manuscript and all authors approved of the final submission.

**The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By
Modulating The Unfolded Protein Response**

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ABSTRACT

Recent studies have shown a link between obesity and endoplasmic reticulum (ER) stress. Perturbations in ER homeostasis cause ER stress and activation of the unfolded protein response (UPR). Adipocyte differentiation is a major contributor to weight gain and we have shown that markers of ER stress/UPR activation, including GRP78, phospho-eIF2 α , and spliced XBP-1 are up-regulated during adipogenesis. Given these findings, the objective of this study was to determine whether attenuation of UPR activation by the chemical chaperone 4-phenylbutyrate (4-PBA) inhibits adipogenesis. Exposure of 3T3-L1 preadipocytes to 4-PBA in the presence of differentiation media decreased expression of ER markers. Concomitant with the suppression of UPR activation, 4-PBA resulted in attenuation of adipogenesis as measured by lipid accumulation and adiponectin secretion. Consistent with these *in vitro* findings, female C57BL/6 mice fed a high fat diet supplemented with 4-PBA showed a significant reduction in weight gain and had reduced fat pad mass, as compared to the high fat diet alone group. Furthermore, 4-PBA supplementation decreased GRP78 expression in the adipose tissue and lowered plasma triglyceride, glucose, leptin and adiponectin levels without altering food intake. Taken together, these results suggest that UPR activation contributes to adipogenesis and that blocking its activation with 4-PBA prevents adipocyte differentiation and weight gain in mice.

Abbreviations: 4-PBA, 4-Phenylbutyrate; CHOP, C/EBP homologous protein; ER stress, Endoplasmic Reticulum Stress; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GRP78, Glucose-Regulated Protein of 78-kDa; HF, High Fat; MDI, isobutyl-methylxanthine dexamethasone and insulin; siRNA, small interfering RNA; UPR, Unfolded Protein Response; XBP1, X-box Binding Protein 1.

INTRODUCTION

Obesity and excess body fat are well known risk factors for diabetes and cardiovascular disease (1). Obesity rates have been increasing at an alarming rate and the obesity epidemic is predicted to worsen given that 22 million children worldwide under the age of five are severely overweight (2). Recently, it has become evident that white adipose tissue is highly dynamic and plays major roles in energy balance and regulation. White adipose tissue also acts as an endocrine organ that carries out various physiological processes and secretes hormones such as leptin and adiponectin, as well as inflammatory factors like MCP-1, IL-6 and TNF α into the circulation (3). Adipogenesis, the recruitment and conversion of a fibroblast-like preadipocyte into mature round fat cells involves the process of adipocyte differentiation (3). In recent years, there has been significant progress in understanding adipocyte differentiation and adipose tissue expansion. Adipogenesis is triggered by a physiological need to increase the number of adipocytes when pre-existing adipocytes reach a critical cell size (4). An increase in the number of cells through adipocyte differentiation (hyperplasia) and an increase in adipocyte size (hypertrophy) lead to adipose tissue expansion (5). The focus of this study

was adipocyte differentiation, a highly regulated cellular process that results in metabolically active adipocytes capable of synthesizing and secreting a number of hormones and cytokines (3, 6, 7).

There is some evidence suggesting that obesity may be a disease related to endoplasmic reticulum (ER) dysfunction (8-11). It has been reported that defective ER signaling in the obese state leads to the retention and accumulation of proteins that are normally susceptible to ER associated degradation (ERAD) (8, 9). The oxidizing environment of the ER provides ideal conditions for membrane proteins as well as secretory proteins to fold into their native conformation (12). However, ER stress occurs when the homeostatic environment of the ER is disrupted due to adverse physiological conditions, such as nutrient deprivation, hypoxia, or viral infection, leading to the accumulation of unfolded or misfolded polypeptides in the ER lumen (9, 10, 13). The majority of cells cope with the increased ER load by activating the unfolded protein response (UPR), an integrated signal transduction pathway consisting of three distinct mediators, inositol requiring enzyme-1 (IRE-1), PKR-like kinase (PERK), and activating transcription factor 6 (ATF6). Activation of these mediators leads to translation attenuation, clearance of misfolded proteins through ERAD, increased ER folding capacity and the up-regulation of ER chaperones that assist in protein folding (9).

The reasons for ER stress and UPR activation in the obese state are not fully understood. ER stress has been linked to conditions such as hyperinsulinemia (14) and hyperglycemia (10, 15), both of which can promote adipocyte differentiation and weight

gain (1, 4). Furthermore, nutrient imbalance, excessive lipid storage and insulin resistance in the hypertrophied adipocyte can lead to ER stress and UPR activation (5, 11). Several studies have reported that there is up-regulation of ER stress markers such as GRP78, phospho-PERK, and phospho-eIF2 α in adipose tissue, liver, and hypothalamus of obese mice, indicative of ER stress in these tissues (10, 14, 16-19). Recent human studies have also demonstrated increased ER stress in adipose tissue of both obese insulin-resistant subjects (20) and obese non-diabetic subjects (21). Furthermore, examination of liver and adipose tissues of obese subjects after weight loss provided evidence of reduced ER stress in these tissues (22). While a direct link between ER homeostasis and transcriptional regulation of metabolic genes such as C/EBP α in the context of fatty liver disease has been established (17, 23), the role of ER stress in adipogenesis and weight gain has not been fully explored. In a recent study, the PERK pathway was shown to be required for lipogenesis and adipogenesis (24). X-box binding protein 1 (XBP-1), a transcription factor up-regulated by the ATF6 pathway and spliced by the IRE1 pathway (25), and phosphorylation of eIF2 α , which occurs downstream of the PERK pathway, were shown to regulate lipogenesis in the liver (17, 26). However, little is known about the role of these UPR pathways in adipogenesis and adipose tissue biology. The ER of cells with a high secretory capacity, such as hepatocytes, plasma B cells, and pancreatic beta cells, is well developed and expanded in order to accommodate the flux of *de novo* proteins into the ER for folding and secretion (9). Since differentiating adipocytes are also highly secretory in nature and are metabolically active cells (3), we hypothesized that UPR activation and up-regulation of ER chaperones is a

critical process that contributes to efficient adipocyte differentiation. Thus, a physiological form of ER stress/UPR activation may be required for adipocyte differentiation in order to allow the cell to cope and adapt to an increased ER folding demand. Furthermore, mice with higher levels of ER stress have an increased rate of weight gain and obesity on a high fat diet (10, 19). Hence we tested the effect of 4-phenylbutyrate (4-PBA), a well-established chemical chaperone and inhibitor of ER stress, on adipogenesis and weight gain in a diet-induced obesity mouse model.

Many recent reports demonstrate that 4-PBA has chaperone properties and by stabilizing protein conformation in the ER, it can repress ER stress/UPR activation *in vitro* and *in vivo* (14, 19, 27-40). 4-PBA was found to restore glucose homeostasis, enhance insulin sensitivity and decrease fatty liver disease in the diabetic *ob/ob* mouse model (14). More recent studies identified the ability of 4-PBA to enhance leptin sensitivity *in vitro* and in obese mice by decreasing ER stress-mediated leptin resistance (19). However, the effect of chemical chaperones such as 4-PBA on adipogenesis and diet-induced weight gain has not been investigated. In the present study, murine 3T3-L1 cells were used to study UPR activation during adipocyte differentiation. Our *in vitro* findings demonstrate that 4-PBA attenuates UPR activation that occurs during 3T3-L1 adipogenesis and prevents their differentiation. We also demonstrate that 4-PBA reduces the expression of the ER chaperone GRP78 in the adipose tissue of mice and decreases weight gain and fat mass, leading to decreased plasma glucose, triglycerides, adiponectin, and leptin concentrations in a diet-induced obesity mouse model. Importantly, these

studies provide a solid foundation for the development of therapeutic approaches aimed at targeting the UPR to reduce the risk of obesity and its complications.

MATERIALS AND METHODS

Cell Culture and Adipocyte Differentiation

Mouse 3T3-L1 cells were purchased from ATCC and cultured in 5% CO₂ at 37°C. Cells were grown in growth media consisting of DMEM (Invitrogen), 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Sigma), 50 units/ml penicillin and 50 µg/ml streptomycin (Sigma). For differentiation experiments, 3T3-L1 preadipocytes were allowed to reach confluence and cultured with stimulation/differentiation media consisting of growth media supplemented with MDI (0.5 mM 3-isobutyl-1-methyl-xanthine, 250 nM dexamethasone, and 10 µg/ml insulin (Sigma)). After 2 days in stimulation media, media was changed to post-stimulation media containing DMEM, 10% fetal bovine serum and 5 µg/ml of insulin. Media was changed every two days until cells were lysed for Western blotting or fixed for Oil red O staining. *Xbp1* ^{-/-} and wild-type mouse embryonic fibroblasts (MEFs) were a kind gift from Dr. Randal Kaufman (University of Michigan). Differentiation was induced using stimulation media with the addition of 5 µM rosiglitazone (Cayman Chemicals) for the initial 48 hours.

Treatment of Cells with ER stress inhibitors: Cells were cultured in stimulation/differentiation media on day 0 and treated with 1 to 20 mM 4-PBA, 0.1 to 2 mg/ml of Tauro-ursodeoxycholic acid (TUDCA), or 5 to 100 µM salubrinal (Calbiochem). On day 2, media was changed to post-stimulation media with re-addition of the chemical chaperone unless otherwise specified.

Oil red O Staining and Lipid Quantification

Staining of Cells with Oil red O: Adherent cells were washed once with PBS and fixed with 3.7% formaldehyde. Oil red O solution, prepared as previously described by Kuri-Harcuch and Green (41), was added to the wells and incubated for 1 hour at room temperature. The solution was removed and the plates were washed with distilled water. Images were taken using a Leica DM1L microscope and Canon PC1192 Powershot S31S camera.

Lipid Quantification: The Oil red O stain was removed and quantified as described previously (42). Equal volumes of 60% isopropanol were added to the culture dishes to de-stain the fixed cells. The solution containing the Oil red O stain was collected and absorbance was measured at 510 nm using a spectrophotometer (SpectraMAX Plus, SOFTmax Pro 4.0).

Metabolic Protein Labelling

To assess *de novo* protein synthesis, 3T3-L1 cells were grown to confluence (day 0) and washed with cysteine/methionine-free DMEM. Cells were then treated with 2 $\mu\text{Ci/ml}$ of L-[^{35}S]-methionine (Perkin Elmer) in cysteine/methionine-free and serum-free DMEM for 4 hours at 37°C. The cells were then washed and left in serum containing cysteine/methionine-free media overnight. The next day the media was collected and frozen (for analysis of labeled secretory proteins) and protein lysates were collected using SDS-lysis buffer for autoradiogram analysis of total labeled protein content. Experiments

were repeated on day 2 and 7 of differentiation. Counts per minute (cpm) were measured using a scintillation counter (Beckman LS 6000LL).

Cell Lysis and Protein Isolation

Cells in culture dishes were washed twice with ice-cold PBS. SDS lysis buffer (60 mM Tris-Cl, pH 6.8; 12.8% Glycerol, 2.05% SDS) containing protease inhibitors (Roche) was added to the cells. The cells were then scraped off and the lysates were frozen at -20°C.

Western Blotting

Total protein concentration in each sample was measured using the Lowry protein assay kit (BioRad). Equivalent amounts of protein lysates were loaded onto SDS-PAGE mini gels. Primary antibody incubations were for 2 hours with gentle shaking at room temperature or overnight at 4°C. The following primary antibodies were used and diluted as per manufacturer's recommendations: Mouse anti-PPAR γ (Upstate), rabbit anti-calreticulin (Affinity Bioreagents), rabbit anti-phospho-eIF2 α , rabbit anti-GAPDH (Cell Signalling), mouse anti-KDEL, rabbit anti-PDI, rabbit anti-NF κ B (Stressgen), mouse anti-GRP78 (BD Transduction), mouse anti-GADD153/CHOP, rabbit anti-eIF2 α , rabbit anti-XBP-1 (Santa Cruz), rabbit anti-peroxiredoxin1 (Enzo Lifesciences), and mouse anti-beta actin antibody (Sigma). The membranes were incubated for 1 hour with either anti-mouse or anti-rabbit horseradish peroxidase IgG secondary antibodies (DAKO EnVision). Chemiluminescence detection using Western Lightning Chemiluminescence Reagent plus (Perkin Elmer, USA) was performed. Membranes were exposed to imaging

film (Kodak Bioflex Econo Scientific) and developed using a Kodak X-OMAT 1000A Processor.

For western blotting using mouse liver or epididymal adipose tissues, frozen tissue was directly homogenized in SDS lysis buffer containing protease inhibitors using a tissue homogenizer. The samples were then centrifuged for 10 minutes at 10,000 rpm and the clear protein layer was carefully removed and used for Western blotting.

ELISA and Cytometric Bead Array Mouse Inflammation Assay

A mouse adiponectin ELISA kit (R&D Systems) was used to assay secreted adiponectin from cultured 3T3-L1 cells and in mouse plasma. Mouse insulin and leptin ELISA kits (Linco Research) were used to detect insulin and leptin in mouse plasma. Inflammatory markers were measured in mouse plasma using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences). Assays were performed as per manufacturer's protocol. All samples were measured in triplicate.

Mouse Studies

Seventeen, 5-6 week old female C57BL/6 mice (Charles River Laboratory) were housed in mouse cages and allowed to acclimatize for at least one week. A high fat diet (21.2% fat by weight and 40% kcal from fat) was purchased from Harlan Teklad (#88137). Pharmaceutical grade 4-PBA, sodium salt was purchased from Scandinavian Formulas Inc. (Sellersville, PA, USA). 4-PBA is soluble in water and thus was added to the drinking water fresh every week. At 10-11 weeks of age, all mice were placed on the high fat diet with or without 4-PBA supplementation for 120 days and were fed *ad*

libitum. Mice were fed a high fat diet without (n=8) or with (n=9) 1g/kg/day of 4-PBA supplemented in the drinking water *ad libitum*. Food and water intake measurements were made and all of the mice were weighed at least once a week. Mice were anaesthetized after 120 days using 4% isoflurone. Blood samples were collected for plasma glucose, cholesterol, and triglyceride measurements (Infinity Thermo Electron Kit), as well as insulin, adiponectin and leptin measurements using ELISA methods. Following cervical dislocation, epididymal fat pads and liver were collected for further study and analysis. None of the mice became ill or died as a result of the treatment. All procedures were approved by the McMaster University Animal Research Ethics Board.

Histology and Adipocyte Cell Sizing

Liver and epididymal fat pads were fixed in formalin and paraffin embedded. Four μm sections of epididymal adipose tissue were de-paraffinized and mounted unstained. Using 10X magnification, 5-10 representative images of each slide were captured. Pictures were analyzed for cell size measurements similar to the study by Sauter *et al.* (43), using the computer imaging program ImagePro. For each group, 4-5 mice were included in the mean cell size and histogram analysis and a minimum of 500 cells were counted and sized for each mouse. For morphological analysis of the liver sections, hematoxylin and eosin staining was performed and representative images were obtained.

Mac-3 Immunohistochemistry: Four micron thick sections were de-paraffinized and the endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 minutes. Sections were blocked with 5% normal rabbit serum, antigen heat-retrieved in

citrate buffer at pH 6.0, and incubated with a rat anti-Mac-3 antibody (Pharmingen), diluted at 1:2000. This was followed by a biotinylated rabbit anti-rat antibody, mouse adsorbed (Vector Laboratories) at a 1:500 dilution. Following streptavidin-peroxidase (Zymed), sections were developed in Nova Red peroxidase substrate (Vector Laboratories) and counterstained with hematoxylin. Pictures were taken with Laborlux S microscope (Leitz) using Olympus DP70 digital camera.

Hepatic Triglyceride and Cholesterol Ester Quantification

A 140-150 mg piece of frozen liver tissue was homogenized in a tissue grinder using buffer containing 10mM HEPES, 20 mM MgCl₂, 10 mM β-Mercaptoethanol, 0.5% Triton X-100, and at pH 7.0. Hexane/isopropanol (3:2, v/v) was added to each liver homogenate in glass tubes. The samples were spun down at 3500 rpm for 3 minutes and the organic layer was carefully removed. The hexane/isopropanol extraction was repeated three times, combining the organic phases in one tube. Following evaporation of the organic phase, triglyceride and cholesterol ester concentrations (mg/ml) were measured using the triglyceride assay kit (Infinity Thermo Electron) or Chol-E kit (Wako Diagnostics).

Statistical Analyses

Statistical analyses were performed using Student's *t*-test assuming unequal variance and two-tailed distribution unless otherwise specified. Statistical significance was defined when $p < 0.05$. All results are shown as mean \pm standard deviation unless otherwise indicated.

RESULTS

Lipid accumulation and adiponectin secretion increase during adipocyte differentiation. 3T3-L1 cells accumulate a significant amount of lipid droplets starting on day 4 of differentiation and gradually increase their lipid content as they differentiate (Figure 1). The lipid droplets were visualized through Oil red O staining (Figure 1A). Quantification of the Oil red O stain allowed for measurement of the extent of lipid accumulation as measured in Figure 1B. Consistent with these findings, a significant and robust increase in the secretion of the adipocytokine adiponectin (marker of differentiated cells) was observed in the culture media on days 4 and 7 of differentiation (Figure 1C).

Markers of ER stress/UPR activation increase during adipocyte differentiation with increased *de novo* protein synthesis. To demonstrate that differentiating adipocytes are metabolically active and produce newly synthesized cytosolic and secreted proteins, *de novo* protein synthesis was studied during 3T3-L1 differentiation. As seen in Figure 2A, day 7 differentiated 3T3-L1 cells showed a significant increase in the secretion of ³⁵S-radiolabelled proteins into the culture media, as compared to preadipocytes. Consistent with these findings, autoradiogram of whole cell lysates indicated an overall increase in the number of bands and in band intensity in day 7 cells (Figure 2B), suggesting an increase in global *de novo* protein synthesis in differentiated cells.

The expression of PPAR γ , the master regulator of adipogenesis, increased by day 5 of differentiation (Figure 2C), consistent with the increase in adiponectin secretion and Oil red O staining, confirming adipogenesis. Markers of UPR activation, including the

ER chaperones GRP78, PDI and calreticulin (data not shown), as well as CHOP, phospho-eIF2 α and spliced XBP1 were modulated during adipocyte differentiation (Figure 2C). The increase in GRP78 ER chaperone levels observed in the later stages of differentiation coincides with the increased *de novo* protein translation/synthesis in day 7 cells (Figure 2A and B). Expression of spliced XBP-1 and phosphorylation of eIF2 α which are downstream of the IRE-1 and PERK pathway, respectively, peaked around day 3, while CHOP followed a biphasic pattern of expression with increased levels in the later stages of differentiation.

The chemical chaperone 4-PBA inhibits adipogenesis and blocks UPR signaling. We sought to block ER stress/UPR signaling during adipogenesis to determine whether activation of the UPR pathways is required for adipocyte differentiation. Treatment of 3T3-L1 cells with the chemical chaperone and ER stress inhibitor 4-PBA on day 0 over a period of five days dose-dependently inhibited differentiation and lipid accumulation in 3T3-L1 cells as compared to untreated control cells (Figure 3A). Similar dose-dependent inhibitory effects on differentiation were observed using another chemical chaperone TUDCA, another well documented chemical chaperone (14, 34, 37) as shown in the supplementary Figure S1. Five days of 4-PBA treatment at doses of 10 or 20 mM significantly attenuated lipid accumulation, as measured by Oil red O staining in day 5 3T3-L1 cells (Figure 3B). To confirm that adipogenesis was blocked in the presence of 4-PBA, adiponectin secretion into the media was measured and a significant dose-dependent reduction in adiponectin concentration was observed in cells treated with 4-PBA, as compared to untreated day 5 cells (Figure 3C). To exclude the possibility that

the reduction in adipocyte differentiation and adiponectin secretion was due to cytotoxicity imposed by 4-PBA treatment, an LDH release/cytotoxicity assay was performed and the result confirmed that 48 hours of 10 mM 4-PBA treatment was not toxic to 3T3-L1 cells (Figure 3D). Additionally, 4-PBA itself did not interfere with the LDH release assay (data not shown).

In order to assess the effects of 4-PBA on UPR activation in 3T3-L1 cells, Western blotting was performed on protein lysates from day 1 of differentiation with 4-PBA (24 hours of treatment in differentiation media). The basal expression of GRP78, GRP94, phospho-eIF2 α , and calreticulin, was reduced with 10 mM 4-PBA treatment (Figure 3E). This confirmed that 4-PBA results in attenuation of UPR signaling and ER stress in 3T3-L1 cells over 24 hours. Importantly, differentiation of 3T3-L1 cells in the presence of 10 mM 4-PBA not only blocked adipogenesis as shown in Figure 3A, but also inhibited the increase in GRP78, CHOP, p-eIF2 α , and spliced XBP1 expression that occurs during adipocyte differentiation (Figure 3F). The reduction in GRP78 expression with 4-PBA treatment was more evident on day 6 of differentiation, when 3T3-L1 cells have up-regulated GRP78. Surprisingly, CHOP, an ER stress marker and negative regulator of adipogenesis was induced in the first 24 hours of 4-PBA treatment (data not shown). Knock-down of CHOP using small interference RNA (siRNA) was performed to determine whether up-regulation of CHOP may be a mechanism by which 4-PBA inhibits adipogenesis. Western blotting confirmed efficient knock-down of CHOP (Figure S2A), however, as shown in Figure S2B and C, CHOP siRNA did not rescue the cells from the inhibitory effects of 4-PBA on adipogenesis. In addition, to rule out other factors that

may be affected by 4-PBA that result in attenuation of adipogenesis, expression of NF κ B or β -catenin, which are known to inhibit adipogenesis (44-46) was assessed by Western blotting (Figure S5A). No significant changes in the expression of these proteins was noted with 4-PBA over 48 hours.

XBP1 deficiency decreases adipogenesis. Others have shown that MEFs deficient in PERK, one of the mediators of UPR signaling, have decreased lipogenic and adipogenic potential (24). Given that XBP1 gene expression and splicing have previously been shown to be important for lipogenesis in the liver, and we have demonstrated that splicing of XBP1 increases during adipogenesis (Figure 2C) we reasoned that deficiency of XBP1 would impair adipogenesis. To illustrate this, *Xbp1*^{-/-} MEFs were stimulated to differentiate for 4 days with the wildtype MEFs as controls. Oil red O staining on days 0, 2 and 4 of differentiation clearly demonstrated that *Xbp1*^{-/-} MEFs have impaired lipogenesis and adipogenesis (Figure 4A and B). To further illustrate that modulating UPR signaling can attenuate adipogenesis, we utilized salubrinal, an inhibitor of ER stress that functions by selectively inhibiting eIF2 α dephosphorylation (47). As shown in Figure S3, salubrinal dose-dependently inhibited adipocyte differentiation as measured by Oil red O staining. At the 100 μ M dose, salubrinal completely blocked lipid accumulation (Figure S3A and B) and decreased XBP-1 splicing in differentiating day 4 3T3-L1 cells (Figure S3C).

The chemical chaperone 4-PBA blocks lipid accumulation during all stages of adipocyte differentiation. To illustrate that the inhibitory effects of 4-PBA on adipocyte

differentiation are more pronounced when UPR activation occurs (as shown in Figure 2C, day 3 onwards), 3T3-L1 cells were treated with 10 mM 4-PBA during various stages of adipogenesis. As shown in Figure 5A, cells that were treated with 4-PBA during the entire timeframe of the experiment (day 0-10) remained fibroblast-like with very little/undetectable lipid droplet formation. Adipocyte differentiation as measured by lipid accumulation was reduced regardless of when the cells were exposed to 4-PBA (Figure 5A and B). Treatment with 4-PBA during day 0-2 with removal of the drug during days 2-10 allowed for some adipogenic conversion and lipid accumulation. Similarly, treatment with 4-PBA during the mid-stages of adipocyte differentiation (day 2-6) and its removal on day 6, allowed for further differentiation to occur between days 6 and 10. The reduction in Oil red O staining on day 10 of the experiment was significant with all of the different treatment conditions as compared to control untreated day 10 cells (Figure 5B). These findings demonstrate that the effect of 4-PBA is reversible once it is washed out and that the reduction in basal levels of UPR markers during early stages (day 0-2) can also impact on the rate of adipogenesis. However, when the initial 4-PBA treatment occurred from day 2-6 or 6-10, it had the most pronounced effect on lipid accumulation ($p < 0.05$ as compared to day 0-2 treatment). These data suggest that the inhibitory effect of 4-PBA is not specific to or dependent on any critical event during adipocyte differentiation, but that its inhibitory effect on adipogenesis is related to the period of time when UPR activation occurs (about 3 days following the onset of differentiation).

Given that rosiglitazone, a potent PPAR γ agonist, can accelerate adipocyte differentiation, we investigated whether driving this process using rosiglitazone could

override the effect of 4-PBA on adipocyte differentiation or vice versa. Figure S4A demonstrates that a combination of 10 mM 4-PBA and 4 μ M rosiglitazone treatment from day 0 to 5 of differentiation led to a mixed cellular phenotype. The cells acquired the rounded phenotype similar to that of day 5 control or 4 μ M rosiglitazone treated cells; however the extent of lipid accumulation was significantly reduced (Figure S4B). Rosiglitazone treatment alone increased the secretion of adiponectin as compared to control day 5 cells as expected, but co-treatment with 4-PBA significantly reduced adiponectin secretion from 3T3-L1 cells. Importantly, adiponectin secretion from the co-treated cells was greater than the 4-PBA treated cells (Figure S4C). These results suggest that the presence of the PPAR γ -agonist rosiglitazone can partially rescue the inhibitory effect of 4-PBA on adipocyte differentiation. Western blotting results in Figure S4D illustrated that 4-PBA treated day 5 adipocytes had blunted expression of GRP78 and GRP94. Rosiglitazone treatment on the other hand, increased these chaperone levels as compared to day 5 control protein lysates. However, rosiglitazone was not found to be an ER stress-inducing agent, as treatment with rosiglitazone in the absence of differentiation did not up-regulate GRP78/GRP94 levels (data not shown). The increase in GRP78 and GRP94 expression observed in differentiating day 5 cells is therefore most likely to be associated with accelerated adipogenesis in day 5 cells exposed to rosiglitazone. Interestingly, co-treatment with rosiglitazone and 4-PBA partially reversed the suppression of chaperone levels observed with 4-PBA treatment alone and led to an increase in GRP78 expression in day 5 3T3-L1 cells (Figure S4D). Thus, the increased Oil red O staining and adiponectin secretion with rosiglitazone and 4-PBA co-treatment

as compared to 4-PBA treatment alone may possibly be due to increased UPR activation that is associated with enhanced adipocyte differentiation.

4-PBA supplementation of C57BL/6 mice fed a high fat diet impairs weight gain, decreases epididymal fat mass and adipocyte size. To test the effect of 4-PBA on adipose tissue growth and weight gain *in vivo*, female C57BL/6 mice were fed a high fat diet for 120 days supplemented with or without 1 g/kg/day of 4-PBA in the drinking water. As seen in Figure 6A, mice fed a high fat diet supplemented with 4-PBA showed a significant resistance to weight gain starting at 63 days after commencing the experiment, while no difference in food intake was noted (Figure 6B). The weight of the epididymal fat pads reflected the total weight of the animals (Figure 6C), with the 4-PBA supplemented mice having significantly lower epididymal fat mass.

To examine the effect of 4-PBA on adipocyte differentiation and cell size, adipose tissues were fixed, paraffin embedded and sectioned for fat cell sizing as described previously (48). Over 500 cells were sized for each mouse and at least 4 mice per group were included in the analysis. The histograms in Figure 6D show the distribution of adipocyte cell sizes in the epididymal adipose tissue of mice supplemented with or without 4-PBA. The distribution was shifted to the left in the mice supplemented with 4-PBA indicating an overall reduction in adipocyte size. Representative images of the cross-section of the epididymal adipose tissue from the two groups of mice are shown in Figure 6E. The mean cell size in the 4-PBA supplemented group was found to be significantly smaller as demonstrated graphically in Figure 6F. Since the epididymal

adipose tissue weights were lower and mean adipocyte size was found to be smaller in 4-PBA supplemented mice, it suggests that 4-PBA is potentially inhibiting the recruitment and differentiation of preadipocytes as well as preventing the hypertrophy of pre-existing adipocytes *in vivo*.

Plasma glucose, triglyceride, adiponectin and leptin levels decreased in mice supplemented with 4-PBA. To assess whether 4-PBA had a general effect on metabolic parameters, blood was collected at the end of the study and total plasma glucose, triglycerides and cholesterol were measured in the fed state (Table 1). Plasma glucose concentrations were significantly lower in the 4-PBA supplemented mice. Mice fed a high fat diet + 4-PBA also had a significant decrease in triglyceride levels. However, cholesterol concentrations did not change following 4-PBA treatment. Furthermore, plasma insulin, adiponectin and leptin concentrations were measured in the fed state (Table 2). Although no significant differences in plasma insulin levels were observed between the groups, both adiponectin and leptin levels were significantly lower in the 4-PBA supplemented mice, proportional to the reduced fat pad mass. To determine whether 4-PBA modulates pro-inflammatory cytokine expression, IL-12p70, TNF α , IFN γ , MCP-1 and IL-6 were measured in the plasma samples (Table 3). No significant differences in circulating cytokines were observed between the groups.

Mice supplemented with 4-PBA have decreased expression of GRP78 in the adipose but not liver tissue. Given our observations *in vitro*, we sought to determine whether 4-PBA decreased body weight by inhibiting ER stress signaling/UPR activation in the

adipose tissue. Western blot analysis of total adipose tissue lysates indicated that mice fed a high fat diet supplemented with 4-PBA had lower expression of the ER stress marker GRP78 (Figure 7A). Protein band quantification in Figure 7B demonstrated that after adjusting for protein loading (GAPDH), mice supplemented with 4-PBA had significantly lower expression of GRP78 as compared to the control group. In contrast to adipose tissue, GRP78 or calreticulin levels were unaffected in the livers of 4-PBA treated versus untreated mice (Figure 7D). Furthermore, as expected with a high fat diet, histological analysis revealed the presence of fatty liver in all mice. However, no differences in liver morphology (Figure 7E) or extent of triglyceride or cholesterol ester accumulation were found with 4-PBA supplementation in C57BL/6 mice (Figure 7F).

Given that inflammation plays an important role in the pathogenesis of obesity (49-52), macrophage infiltration into the adipose tissue was assessed using immunohistochemical staining with a Mac-3 antibody. As shown in the representative images in Figure 7C, no differences in the extent of macrophage infiltration or crown-like structures were noted between the two groups of mice. Furthermore, Western blotting was performed to examine the effect of 4-PBA on inflammation or oxidative stress in 3T3-L1 cells, as well as in the liver and adipose tissue lysates. As shown in supplemental Figure S5A, 4-PBA treatment of 3T3-L1 cells did not alter the expression of NF κ B or peroxiredoxin-1, markers of inflammation and oxidative stress, respectively. Supplementation of mice with 4-PBA also did not alter the expression of NF κ B or peroxiredoxin-1 in the adipose or liver tissues of mice (Figure S5B).

DISCUSSION

In this study we report that a physiological form of UPR activation and the subsequent up-regulation of ER chaperones occur during adipocyte differentiation. Currently, there are two forms of UPR activation: acute and chronic/physiological. The acute form of the UPR occurs in response to adverse physiological conditions such as hypoxia, hyperglycemia, or viral infection that lead to ER stress. At the tissue level, nutrient excess, chronic inflammation, hypoxia, oxidative as well as mechanical stress due to adipocyte hypertrophy within the adipose tissue of obese individuals may lead to ER stress (11). This form of acute ER stress could adversely affect adipose function and contribute to type 2 diabetes, insulin resistance, leptin resistance and lipotoxicity (10, 11, 19). The latter type of UPR activation occurs during cellular processes such as differentiation. This long-term physiological form of UPR activation likely takes place as a natural adaptive mechanism to ensure cell survival and efficient ER function during cell processes such as differentiation of B cells into antibody producing plasma cells (53-55).

In the present study, we demonstrate that a physiological form of UPR activation occurs during the process of adipogenesis. Expression of ER chaperones GRP78 and PDI increased during adipocyte differentiation. However, early markers of ER stress/UPR activation such as phospho-eIF2 α and spliced XBP-1 peaked around day 3 and their expression declined in the later stages. Given that adipocytes synthesize and secrete large quantities of protein, it is intuitive that eIF2 α is de-phosphorylated in late adipogenesis to allow for efficient protein synthesis to occur since increased or prolonged

phosphorylation of eIF2 α would prevent the global translation of proteins that may be necessary for cellular differentiation (56). In addition, the extent and duration of phosphorylated eIF2 α and its downstream integrated stress response regulate PPAR γ , C/EBP α and β expression (17, 57, 58), which are pivotal transcription factors in adipogenesis. The transcription factor XBP-1, on the other hand, is downstream of the IRE-1 pathway and has been shown to be involved in hepatic lipogenesis (26). While there was an increase in spliced XBP1 during differentiation, it is unclear why the expression of both the spliced and unspliced forms of XBP-1 decreased in the late stages of adipogenesis. In contrast, CHOP, exhibited a biphasic pattern of expression similar to the finding of a microarray analysis of 3T3-L1 cells (59). This finding is reasonable given that CHOP is a known negative-regulator of adipogenesis (60), and early up-regulation of CHOP following treatment with ER stress inducing agents can block adipogenesis (61). While changes in the adipocyte during differentiation, such as alterations in the expression of specific transcription factors and kinases may be modulating the UPR response, another potential underlying stimulus is the increased *de novo* protein synthesis and secretion that must occur in the mature adipocyte. Activation of the UPR primes the cell for the increased demand on the ER for proper folding and secretion of proteins. Therefore, an increase in ER chaperone expression would enable the cell to cope as it differentiates into a mature, metabolically active adipocyte. Since UPR activation occurs during adipocyte differentiation and adipogenesis is triggered during weight gain, mechanisms that relieve ER stress in the dysfunctional adipose tissue could be useful pharmacological strategies for preventing weight gain and obesity co-

morbidities. Thus, we explored the effect of an ER stress inhibitor, 4-PBA on adipogenic conversion of 3T3-L1 cells and weight gain in a high-fat diet induced obesity mouse model.

A number of studies have previously demonstrated that the low molecular weight fatty acid, 4-PBA, can act as a chemical chaperone, assisting with protein folding, and thus relieving the cell of ER stress (14, 27-32, 62, 63). In the present study we have demonstrated that 4-PBA can attenuate ER stress/UPR activation by decreasing the expression of GRP78, phospho-eIF2 α , and spliced XBP1 in 3T3-L1 cells and results in diminished adipogenesis. The observation that 4-PBA could modulate lipid accumulation at any stage of the differentiation process, but more strikingly in the later stages, is important as it indicates that 4-PBA is not affecting the clonal expansion or preadipocyte growth arrest that occurs during the early stages of adipogenesis. Moreover, 4-PBA treatment at a stage when UPR activation naturally occurs (day 2 onwards) had the most potent effect on lipid accumulation and adipocyte differentiation, confirming our hypothesis that 4-PBA likely blocks adipogenesis through attenuation of UPR signaling. Furthermore, co-treatment of 4-PBA with the PPAR γ agonist rosiglitazone, drives the adipogenic pathway and this alone results in enhanced UPR activation and GRP78 expression. Consequently, rosiglitazone can partially rescue the 4-PBA-mediated repression in adipogenesis.

It was surprising to find CHOP expression up-regulated with 4-PBA treatment in 3T3-L1 cells and loss of function studies revealed that up-regulation of CHOP was not

the mechanism by which 4-PBA inhibits adipogenesis *in vitro*. Additionally, CHOP was not up-regulated in the adipose and liver tissues of 4-PBA supplemented mice (data not shown). We explored the possibility that 4-PBA may be blocking adipogenesis through mechanisms unrelated to UPR signaling, such as changes in NF κ B or β -catenin, both of which have been shown to inhibit adipocyte differentiation (44-46). However, these proteins were unaffected by 4-PBA treatment. A well-documented mechanism of action of 4-PBA is its function as a HDAC inhibitor (64, 65). It is therefore possible that its histone acetylase activity may be partially responsible for the inhibition of adipogenesis. However, recent findings on the effect of HDAC inhibitors on adipocyte differentiation are not conclusive. Most studies indicate that HDAC inhibitors stimulate adipocyte differentiation in 3T3-L1 cells (66-70), while one study suggests that inhibition of HDAC blocks adipogenesis (62). Because the overall findings of these studies imply that HDAC inhibitors act as stimulators of adipogenesis, and we found 4-PBA to have an inhibitory effect on differentiation, a HDAC-related mechanism of action can be excluded in the present study. We suggest that reduced UPR signaling with 4-PBA treatment through diminished splicing of XBP-1 or decreased phosphorylation of eIF2 α play a significant role in its ability to block differentiation, as these genes are modulated during the normal course of adipogenesis and cells deficient in PERK and XBP1 exhibit impaired adipogenesis. Further, decreased ER chaperone expression likely potentiates these effects as the cells become deficient in the ER folding machinery. Further studies will help shed light on the specific mechanisms by which 4-PBA inhibits adipogenesis.

It is noteworthy that 4-PBA has other physiological functions in addition to its chaperone properties. It has been clinically used to treat urea cycle disorders in children, as well as sickle cell disease and thalassemia (27). 4-PBA was shown to be an efficient ammonia scavenger in C57BL/6 mice with hyperargininemia (71). Its function as a histone deacetylase (HDAC) inhibitor and anti-cancer agent (64, 65, 72-74) as well as its potential as a therapeutic approach for the treatment of amyotrophic lateral sclerosis (28) have also been demonstrated. We chose to use the 10 mM dose of 4-PBA *in vitro* and 1 g/kg/day of 4-PBA *in vivo*, based on the study by Ozcan *et al.* demonstrating that 4-PBA can restore glucose homeostasis, improve insulin sensitivity and resolve fatty liver disease in *ob/ob* mice, a model of type 2 diabetes (14). Though Ozcan and colleagues did not find an effect of 4-PBA on body weight over the short duration of their study, we were interested to explore the ability of 4-PBA to affect diet-induced obesity over a much longer time frame. In addition, since 4-PBA is currently an orally administered drug (75-78), we tested its efficacy by dissolving it in the drinking water. Consistent with our *in vitro* findings, 4-PBA supplemented mice had decreased GRP78 expression in the epididymal adipose tissue indicating decreased ER stress/UPR activation. Interestingly, a recent report indicated that 4-PBA can decrease ER stress in the hypothalamus of obese mice and enhance leptin sensitivity (19). Therefore, this may represent an additional axis through which 4-PBA can be utilized as an anti-obesity treatment. Plasma leptin and adiponectin concentrations were measured in our study and the decrease in these hormones was directly proportional to the adipose tissue mass, reflecting a decline in adipogenesis *in vivo*. The decrease in plasma glucose and triglyceride concentrations in

the 4-PBA supplemented mice was likely a reflection of the lower body mass and adipose tissue mass in this group. Taken together, these results confirm the *in vitro* finding that 4-PBA can attenuate ER stress and inhibit adipocyte differentiation/maturation, lipid accumulation and adipocyte hypertrophy.

A combination of adipocyte hypertrophy and hyperplasia lead to an increase in adipose tissue mass and obesity. Our findings show that 4-PBA supplemented mice on average had significantly smaller adipocytes and fat pad weight. We hypothesize that similar to our *in vitro* findings where 4-PBA treatment at various stages of adipogenesis affected lipid storage and adipogenesis, 4-PBA treatment *in vivo* is likely not only affecting adipocyte differentiation, but also playing a role in the maintenance of adipocytes at various sizes and stages of maturation. Therefore, it is possible that 4-PBA is directly affecting the dynamics of adipose tissue remodeling during the high fat diet feeding. In contrast to the adipose tissue, we found no differences in plasma inflammatory cytokines or liver morphology, liver ER stress markers or extent of fatty liver disease in the two groups of mice. Importantly, since adipose mass was reduced with 4-PBA treatment and hepatic TG and cholesterol esters were not affected, it suggests that the lower plasma TG concentration in these mice is not due to enhanced storage in the liver or adipose tissue. A likely explanation is that 4-PBA has lipolytic properties and may inhibit TG formation or enhance its breakdown by rapid lipolysis or beta-oxidation in the adipose or liver tissue. Alternatively, decreased UPR activation in the adipose tissue may be decreasing the activation of SREBP-1 and/or 2, proteins involved in *de novo* lipid biosynthesis (79, 80). Therefore, a SREBP-mediated mechanism of action can

also be a potential pathway to investigate, as decreased SREBP activation may attenuate lipid accumulation.

In summary, we have demonstrated that UPR activation contributes to adipocyte differentiation and hypertrophy. Through the use of the chemical chaperone 4-PBA we have demonstrated that blocking UPR signaling, can prevent adipocyte differentiation in 3T3-L1 cells and in the adipose tissue of high fat diet fed C57BL/6 mice. Further research into the role of 4-PBA on other cellular pathways and processes may shed more light on its potential as an anti-obesity and anti-diabetic drug. Based on our findings, a better understanding of the relation between UPR activation and adipogenesis *in vivo* would enhance the discovery of unique pharmacological and therapeutic approaches aimed at reducing the risk of obesity and its complications.

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DISCLOSURES

None.

TABLES**Table 1. Total Plasma Concentrations of Glucose, Triglyceride and Cholesterol in High Fat Diet Fed Mice Supplemented With or Without 4-PBA**

Metabolic Parameters (mg/ml)	Glucose	Triglyceride	Cholesterol
High Fat	1.259±0.183	0.555±0.100	3.115±0.241
High Fat + 4-PBA	1.087±0.067*	0.377±0.096**	3.223±0.389

Plasma was collected from C57BL/6 mice fed a high fat diet with or without 4-PBA supplementation for 120 days. Non-fasting glucose (n=7), triglyceride (n=8) and cholesterol (n=7) concentrations were determined as per manufacturer's instructions. * p≤0.05; **p≤0.005 as compared to 4-PBA non-supplemented mice on the same diet.

Table 2. Total Plasma Concentration of Insulin, Adiponectin and Leptin in High Fat Diet Fed Mice Supplemented With or Without 4-PBA

Variables	Insulin (ng/ml)	Adiponectin (µg/ml)	Leptin (ng/ml)
High Fat	4.284±2.437	9.560±1.156	47.356±11.18
High Fat+4-PBA	6.099±2.196	8.200±0.827	35.873±3.893

Plasma was collected from C57BL/6 mice fed a high fat diet with or without 4-PBA supplementation for 120 days. Insulin (n=7), adiponectin (n=6), and leptin (n≥7) concentrations were determined using ELISA kits (*p≤0.05).

Table 3. Measurement of Plasma Pro-inflammatory Markers in High Fat Diet Fed Mice Supplemented With or Without 4-PBA

Inflammatory Variables (pg/ml)	IL-12p70	TNFα	IFNγ	MCP-1	IL-6
High Fat	9.69±3.23	6.41±0.67	2.17±0.49	61.05±24.66	2.44±0.71
High Fat+4-PBA	9.13±4.58	6.53±1.78	1.96±0.38	40.87±14.83	2.72±1.11

Plasma was collected from C57BL/6 mice fed a high fat diet with or without 4-PBA supplementation. Using bead flow cytometry, the concentration of each inflammatory marker was measured. IL-12p70 (n≥6); TNFα (n=7); IFNγ (n=7); MCP-1 (n≥6); IL-6 (n=7). No significant differences were found.

FIGURES

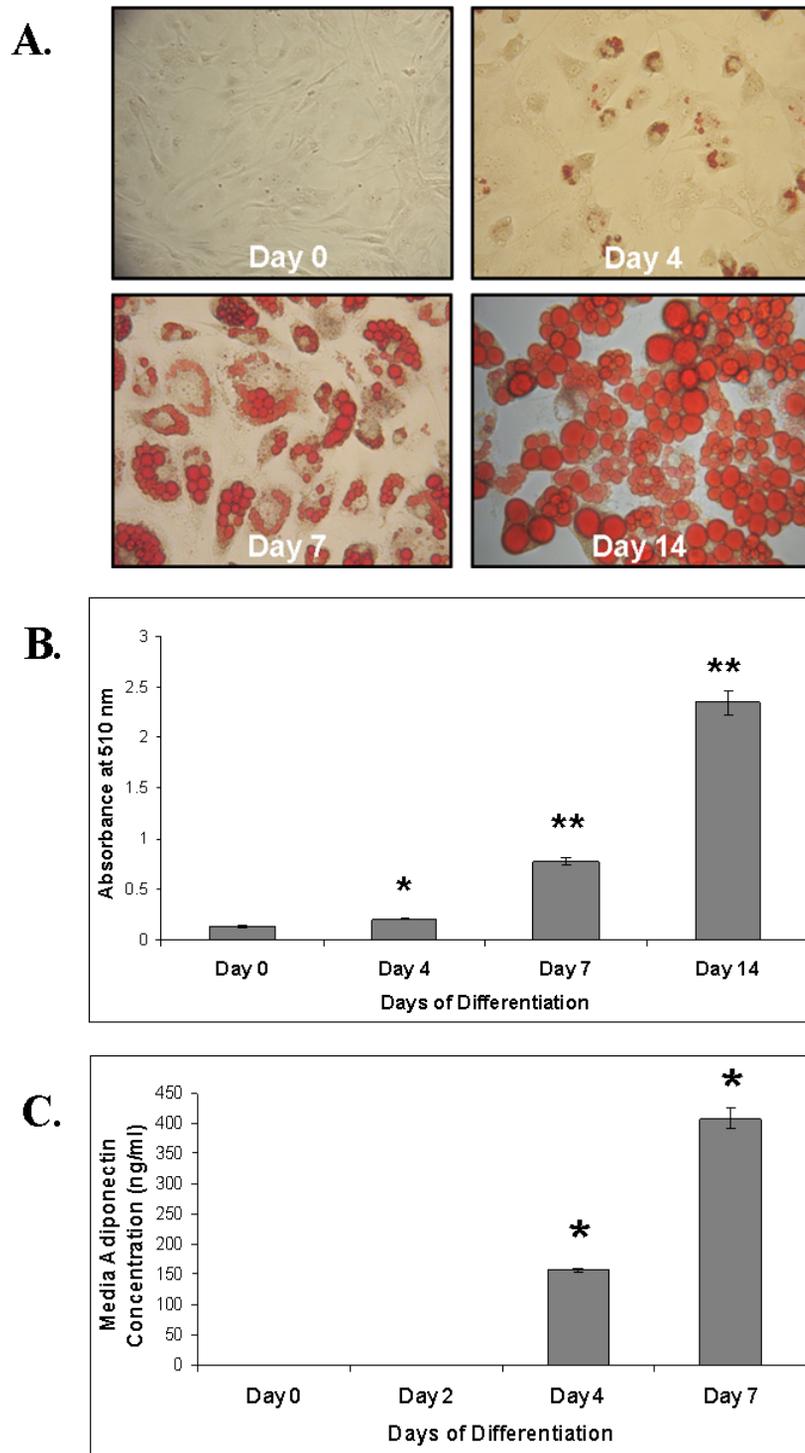
Figure 1.**Basseri et al Fig. 1**

Figure 1. Lipid accumulation and adiponectin secretion increase during adipocyte**differentiation. A) Lipid accumulation increases with differentiation of 3T3-L1 cells.**

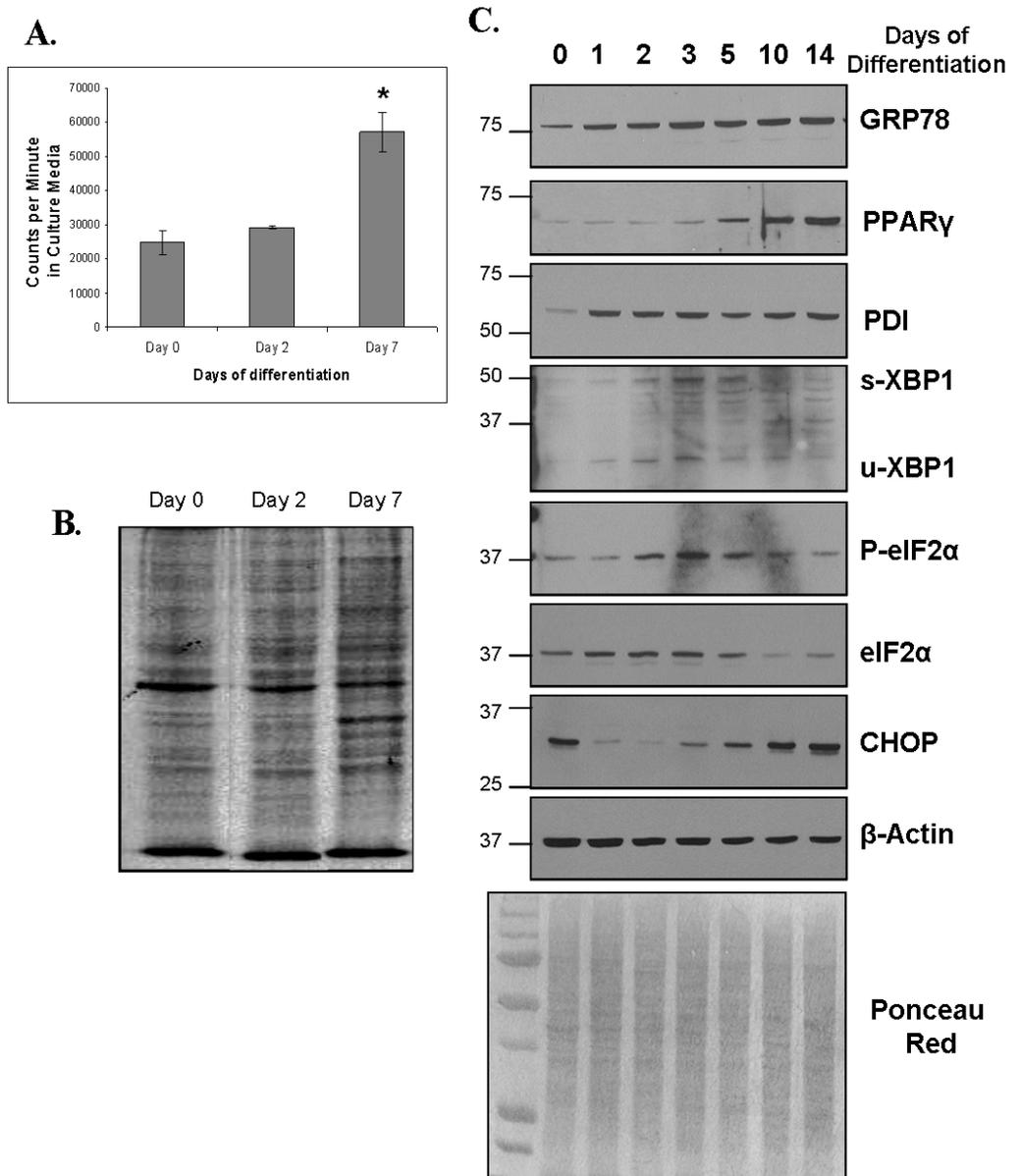
3T3-L1 cells grown to confluence were differentiated in growth media containing MDI (isobutyl methylxanthine, dexamethasone and insulin). Cells were washed in PBS, fixed with 3.7% formaldehyde, stained with Oil red O for 1 hour and photographed on day 0 (preadipocytes) and days 4, 7 and 14 of differentiation. **B) Quantification of Oil red O staining in differentiating 3T3-L1 cells.**

Oil red O was extracted from the cells using 60% isopropanol, collected and the absorbance was measured at 510 nm from cells on days 0, 4, 7 and 14 of differentiation (* $p < 0.0005$, ** $p < 0.00001$ compared to day 0; $n=3$).

C) Adiponectin secretion from 3T3-L1 cells increases significantly following differentiation.

3T3-L1 cells were grown to confluence and cultured in differentiation media. Media was collected on days 0, 2, 4 and 7, and adiponectin levels were measured using ELISA. Concentrations in the samples were determined using a standard curve (* $p \leq 0.000001$ compared to day 0; $n=3$).

Figure 2.

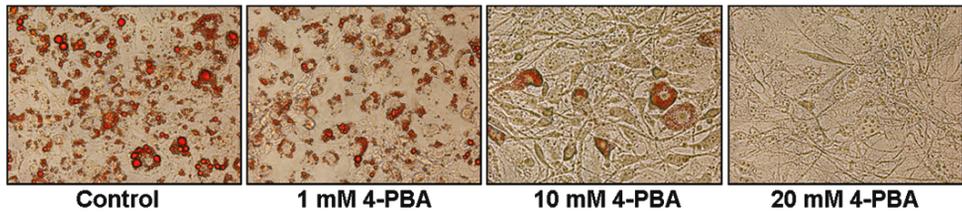


Basseri et al Fig. 2

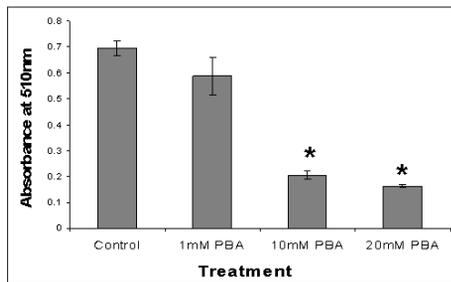
Figure 2. UPR activation correlates with increased *de novo* protein synthesis during adipocyte differentiation. *A) Differentiating 3T3-L1 cells display increased *de novo* protein synthesis and secretion.* 3T3-L1 cells on day 0, 2 and 7 of differentiation were cultured for 4 hours in cysteine/methionine-free media containing ^{35}S -methionine, washed and incubated overnight in cysteine/methionine-free media. Media was collected the next day and counts per minute (cpm) of radioactivity were measured using a scintillation counter (* $p \leq 0.001$ compared to day 0; $n=3$). *B) Total protein lysates from the ^{35}S -labeling experiment were collected and equivalent concentrations were loaded onto a 10% SDS polyacrylamide gel. The gel was subsequently dried and exposed to x-ray film for 48 hours. The autoradiogram is shown here.* *C) Up-regulation of ER stress/UPR markers occurs during 3T3-L1 differentiation.* Cells were allowed to differentiate up to 14 days and total protein lysates were collected in SDS lysis buffer at various time points. Protein was quantified using a Lowry protein assay and equivalent amounts (30 μg) of protein were loaded into each well. Western blotting was performed to detect the following ER stress/UPR markers: GRP78, PDI, spliced and unspliced XBP1, phosphorylated and total eIF2 α , CHOP, a marker of adipocyte differentiation PPAR γ , and β -actin to control for protein loading. As another loading control, the ponceau red stained membrane is shown.

Figure 3.

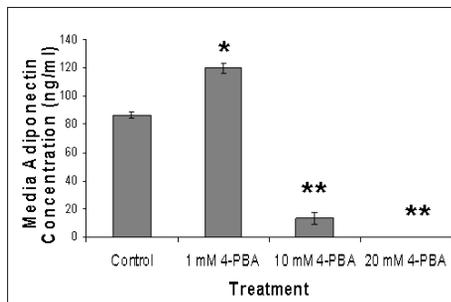
A.



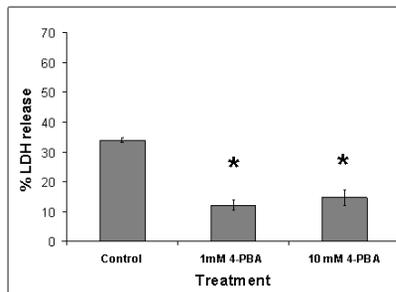
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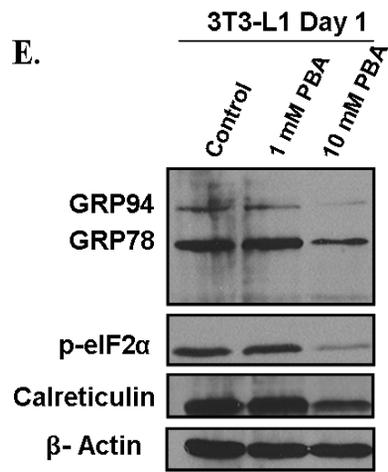
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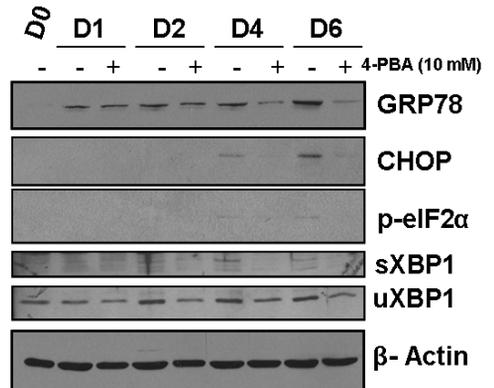
D.



E.



F.



Basseri et al Fig. 3

Figure 3. Treatment of 3T3-L1 cells with 4-PBA inhibits UPR activation and blocks differentiation in a dose-dependent manner. *A) Inhibition of Oil red O staining in differentiating 3T3-L1 cells by 4-PBA.* Confluent 3T3-L1 cells were cultured in differentiation media with increasing concentrations of 4-PBA. On day 5, cells were fixed and stained with Oil red O. Representative images of Oil red O stained cells are shown for each condition. *B) Quantification of Oil red O indicates a significant decrease in lipid droplets with 4-PBA treatment.* Following extraction and collection of Oil red O in isopropanol, absorbance was measured at 510 nm (* $p < 0.001$ as compared to control; $n = 3$). *C) Adiponectin secretion of 3T3-L1 cells is inhibited by 4-PBA.* Media was collected on day 5 of differentiation and adiponectin levels were measured using an ELISA (* $p \leq 0.0005$, ** $p \leq 0.00001$ as compared to control; $n = 3$). *D) 4-PBA treatment is not cytotoxic to 3T3-L1 cells.* Confluent 3T3-L1 cells were treated with 1 mM or 10 mM 4-PBA in 1% FBS media for up to 48 hours. Media was collected and LDH release was measured (* $p < 0.0005$; $n = 3$). *E) Down-regulation of UPR markers in 3T3-L1 cells following treatment with 4-PBA.* Confluent 3T3-L1 cells were treated with 1 or 10 mM 4-PBA. After 24 hrs, total cell lysates were collected and run on a 10% SDS polyacrylamide gel. Antibodies against KDEL (to detect GRP78 and GRP94), calreticulin, phospho-eIF2 α , or β -actin were used for Western blotting. *F) Differentiation of 3T3-L1 cells in the presence of 4-PBA suppresses GRP78 expression.* 3T3-L1 cells were stimulated to differentiate in the presence or absence of 10 mM 4-PBA. Protein was collected on day 0 (confluent cells with no 4-PBA treatment), or days 1, 2, 4, and 6 of

differentiation and Western blotting was used to detect GRP78, CHOP, phospho-eIF2 α and spliced XBP1. As a loading control, the membrane was probed for β -actin.

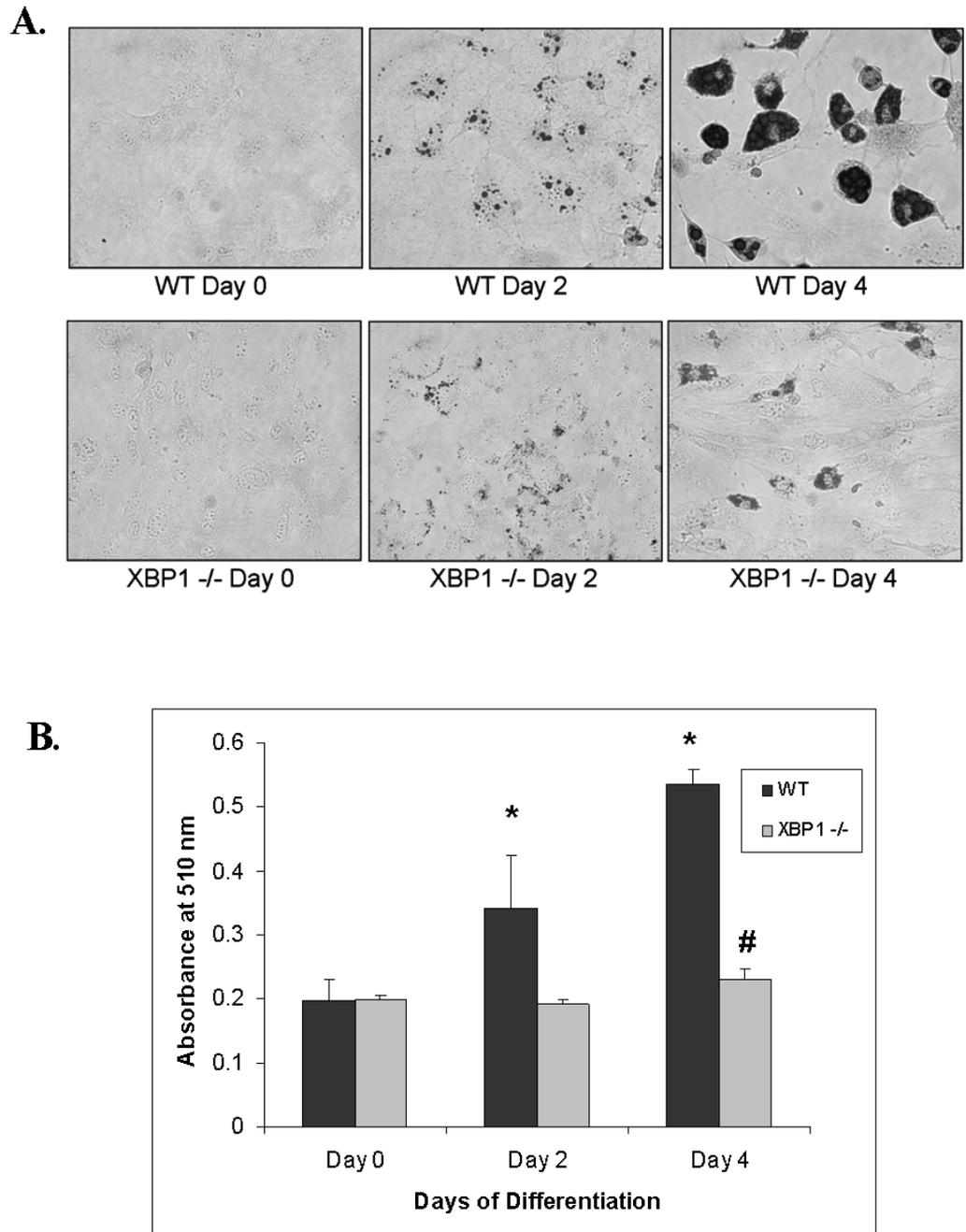
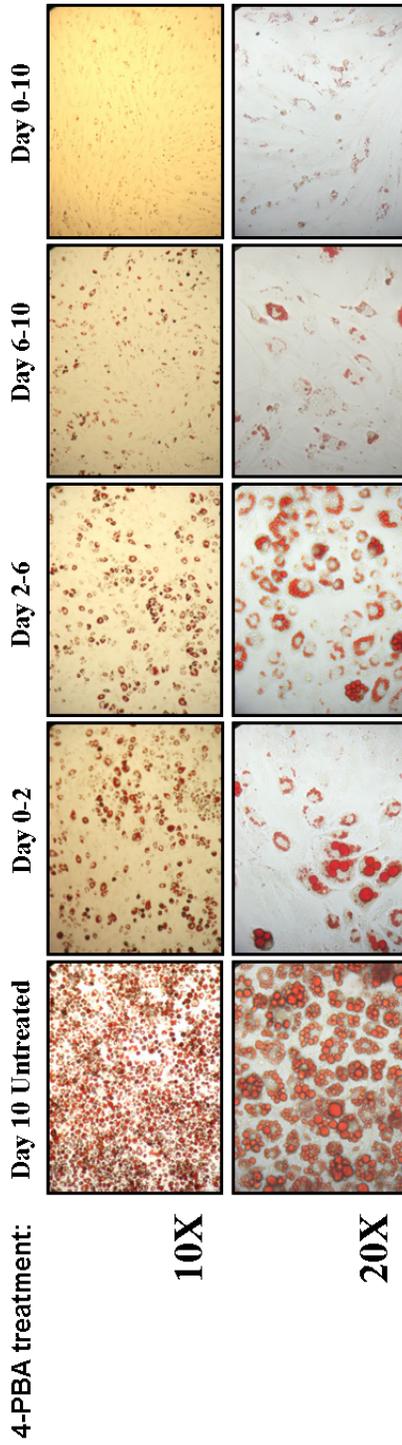
Figure 4.**Basseri et al Fig. 4**

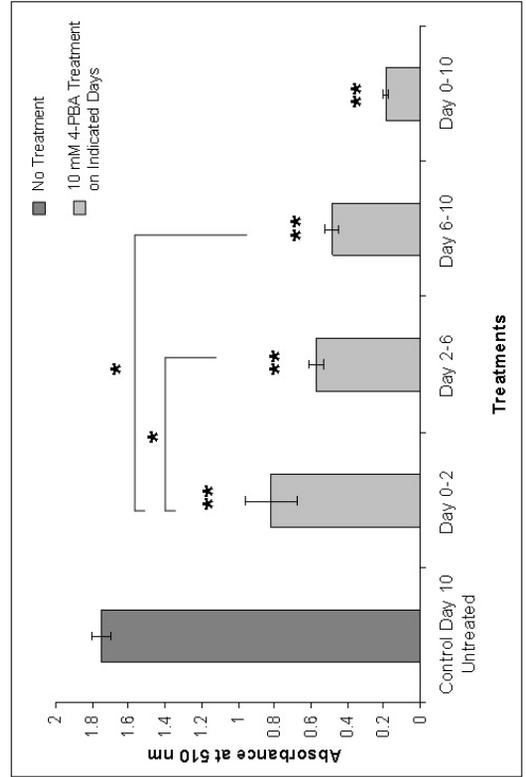
Figure 4. XBP1 deficient mouse embryonic fibroblasts have reduced adipogenic potential. **A)** *Xbp1*^{-/-} MEFs exhibit impaired adipogenesis. Wild-type (WT) or *Xbp1*^{-/-} MEFs were stimulated to differentiate into adipocytes for 4 days. Cells on day 0, 2 or 4 of differentiation were fixed and stained with Oil red O. **B)** *Quantification of Oil red O staining.* WT MEFs accumulate lipid and demonstrate a significant increase in Oil red O staining while deficiency of XBP1 prevents lipid accumulation as compared to WT MEFs (*p<0.01 as compared to WT day 0 cells, #p<0.05 as compared to day 0 *Xbp1*^{-/-} MEFs; n=6).

Figure 5.

A.



B.

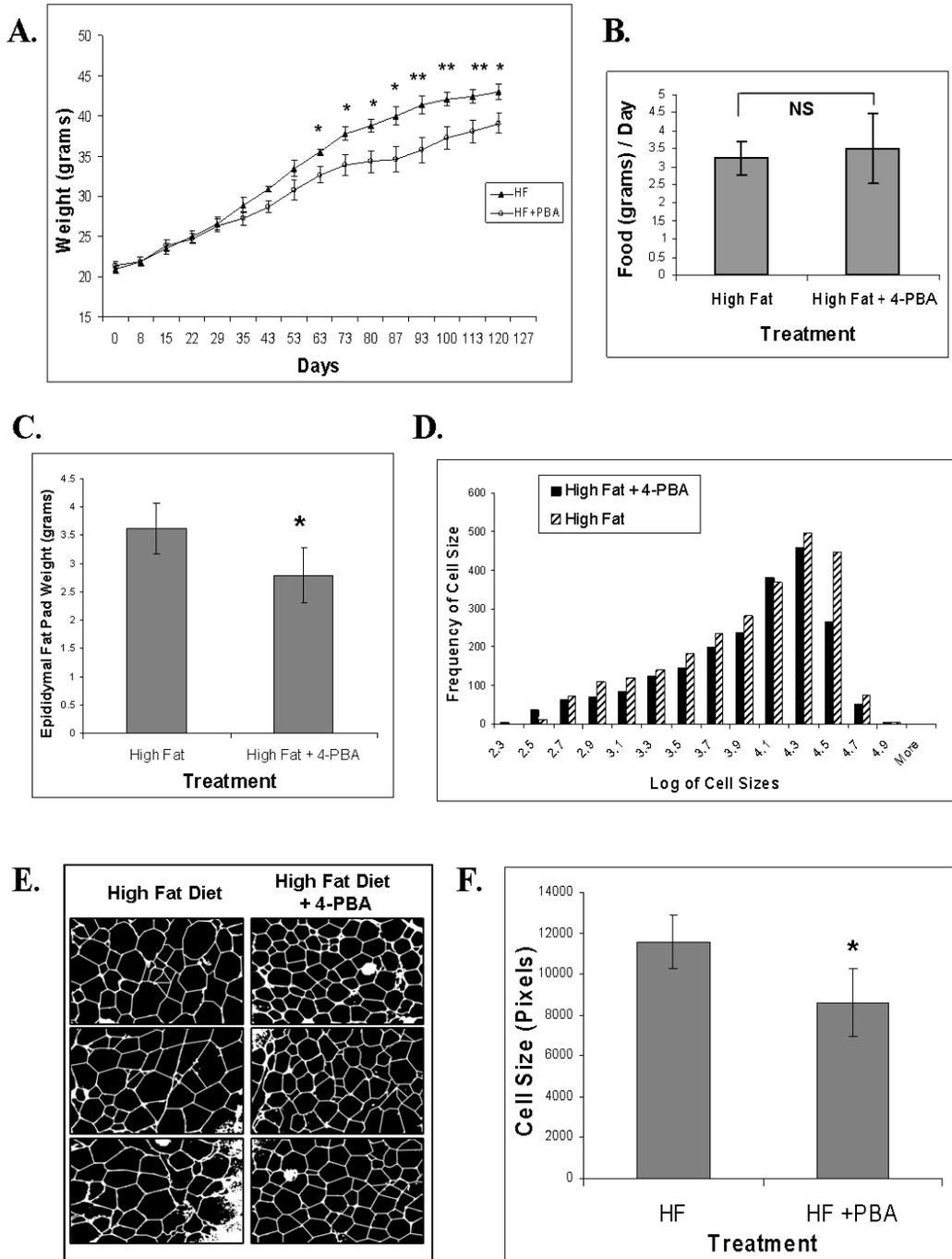


Basseri et al Fig. 5

Figure 5. 4-PBA inhibits lipid accumulation during all stages of differentiation. A)

Effect of 4-PBA on Oil red O staining in differentiating 3T3-L1 cells. 3T3-L1 cells were treated with 10 mM 4-PBA at the onset of differentiation in the presence of MDI (day 0-2), during mid-stages after the MDI was removed (day 2-6), and during late stages after the majority of cells had differentiated (day 6-10). All cells were fixed and stained with Oil red O 10 days after commencing the experiment. As controls, 4-PBA treatment from day 0-10 and no 4-PBA treatment (day 10 untreated) were used at 10 and 20X magnifications. **B) Quantification of Oil red O staining in 3T3-L1 cells treated without or with 4-PBA treatment at all stages of adipogenesis.** Oil red O stain was quantified and a significant reduction in lipid accumulation in all conditions was observed with 4-PBA treatment as compared to day 10 untreated control (** $p < 0.001$; $n=3$). The decline in lipid content was most significant in cells treated with 4-PBA during the later stages of adipogenesis; on days 2-6 or 6-10 ($*p < 0.05$ as compared to day 0-2).

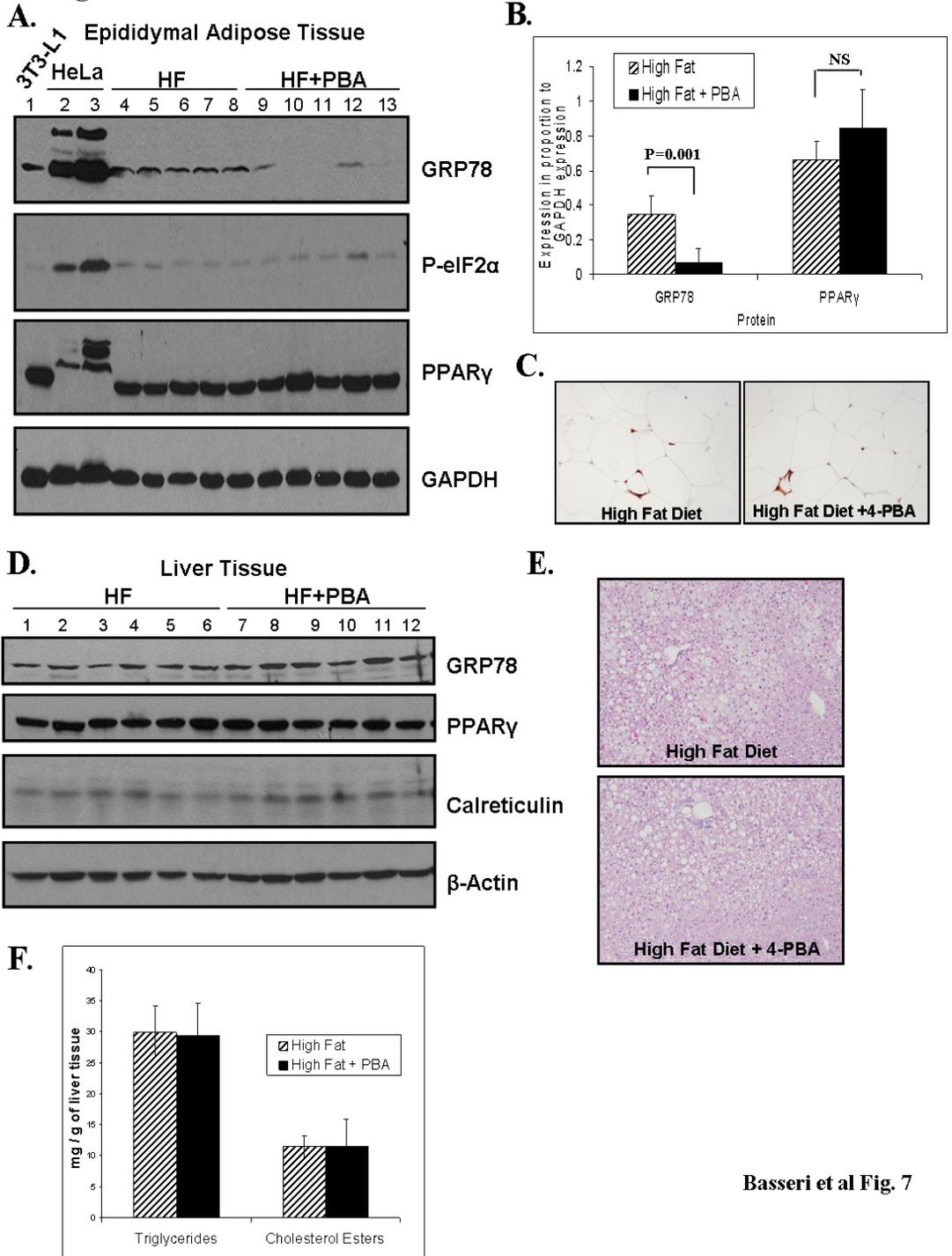
Figure 6.



Basseri et al Fig. 6

Figure 6. Effect of 4-PBA on weight gain and adipogenesis in mice fed a high fat diet. C57BL/6 mice were fed a high fat diet supplemented without (n=8) or with 4-PBA (n=9) at a dose of 1g/kg/day. **A) Effect of 4-PBA on weight gain.** Starting at day 63 and for the remainder of the study, mice supplemented with 4-PBA had significantly lower body weights as compared to controls (*p≤0.05, **p≤ 0.01 as compared to the mice supplemented with 4-PBA using a rank-sum test of significance; n=8-9). **B) Food intake measurements.** No significant difference in food intake was noted between the two groups. **C) Effect of 4-PBA on epididymal fat mass.** Epididymal fat pads were removed at the conclusion of the study and weighed. Mice on the high fat + 4-PBA diet had significantly lower mean fat pad mass (*p<0.01 using a rank-sum test of significance; n=8-9). **D) Adipocyte cell size distribution from epididymal adipose tissue of C57BL/6 mice.** The histograms represent the distribution of cell sizes in mice fed a high fat diet with or without 4-PBA supplementation. The x-axis represents the logarithm of cell sizes in pixels while the y-axis shows the frequency of a given cell size in the population of cells for each group of mice. **E) Representative cross-sections of epididymal fat pads.** Images of the sectioned tissue were taken and representative pictures from the two groups of mice are shown. **F) Mean adipocyte size is reduced with 4-PBA supplementation.** Mice fed a high fat diet supplemented with 4-PBA had a significant reduction in average cell size as compared to the high fat diet alone group (*p<0.05). For average cell size measurements, n=5 for the high fat group and n=4 for the high fat + 4-PBA group.

Figure 7.

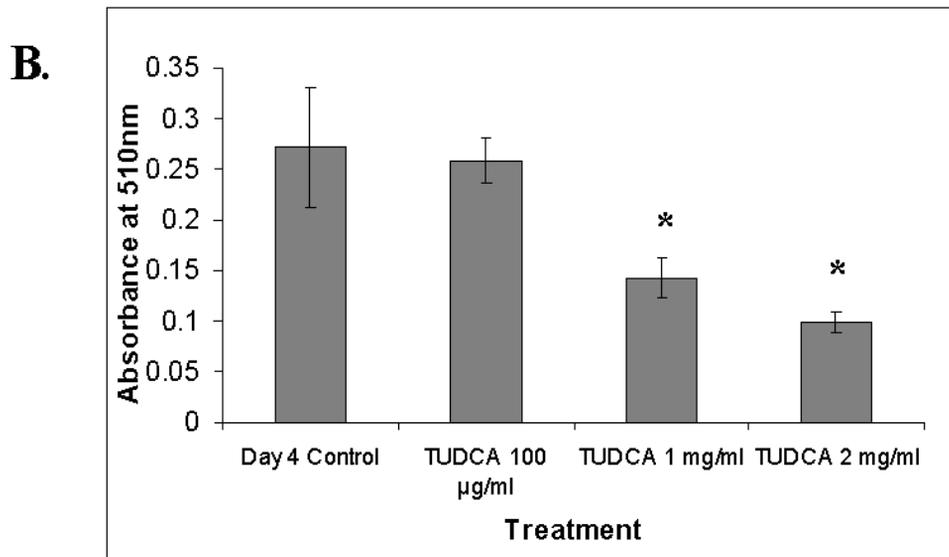
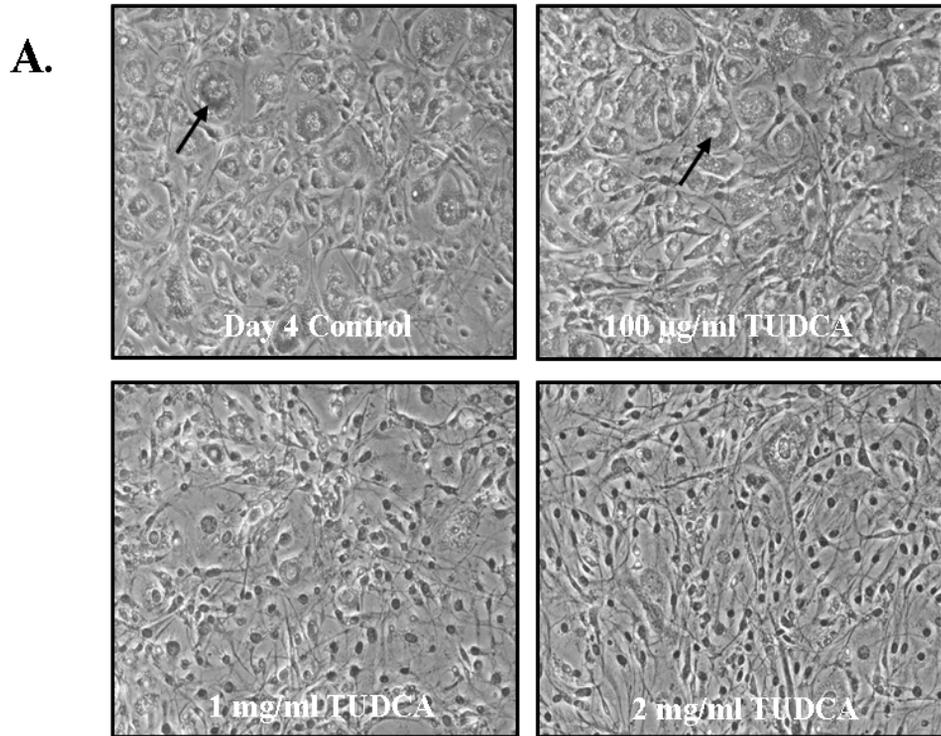


Basseri et al Fig. 7

Figure 7. 4-PBA treatment reduces GRP78 expression in the adipose but not liver tissue. **A)** Protein lysates from the epididymal adipose tissue of mice on high fat diet (lanes 4 to 8) or high fat diet + 4-PBA (lanes 9 to 13) were separated on a 10% SDS polyacrylamide gel. Membranes were probed for KDEL (to detect GRP78), PPAR γ or phospho-eIF2 α . GAPDH was used as a loading control. Differentiated 3T3-L1 cell lysate (lane 1) and protein lysates from HeLa cells treated with the ER stress inducing agent tunicamycin (lanes 2 and 3) were also loaded as positive controls for PPAR γ , GRP78 and phospho-eIF2 α expression, respectively. **B)** Band intensity quantification analysis demonstrated a significant decrease in GRP78 in the adipose tissues of 4-PBA treated mice (*p=0.001; n=5). No significant difference was seen in PPAR γ or phospho-eIF2 α expression between the two groups of mice. **C)** *Mac-3 staining of the adipose tissue.* The epididymal adipose tissue was stained with an antibody against Mac-3 to detect infiltrated macrophages. Representative images are shown. **D)** Total liver lysates from mice on high fat diet (lanes 1 to 6) or high fat diet + 4-PBA (lanes 7 to 12) were separated on a 10% SDS polyacrylamide gel. Western blot analysis was used to detect the ER stress markers GRP78 and calreticulin, as well as PPAR γ and β -actin. No significant differences were seen between the two groups. **E)** *Detection of fatty liver in mice fed high fat or high fat diet + 4-PBA.* Fixed and paraffin-embedded liver samples were sectioned, deparaffinized and stained with hematoxylin and eosin. Representative images from each group showed similar degrees of hepatic steatosis. **F)** *Quantification of hepatic triglycerides and cholesterol esters.* Approximately 150 mg of frozen liver tissue was homogenized and triglycerides and cholesterol esters were extracted. No differences were

found in triglyceride or cholesterol ester concentrations between the two groups of mice (n=4).

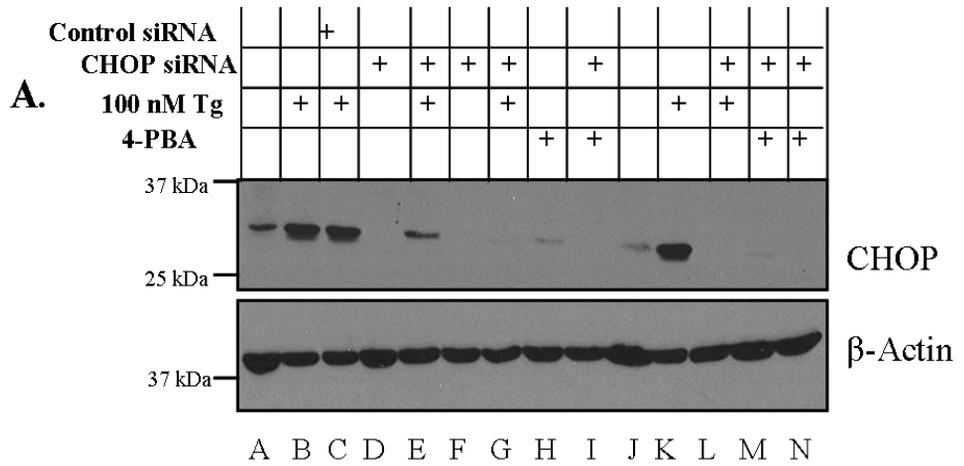
SUPPLEMENTARY FIGURES

Supplementary Figure S1.

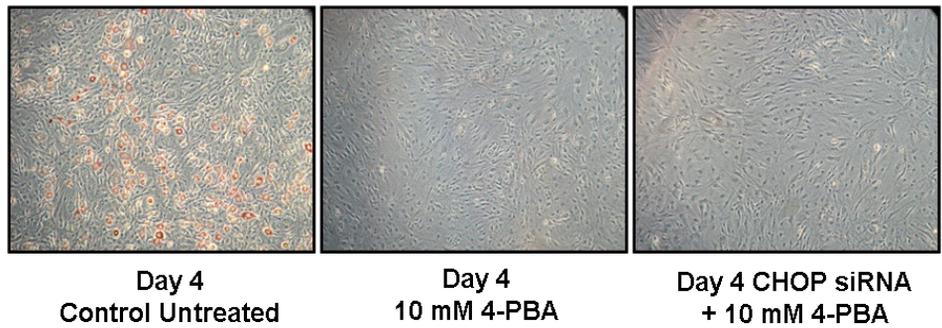
Basseri S et al. The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By Modulating The Unfolded Protein Response.

Figure S1. Treatment of 3T3-L1 cells with tauro-ursodeoxycholic acid (TUDCA) blocks 3T3-L1 differentiation in a dose-dependent manner. **A)** Confluent day 0 3T3-L1 cells were cultured in differentiation media with increasing concentrations of TUDCA (0.1, 1, or 2 mg/ml). On day 4, cells were fixed and stained with Oil red O. Representative images of Oil red O stained cells are shown for each condition, with arrows indicating differentiated adipocytes. **B)** *Quantification of Oil red O indicated a significant decrease in lipid droplets with TUDCA treatment.* Following extraction and collection of Oil red O in isopropanol, absorbance was measured at 510 nm. Data are presented as the mean values +/- standard deviation (n=3). (**p<0.01*)

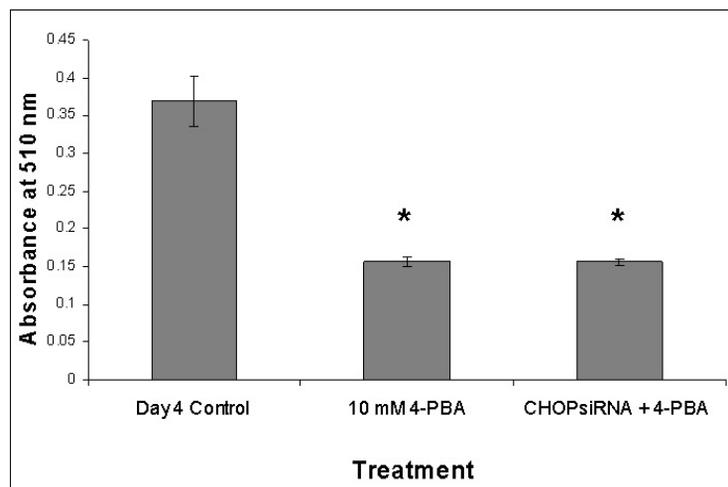
Supplementary Figure S2.



B.



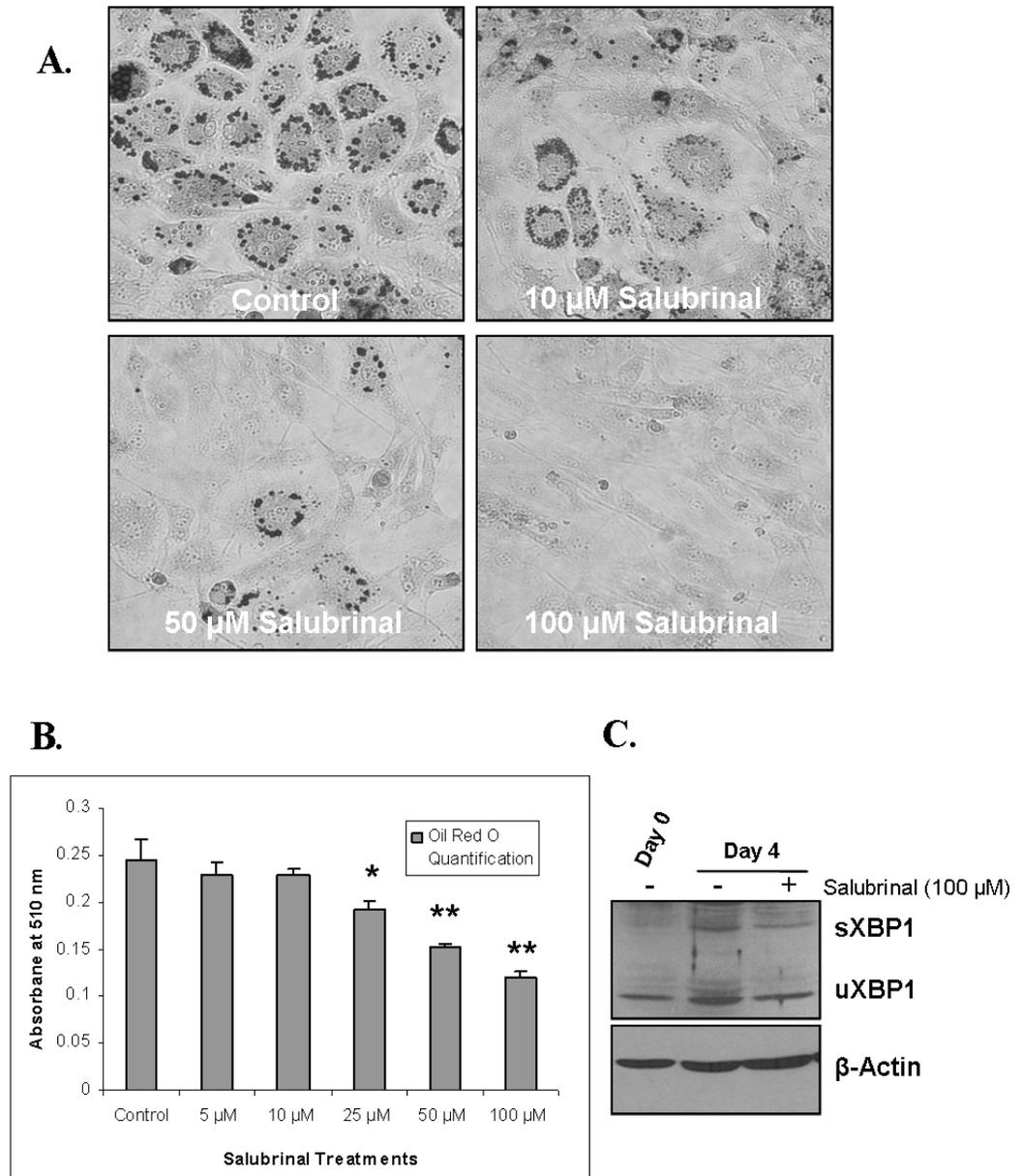
C.



Basseri S et al. The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By Modulating The Unfolded Protein Response.

Figure S2. 4-PBA treatment inhibits adipocyte differentiation through a mechanism independent of CHOP. *A) CHOP protein expression is repressed in CHOP siRNA transfected 3T3-L1 cells when treated with 100 nM thapsigargin or 10 mM 4-PBA.* Cells were treated with 100 nM CHOP siRNA in penicillin/streptomycin-free growth media (day -2). Differentiation media was added on day 0 (48 hrs post-initial siRNA transfection) and post-stimulation media was added on day 2. Cells were transfected with CHOP siRNA on day -2 and 0. Cells were treated with 100 nM of the ER stress inducer thapsigargin (Tg) on day -2, day 0, and day 2 for 48 hrs to induce CHOP expression. On day 0 and 2, 10 mM 4-PBA was added to the media. Protein was collected at various time points during the experiment and run on a 10% SDS polyacrylamide gel. Western blotting was performed and the membrane was probed with an anti-CHOP antibody as well as anti- β -actin as a loading control. Loading order including the day protein was collected: **A-** Day 0 Control, **B-** Day 0 after 48 hr 100 nM Tg, **C-** Negative Control siRNA + 100 nM Tg, **D-** Day 0 CHOP siRNA (48 hrs), **E-** Day 0 CHOP siRNA + 100 nMTg (48 hrs), **F-** Day 2 CHOP siRNA (96 hrs), **G-** Day 2 CHOP siRNA (96 hrs) + 100 nM Tg (48 hrs), **H-** Day 2 48 hrs 4-PBA treated, **I-** Day 2 CHOP siRNA (96 hrs) + 48 hrs 4-PBA treated, **J-** Day 4 Control, **K-** Day 4 after 48 hrs 100 nM Tg, **L-** Day 4 CHOP siRNA treated day -2 to day 2 + 48 hrs 100 nM Tg, **M-** Day 4 96 hrs 4-PBA treated, **N-** Day 4 CHOP siRNA treated day -2 to day 2 + 96 hrs 4-PBA treated. **B) Knock-down of CHOP using CHOP siRNA, did not rescue the cells from the anti-adipogenic effects of 4-PBA.** 3T3-L1 cells were seeded at 70% confluence and transfected with CHOP siRNA. Two days later on day 0, cells were transfected again and differentiation media was added

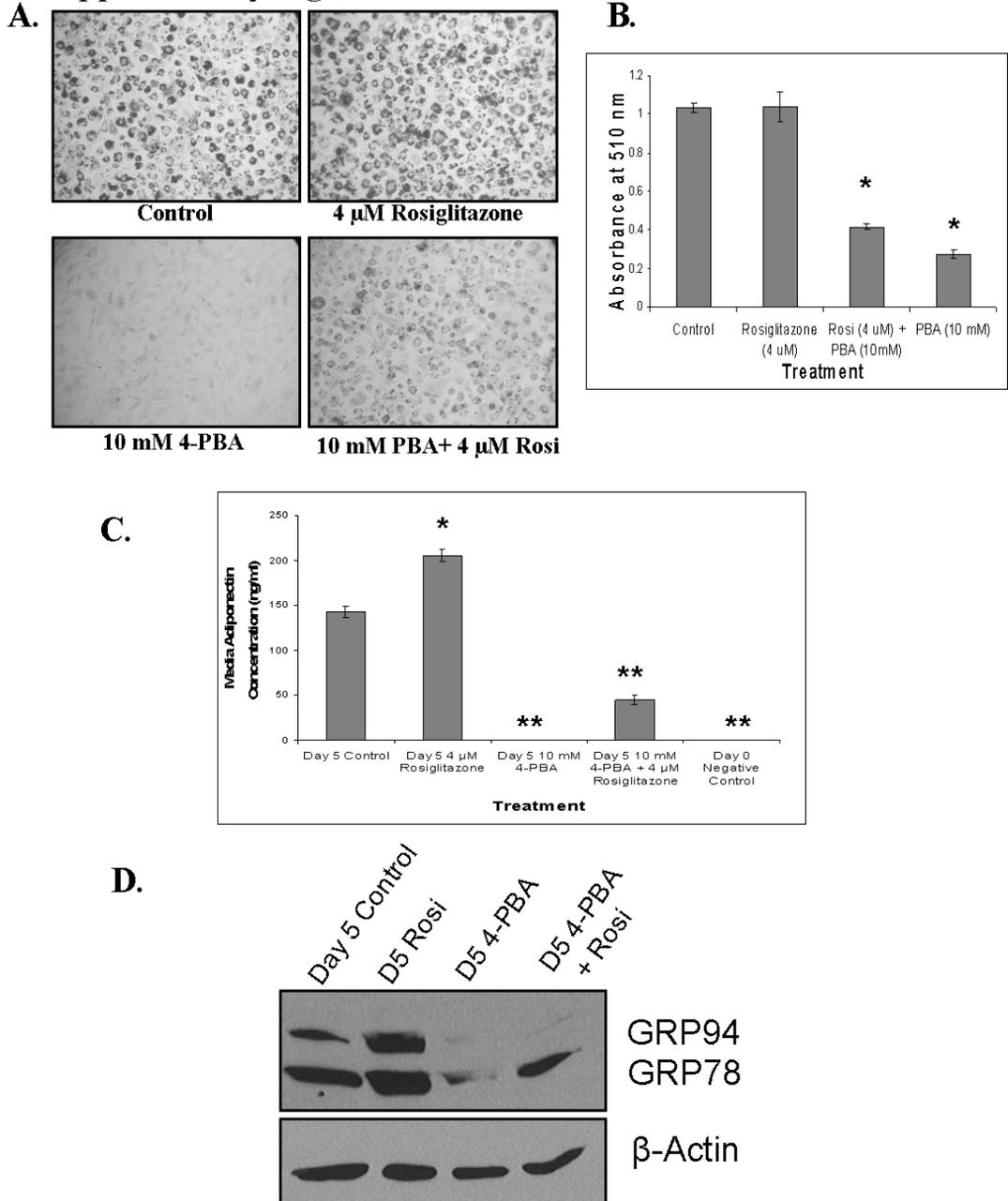
in the presence or absence of 10 mM 4-PBA. On day 2, cells were washed and post-stimulation media with or without 10 mM 4-PBA was added. Cells were fixed and stained with Oil red O on day 4. Images were taken at 20X magnification. C) Oil red O quantification indicated a significant decrease in lipid content in 4-PBA and CHOP siRNA+4-PBA treated cells as compared to untreated control cells. However, there was no difference in adipogenic differentiation with 4-PBA when CHOP was knocked down versus non-transfected cells ($*p < 0.001$).

Supplementary Figure S3.

Basseri S et al. The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By Modulating The Unfolded Protein Response.

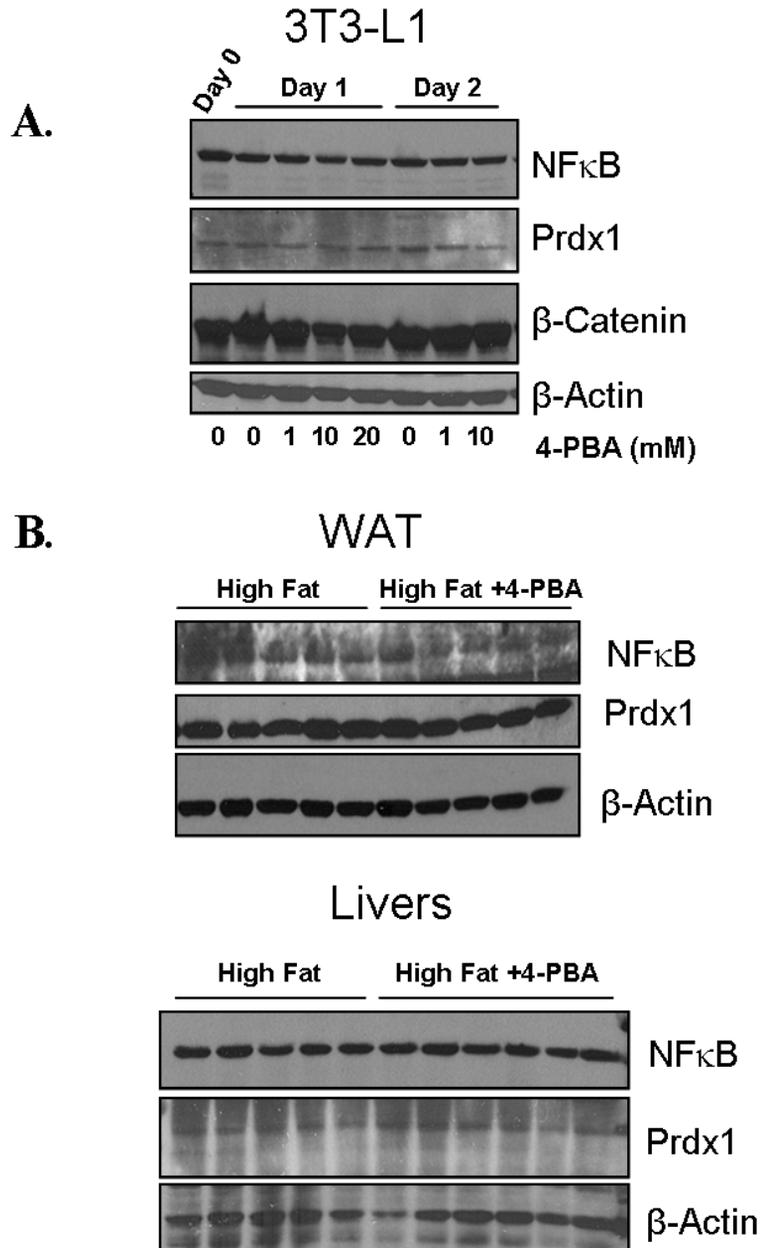
Figure S3. Salubrinal, a selective inhibitor of eIF2 α phosphorylation, blocks adipogenesis in a dose-dependent manner. **A)** Day 0 3T3-L1 cells were stimulated to differentiate in the presence or absence of 10, 50 or 100 μ M salubrinal. On day 4, the cells were fixed and stained with Oil red O. Representative images are shown. **B)** *Salubrinal dose-dependently inhibited lipid accumulation.* Oil red O staining was quantified and a significant decrease in staining intensity was observed with increasing dose of salubrinal ($*p < 0.05$, $**p < 0.01$). **C)** Western blotting confirmed that 100 μ M salubrinal decreases splicing of XBP1 in differentiating day 4 adipocytes.

Supplementary Figure S4.



Basseri S et al. The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By Modulating The Unfolded Protein Response.

Figure S4. Rosiglitazone can partially rescue the differentiation of 3T3-L1 cells in the presence of 4-PBA by enhancing GRP78/GRP94 expression levels. *A)* Day 0 cells were treated with 4 μ M rosiglitazone, 10 mM 4-PBA or a combination of both in differentiation media. Cells were re-supplemented with these drugs on day 2. On day 5, cells were fixed and stained with Oil red O. *B)* 4-PBA alone and the rosiglitazone/4-PBA co-treatment resulted in a significant decrease in Oil red O staining ($*p < 0.001$) though co-treatment allowed for some adipogenic conversion to occur. *C)* *Rosiglitazone and 4-PBA co-treatment allows for some adiponectin secretion by 3T3-L1 cells that was completely blocked by 4-PBA treatment alone.* Media was collected on day 5 of differentiation and an ELISA was performed to detect secreted adiponectin protein. Media from day 0 untreated and undifferentiated 3T3-L1 cells was used as a negative control. Data represent the mean values \pm standard deviation. ($*p = 0.0003$, $**p \leq 0.0001$) *D)* *Rosiglitazone exerts its pro-adipogenic effect in the presence of 4-PBA by enhancing GRP78 expression in differentiating adipocytes.* Western blotting using lysates collected on day 5 of differentiation with various treatments. Rosiglitazone treatment during 5 days of differentiation (day 0-5) resulted in an increase in GRP78 expression. 4-PBA blocked GRP78 expression, while rosiglitazone and 4-PBA co-treatment enhanced GRP78 expression.

Supplementary Figure S5.

Basseri S et al. The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By Modulating The Unfolded Protein Response.

Figure S5. Markers of inflammation or oxidative stress were not altered with 4-PBA treatment. *A)* 3T3-L1 cells treated with varying doses of 4-PBA over 48 hours did not indicate any changes in the expression of NF κ B or peroxiredoxin-1 on Western blots. Expression of β -catenin, a known inhibitor of adipogenesis was also not affected by 4-PBA. *B)* Analysis of the WAT and liver lysates from the 4-PBA supplemented mice did not indicate any changes in NF κ B or peroxiredoxin-1 expression as compared to the non-supplemented group. β -actin was used as a loading control.

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CHAPTER 3

T-cell death associated gene 51 (TDAG51) is suppressed by ER stress and its absence accelerates early adipogenic events in preadipocytes

Preamble

This chapter consists of a research article parts of which have been submitted for publication:

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Authors' Contribution

S. Basseri designed and conducted the experiments, analyzed data and wrote the manuscript. R.C. Austin assisted in designing experiments, edited the manuscript and provided intellectual input. We acknowledge the assistance of Anthony Bruce with packaging and production of retroviral stocks and Rachel Filice for contributing to some of the Western blotting data shown in this chapter, but not included in the current manuscript submission.

T-cell death associated gene 51 (TDAG51) is suppressed by ER stress and its absence accelerates early adipogenic events in preadipocytes*

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Abstract

Recent studies have shown that ER stress/UPR pathways are important for adipogenesis and lipogenesis. A microarray study in preadipocytes identified T-cell death associated gene 51 (TDAG51) among genes that are differentially regulated during adipogenesis. TDAG51 was shown to be an ER stress responsive gene in endothelial cells, however the role of TDAG51 in adipogenesis has not been previously explored. Our findings reveal that TDAG51 is expressed in white adipose tissue and its expression is highest in preadipocytes and repressed in mature adipocytes. Furthermore, TDAG51 was down-regulated during adipogenesis when cells begin to accumulate lipid droplets. Severe/acute ER stress caused by tunicamycin, thapsigargin or DTT treatment suppressed TDAG51 protein expression. Knock-down of TDAG51 in 3T3-L1 cells using siRNA and shRNA accelerated early adipogenic events and increased both lipid accumulation and secretion of the adipocyte-specific marker adiponectin. Furthermore, primary preadipocytes derived from TDAG51-deficient (*TDAG51*^{-/-}) mice exhibited greater lipid accumulation, and increased expression of adipogenic markers. These results suggest that TDAG51 is a novel regulator of early adipogenic events and may therefore be a useful therapeutic target in the treatment of obesity and dyslipidemia.

Introduction

Obesity results from an increase in white adipose tissue (WAT) mass, which occurs as a result of two processes, adipocyte hypertrophy and hyperplasia. The former process indicates an increase in adipocyte cell size, while the latter is the result of an increase in mature adipocyte cell number through preadipocyte recruitment and adipocyte differentiation (1, 2). Two important transcription factors that are crucial during adipocyte differentiation are peroxisome proliferator-activated receptor γ (PPAR γ) (3, 4) and CCAAT/enhancer binding protein α (C/EBP- α) (5-7). Several changes in the extracellular matrix (reduction in collagen and fibronectin) and cytoskeletal remodeling (decline in actin and tubulin expression) are required for adipocytes to differentiate (8, 9). In the later stages of terminal differentiation, proteins such as adipocyte-specific fatty acid binding protein (aP2), adiponectin, and leptin are expressed (10). A fine balance between adipocyte hypertrophy and adipogenesis must exist to prevent the formation of dysfunctional adipose tissue, since large cells are more likely to be insulin resistant (11, 12), and therefore can influence adipose tissue metabolism and whole body energy homeostasis. Despite significant progress that has been made over the past several years, novel players in adipogenesis are still being uncovered, and these additional cellular factors and signalling pathways that influence adipogenesis and adipocyte function require further investigation.

We have previously shown that the unfolded protein response (UPR) is activated during adipocyte differentiation (13). Our findings demonstrated that adipocyte

differentiation involves a chronic/physiological form of ER stress and UPR activation that occurs over a number of days and this response primes the cell for an increased secretory protein load. This is in contrast to an acute form of ER stress/UPR that results from sudden unfavourable conditions in the cell and can lead to unresolved ER stress conditions. In support of these findings, recent studies have demonstrated that deficiency in components of the UPR signaling pathway attenuates adipogenesis *in vitro* (14-16). These studies illustrated that all three arms of the UPR (IRE1-XBP1, PERK-eIF2 α , and ATF6 α) need to be intact for adipogenesis to occur.

We and others have previously identified T-cell death associated gene (TDAG) 51 as an ER stress-inducible gene in endothelial cells (17), atherosclerotic lesion-resident macrophages (18), and lung carcinoma cells (19). TDAG51 is a member of the pleckstrin homology (PH)-like domain family (human homolog PHLDA1) (20) and was first demonstrated to be induced upon T cell activation-mediated apoptosis in culture (21). However, in contrast to the *in vitro* findings, absence of TDAG51 *in vivo* had no apparent effect on T cell apoptosis, number or function (22). In addition to its PH-like domain, TDAG51 possesses distinct C-terminal proline-glutamine and proline-histidine repeats (23), which may be involved in its pro-apoptotic function in some cell types (24). TDAG51 has been suggested to play a role in tumorigenesis (25, 26), and we have previously shown its expression in atherosclerotic lesions of hyperhomocysteinemic mice (17), though the precise physiological role of TDAG51 is not well understood.

To date, the role of TDAG51 in adipogenesis and energy metabolism has not been examined. Microarray analysis of mouse embryonic fibroblasts (MEFs) from *TDAG51*^{-/-} mice found *PPAR* γ to be induced 29 fold in the *TDAG51*^{-/-} MEFs as compared to wild-type (WT) cells from littermate mice (Hossain and Austin, unpublished data). This finding raised the possibility that TDAG51 may be important for adipogenesis, a process that requires and relies upon *PPAR* γ and its downstream target genes. A recent microarray analysis of differentiating 3T3-L1 preadipocytes found *TDAG51*, among other genes, to be induced within the first 8 hours of adipogenesis (27) and was categorized in the same gene cluster as *C/EBP* β and *C/EBP* δ , early adipogenic genes crucial for initiating the adipogenic gene expression cascade (28). Given the importance of the first 24 hours in adipogenesis and determination of adipocyte cell fate decisions (29, 30), this finding suggests that TDAG51 may be a novel candidate gene involved in adipocyte differentiation. In this study, we show that TDAG51 protein is expressed in WAT and preadipocytes, but its expression declines during adipogenesis. Thus, TDAG51 expression becomes undetectable in mature lipid-laden adipocytes. Furthermore, knock-down or absence of TDAG51 accelerates early adipogenic events and lipid accumulation in preadipocytes.

Results

TDAG51 is expressed in preadipocytes and is down-regulated during adipogenesis.

Expression of TDAG51 had not previously been examined in WAT or brown adipose tissue (BAT), thus we examined TDAG51 protein expression pattern in various tissues from adult C57BL/6 mice. In addition to the liver and lung which had previously been shown to have abundant TDAG51 levels at the mRNA and protein level (20, 22), WAT also expresses TDAG51 protein (**Figure 1A**). This finding suggests that TDAG51 may have a regulatory function in the cells residing in WAT. Although most of adipose tissue is composed of preadipocytes and mature adipocytes, other cell types including endothelial cells and fibroblasts are also present. Therefore, closer examination of the preadipocytes was needed to determine whether it is these precursor cells or differentiated adipocytes that express TDAG51, and how its expression may be regulated during adipogenesis. Microarray analysis of gene expression during adipogenesis indicated that *TDAG51* expression, among other genes, is modulated in early adipogenesis (27). Therefore, we carried out quantitative RT-PCR analysis examining *TDAG51* mRNA levels, which confirmed that it is induced in the first 2 hours of adipogenesis followed by a sharp decline in expression (**Figure 1B**). We next sought to evaluate the expression profile of TDAG51 at the protein level during differentiation. **Figure 1C** demonstrates the expression pattern of TDAG51 during the differentiation of 3T3-L1 cells, with up-regulated protein expression in the first 4 days post-stimulation, a gradual decline after day 4, and diminished expression by day 14 of differentiation. TDAG51 expression showed an inverse correlation with PPAR γ expression, an adipocyte

marker and master regulator of adipogenesis. By day 14 of differentiation the vast majority of cells have accumulated large lipid droplets as shown by Oil red O staining in the top panel of **Figure 1C**. Given that TDAG51 mRNA peaked at 2 hours and returned to baseline expression by 8 hours, the protein expression data suggests that TDAG51 protein stability or turnover may be altered in undifferentiated and differentiating cells, which would explain the differences in mRNA and protein expression patterns. This was confirmed by treating cells with cycloheximide to block *de novo* protein translation and then comparing TDAG51 expression over time. Day 3 cells exhibited a greater reduction in TDAG51 expression in comparison to DMSO vehicle control cells, suggesting that TDAG51 protein stability is reduced in differentiating cells as compared to day 0 undifferentiated cells (**Supplemental Figure 1**).

In support of the findings in 3T3-L1 cells, an inverse correlation between TDAG51 protein levels and extent of differentiation was also observed in mouse embryonic fibroblasts (MEFs) induced to differentiate in culture (**Figure 1D**). We next examined protein extracts from human preadipocytes isolated from 4 subjects and differentiated mature adipocytes from corresponding subjects, which also showed disappearance of TDAG51 expression in mature adipocytes (**Figure 1E**). These findings indicate that TDAG51 is down-regulated in differentiated lipid-laden adipocytes, suggesting that TDAG51 may play an important role in regulating critical cell fate decisions during adipogenesis.

TDAG51 is repressed by ER stress-inducing agents in 3T3-L1 preadipocytes. Previous studies in our laboratory indicated that TDAG51 expression was increased by ER stress-inducing agents such as homocysteine and DTT in endothelial cells (17). Given that TDAG51 expression is down-regulated in differentiating adipocytes concomitant with the time frame when ER stress responsive genes and chaperones such as GRP78 are induced (13), we next examined the effect of ER stress on TDAG51 expression in 3T3-L1 preadipocytes. Cells were treated with ER stress inducing agents tunicamycin, an inhibitor of N-linked glycosylation, thapsigargin, an inhibitor of the sarco/endoplasmic reticulum calcium ATPase, and DTT which induces protein misfolding by reducing disulphide bonds. While ER stress markers such as GRP78 and CHOP were induced as expected over the 48 hours of treatment, TDAG51 protein expression was drastically down-regulated after 16 hours and remained so 48 hours after treatment with tunicamycin, thapsigargin, or DTT (**Figure 2A**).

Given our previous finding that 4-PBA can attenuate ER stress/UPR activation in 3T3-L1 cells and decrease expression of ER stress markers (13), we sought to determine whether 4-PBA can modulate TDAG51 protein expression. However, as shown in **Figure 2B**, 24 hours of 4-PBA treatment did not appear to have an effect on TDAG51 protein expression.

Absence of TDAG51 accelerates adipogenesis by altering early adipogenic gene expression. In order to examine the role of TDAG51 in adipogenesis, primary stromal-vascular cells (SVCs) were isolated from white adipose tissue of WT and *TDAG51*^{-/-} mice

and induced to differentiate in culture. *TDAG51*^{-/-} SVCs had an increased potential for adipogenesis as shown by Oil red O staining (**Figure 3A, top panel**) and measured by quantification of the Oil red O-positive cells (**Figure 3A, bottom panel**). Furthermore, *TDAG51*^{-/-} SVCs exhibited greater adiponectin secretion, a marker of mature adipocytes, into the media on day 8 (**Figure 3B**), and earlier and/or greater expression of adipogenic markers such as PPAR γ 2, aP2, fatty acid synthase (FAS), perilipin, and C/EBP α on day 2 (**Figure 3C**).

To complement these findings, both silencing/short interfering (si)-RNA and short hairpin (sh)-RNA targeting mouse *TDAG51* were used to knock-down TDAG51 in 3T3-L1 cells. Similar to *TDAG51*^{-/-} SVCs, siTDAG51 transfected cells had greater lipid accumulation as compared to siControl transfected cells (**Figure 4A**). These cells also exhibited higher expression of C/EBP α and hormone sensitive lipase (HSL) on day 3, and enhanced C/EBP β expression on day 2, as assessed by Western blotting (**Figure 4B**). To examine the long-term effects of down-regulated TDAG51 on differentiating 3T3-L1 cells, shTDAG51 constructs were used to transfect 3T3-L1 cells and a stable 30% knockdown was achieved (**Figure 4C**). Even this modest decrease in TDAG51 expression had a noteworthy effect on adipogenesis as indicated by the significant increase in adiponectin levels on day 3 (**Figure 4D**) and changes in early adipogenic gene expression. Expression of *C/EBP δ* and *C/EBP β* , both very early markers of adipogenesis, was higher within the first 8 hours of differentiation but dropped off more quickly by day 1 in the stable shTDAG51 cells as compared to shControl cells (**Figure 4E**). *Pref-1*, a preadipocyte marker, declined more quickly in the shTDAG51 cells on day 1. Analysis of

late markers of adipogenesis such as *PPAR γ* , *aP2* and *C/EBP α* indicated that they are expressed earlier (day 2) and to a greater extent in shTDAG51 cells (**Figure 4F**). Interestingly, however, 3T3-L1 cells stably over-expressing TDAG51 using a retroviral construct did not exhibit any significant differences in lipid accumulation as assessed by Oil red O staining, and adipogenic markers examined through ELISA and Western blotting (**Supplemental Figure 2A-D**).

Given the inverse correlation between TDAG51 and *PPAR γ* during adipogenesis (**Figure 1C**), and the central role of *PPAR γ* as a nuclear receptor transcription factor that is required to drive adipogenesis (3), the effect of TDAG51 knock-down on *PPAR γ* localization was evaluated. Nuclear *PPAR γ* expression was first observed on day 2 of 3T3-L1 differentiation (**Figure 5A**), therefore, nuclear and cytoplasmic protein extracts were isolated from siTDAG51 and siControl transfected cells on day 2. No difference in the extent of nuclear *PPAR γ* localization was observed with TDAG51 knockdown (**Figure 5B**). These findings suggest that *PPAR γ* localization is not a factor contributing to the increased adipogenesis observed with knockdown of TDAG51, however a change in *PPAR γ* activity cannot be ruled out. Furthermore, TDAG51 expression was undetectable in the nuclear fractions (data not shown), making it unlikely that TDAG51 plays a role as a nuclear protein in differentiating 3T3-L1 cells.

Discussion

Understanding adipogenesis and identification of novel players involved in adipocyte differentiation are important for development of pharmacological treatments to combat obesity and its metabolic co-morbidities such as type 2 diabetes and dyslipidemia. The findings of this study show for the first time that TDAG51 is expressed in white adipose tissue and that its expression is modulated during adipogenesis. At the mRNA level *TDAG51* is induced at two hours following adipogenic stimulation, however its protein expression is sustained for several days before declining as TDAG51 protein stability decreases in undifferentiated cells. Other adipogenic transcription factors such as *C/EBP β* and δ are also induced in such a manner with transcriptional repression following the first two hours, and TDAG51 was categorized in the same gene cluster in the microarray study examining gene expression during 3T3-L1 differentiation (27). Given that TDAG51 protein expression is minimal or undetectable in mature mouse and human adipocytes, TDAG51 may be utilized as a novel marker of preadipocytes in future studies to differentiate between pre- and mature adipocytes. The absence of TDAG51 in mature adipocytes may allow for enhanced lipid accumulation and/or insulin mediated *de novo* lipogenesis. This may be required to allow the cells to cope with the nutrient excess and efficiently store TG as lipid droplets.

Absence or knock-down of TDAG51 accelerates lipid accumulation and adipogenic gene expression in 3T3-L1 cells and primary preadipocytes. Knock-down of TDAG51 using siRNA did not appear to affect the localization of PPAR γ within the first

2 days of differentiation when PPAR γ appears in the nucleus. This suggests that at least in preadipocytes increased nuclear localization of PPAR γ cannot explain the increased potential for adipogenesis. In fact, significant differences in *C/EBP β* , *C/EBP δ* and *Pref1* gene expression were found much earlier (within the first 24 hours of differentiation) in stable 3T3-L1 cells with knock-down of TDAG51. Given that TDAG51 expression was undetectable in the nuclear protein fractions of differentiating 3T3-L1 cells, it would be worth investigating the function of TDAG51 or its potential binding partners in the cytoplasm through co-immunoprecipitation pull-down studies. Furthermore, our finding that TDAG51 over-expression had no obvious effect on adipogenic markers or lipid accumulation in cultured preadipocytes, raises the possibility that TDAG51 expression beyond a certain threshold does not produce a physiological outcome in differentiating adipocytes. This could be due to a limited supply or tightly regulated proteins (such as binding partners or complexes), which may be required for mediating the effects of TDAG51 on cell function.

Interestingly, TDAG51 was not induced by ER stress-inducing agents in 3T3-L1 preadipocytes and its over-expression did not lead to apoptotic cell death under non-stressed conditions and in cells undergoing adipogenesis. This is in contrast to previous findings in endothelial cells where it was identified as an ER stress-inducible pro-apoptotic gene (17), raising the possibility that TDAG51 may have different binding partners or be involved in different signaling pathways depending on the cell type. Such a cell-type dependent mode of action would also explain its non-ubiquitous protein expression pattern across various tissues and organs.

TDAG51 possesses a PH-like domain which may allow it to function in cellular signaling processes. Proteins with PH domains are important in recruitment of their target proteins to cell membranes by binding to phospholipids such as phosphatidylinositol (3,4,5)-triphosphate (PIP3) and phosphatidylinositol (4,5)-bisphosphate (PIP2) (31, 32). Indeed, previous studies suggest that ERK and p38 MAPK signaling can modulate TDAG51 expression (19, 33, 34). TDAG51 was identified as a specific IGF-1 target gene in NIH-3T3 fibroblasts and shown to mediate the effects of IGF-1 on cell survival (34). The exact cellular mechanism by which IGF-1/p38 MAPK induces TDAG51 expression is unknown, but this result suggests that TDAG51 may play a role in regulating growth factor-mediated cell signaling. It is worth noting that chemical inhibition of p38 MAPK impaired adipogenesis in 3T3-L1 and primary human preadipocytes (35, 36), but a contrasting study showed that p38MAPK inhibition or absence of p38MAPK in preadipocytes enhanced adipogenesis (37). The precise role of p38MAPK in adipocyte determination and differentiation stages appears to be controversial and was therefore not specifically examined in this study.

Interestingly, TDAG51 has a high stringency PIP3-binding site in its PH-like domain predicted by ScanSite (38). Furthermore, the PH-like domain of TDAG51 exhibits a high degree of homology to that of phosphatidylinositol 3-kinase (PI3K) enhancer (PIKE), as predicted by Protein Homology/Analog Y Recognition Engine (Phyre2) (39). Given the role of PIKE family members in PI3K/Akt signaling, this prediction raises the possibility that TDAG51 and PIKE may be competing with each other for binding to PI3K, Akt or other enzymes. TDAG51 was previously shown to be

induced by IGF-1, an activator of PI3K/Akt signaling, which regulate post-transcriptional events during adipogenesis (40). Thus, the relationship between TDAG51 and PI3K/Akt activity clearly warrants further investigation. Similar to TDAG51, the PH domain in PIKE contains a nuclear localization sequence (NLS) which allows for it to be shuttled between the cytoplasm and nucleus depending on the concentration of PIP at the cell membrane (41). In contrast to *TDAG51*^{-/-} preadipocytes, a recent study has shown that *PIKE-A*^{-/-} MEFs fail to differentiate into adipocytes (42). Interestingly, a novel PH-domain containing protein PHLDB1 was shown to enhance insulin-stimulated Akt phosphorylation in adipocytes by binding to PIP3 through its PH domain (43). Whether the PH-like domain of TDAG51 plays a role in insulin/growth factor signaling and in mediating the effects of TDAG51 on adipogenic regulation is the subject of current investigation in our laboratory.

Methods

Cell Culture. Mouse 3T3-L1 cells were cultured, differentiated and stained with Oil red O as previously described (Basseri et al. 2009). Pooled protein extracts were obtained from isolated human preadipocytes and their corresponding differentiated adipocytes from four female subjects (Zenbio). Cells were treated with ER stress-inducing agents 2.5 µg/ml tunicamycin (Sigma), 100 nM thapsigargin (Sigma), and 1 mM DTT (Invitrogen) for up to 48 hours, or treated with 1-20 mM of the ER stress inhibitor 4-PBA (Calbiochem) for 24 hours. To inhibit protein synthesis cells were treated with 20 µg/ml cycloheximide (Sigma).

Isolation and culturing of primary cells. Primary preadipocytes were cultured by isolating the stromal-vascular fraction from mouse WAT. Briefly, WAT was minced, collagenase digested (Sigma) and centrifuged to separate the SVF from the mature adipocyte fraction. The cells were then plated and induced to differentiate in DMEM/F12 containing 2% FBS, 1 µg/ml insulin, 250 nM dexamethasone, 0.5 mM IBMX, 60 µM indomethacin, and 2.5 µM rosiglitazone. Cells were stained with Oil red O as previously described (13) or protein lysates were collected for Western blotting.

TDAG51 Knock-down Studies. 3T3-L1 cells were transfected with siRNA targeting TDAG51 or control scrambled siRNA (Dharmacon) following the manufacturer's protocol. Once the cells reached confluence, the transfected cells were stimulated to differentiate using the media described above. Stable knock-down of TDAG51 was achieved by infecting cells with shTDAG51 encoding lentiviral particles or shControl

particles as a control. Following 7 days of puromycin (2 µg/ml) selection, 3T3-L1 cells with stable down-regulation of TDAG51 were generated.

TDAG51 Over-expression Studies. 3T3-L1 cells stably over-expressing TDAG51 were obtained by infecting cells with retroviral particles containing human TDAG51-pBabe (TDAG51-pBabe) or a control pBabe empty vector (EV-pBabe). Briefly, viral particles were harvested by growing 293T cells and performing a calcium phosphate transfection as previously described (44). A mixture of plasmids (10 µg VSV, 10 µg GP and 10 µg pBabe retroviral plasmid with or without the human TDAG51 open reading frame) in 2.5 M CaCl₂ was added to 293T cells and incubated for 48 hours. Viral particles in the media were harvested, filtered to remove debris, centrifuged, and the pelleted viral particles were then resuspended in media containing 1 µg/ml of polybrene and used to infect 3T3-L1 cells. To generate a stable 3T3-L1 cell line, cells were selected with puromycin (2 µg/ml) for 7-10 days.

mRNA Quantification by Real-Time PCR. Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's protocol. Using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) total RNA was reverse transcribed to obtain cDNA. qPCR was performed using Fast SYBR Green PCR master mix (Applied Biosystems) in the AB7900 HT Fast Real Time PCR System. Primer sequences were as follows: *aP2*, forward, TTC GAT GAA ATC ACC GCA GA, reverse AGG GCC CCG CCA TCT; *Cebpa*, forward, TGT GCG AGC ACG AGA CGT C, reverse, AAC TCG TCG TTG AAG GCG G, *Cebpβ*, forward, CTA TTT CTA TGA GAA AAG AGG CGT

ATG TAT, reverse, ATT CTC CCA AAA AAG TTT ATT AAA ATG TCT, *Cebpd*, forward, TGC CCA CCC TAG AGC TGT G, reverse, CGC TTT GTG GTT GCT GTT GA, *Cyclophilin*, forward, GCA TAC AGG TCC TGG CAT CT, reverse, TTC ACC TTC CCA AAG ACC AC, *Ppar γ* , forward, GGC CAT GAG GGA GTT AGA AG, reverse, CCC TTT ACC ACA GTT GAT TT, *Prefl*, forward, GAA ATA GAC GTT CGG GCT TG, reverse, AGG GGT ACA GCT GTT GGT TG. Data analysis was performed using the $\Delta\Delta C(T)$ method and normalized to cyclophilin as previously published (45, 46).

Immunoblotting. Cells and tissues were homogenized in 4x SDS lysis buffer and protein lysates were subjected to immunoblotting as previously described (13). Antibodies were used to detect the following proteins: C/EBP α , CHOP, PPAR α , SREBP-1, TDAG51 (Santa Cruz Biotechnology), aP2, C/EBP β , phospho-eIF2 α , GAPDH, HSL, perilipin, PPAR γ (Cell Signalling), GRP78 (BD Biosciences), and β -actin (Sigma). Nuclear and cytosolic protein extracts were prepared as previously described (47). Briefly, cells were lysed in a hypotonic buffer containing protease and phosphatase inhibitors, homogenized and centrifuged at 16,000xg for 20 min at 4°C. The supernatant (cytosolic fraction) was collected and the nuclei pellet was washed with PBS and then resuspended in a hypotonic buffer containing 0.42 M NaCl and 20% glycerol, rotated for 30 min at 4°C and centrifuged again as in the first step. The supernatant containing nuclear proteins was collected and the pellet was discarded.

Adiponectin ELISA. Media was collected from cells grown in culture and mouse adiponectin concentrations were measured using an ELISA (R&D) as per manufacturer's instructions. Media from differentiated cells required a dilution between 1:10-1:100.

Acknowledgements

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FIGURES

Figure 1.

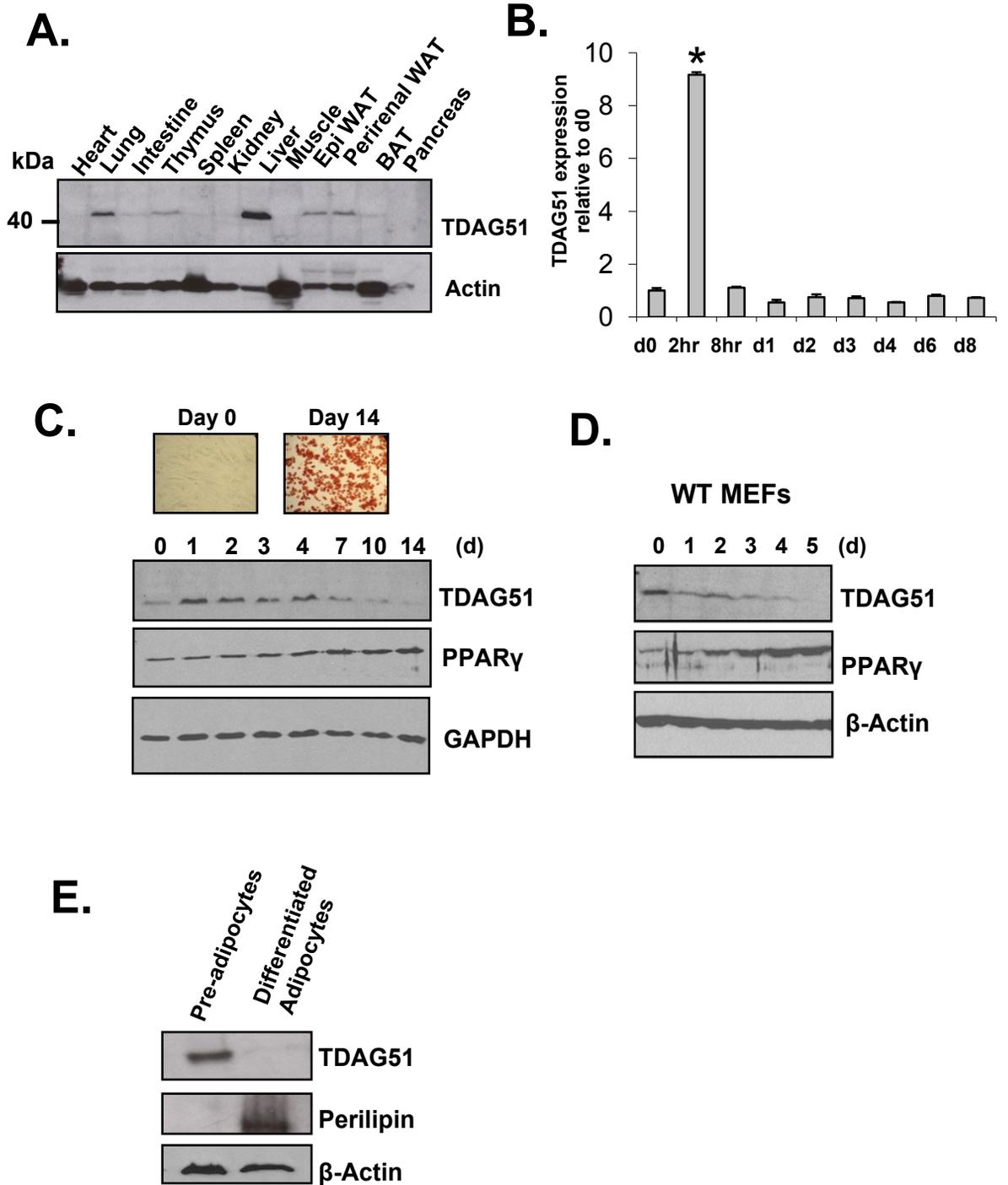
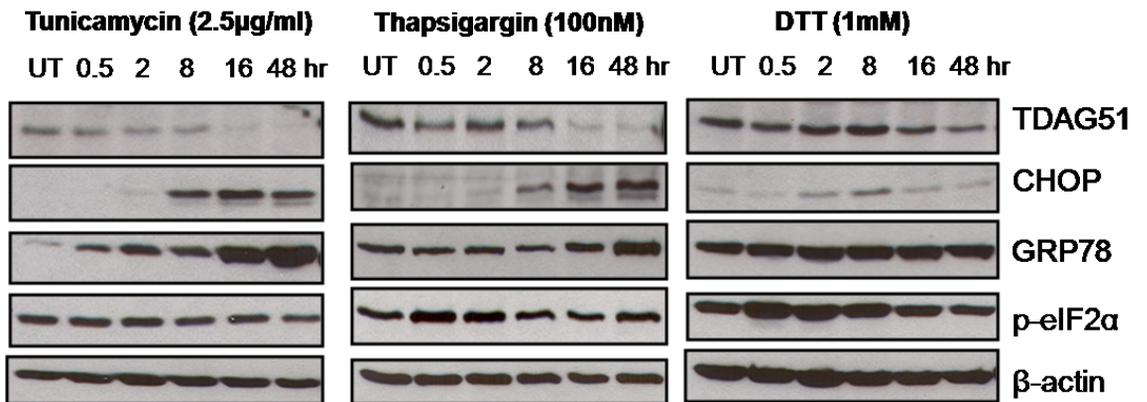


Figure 1. TDAG51 is expressed in preadipocytes and is down-regulated during adipogenesis. (A) Western blotting for TDAG51 using whole tissue lysates from WT C57BL/6 mice. (B) RT-qPCR for *TDAG51* mRNA expression in 3T3-L1 preadipocytes during differentiation. Data represent means \pm standard deviation (SD). (C) 3T3-L1 cells were induced to differentiate for 14 days and stained with Oil red O to detect neutral lipids (upper panel). Total lysates were assessed for PPAR γ , TDAG51, and β -actin (loading control) by immunoblotting (lower panel). (D) MEFs isolated from wild-type C57BL/6 mice were induced to differentiate (at passage 5) and protein extracts were collected and subjected to immunoblotting. (E) Immunoblotting for TDAG51, perilipin (positive control for mature adipocytes) and β -actin in human preadipocyte and corresponding mature adipocyte protein extracts.

Figure 2.

A.



B.

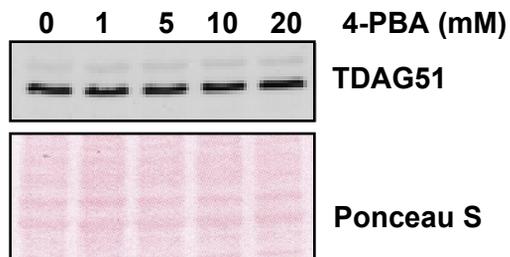


Figure 2. TDAG51 is repressed by ER stress-inducing agents in 3T3-L1 preadipocytes. (A) Undifferentiated 3T3-L1 cells were treated with tunicamycin, thapsigargin, or DTT for up to 48 hours and protein lysates were collected at the indicated time points. Lysates were subjected to immunoblotting using antibodies against TDAG51, CHOP, GRP78, phospho-eIF2 α and β -actin. (B) 3T3-L1 cells were treated with various doses of 4-PBA (1-20 mM) for 24 hours to examine TDAG51 protein expression. Ponceau S staining of the nitrocellulose membrane is shown to illustrate protein loading.

Figure 3.

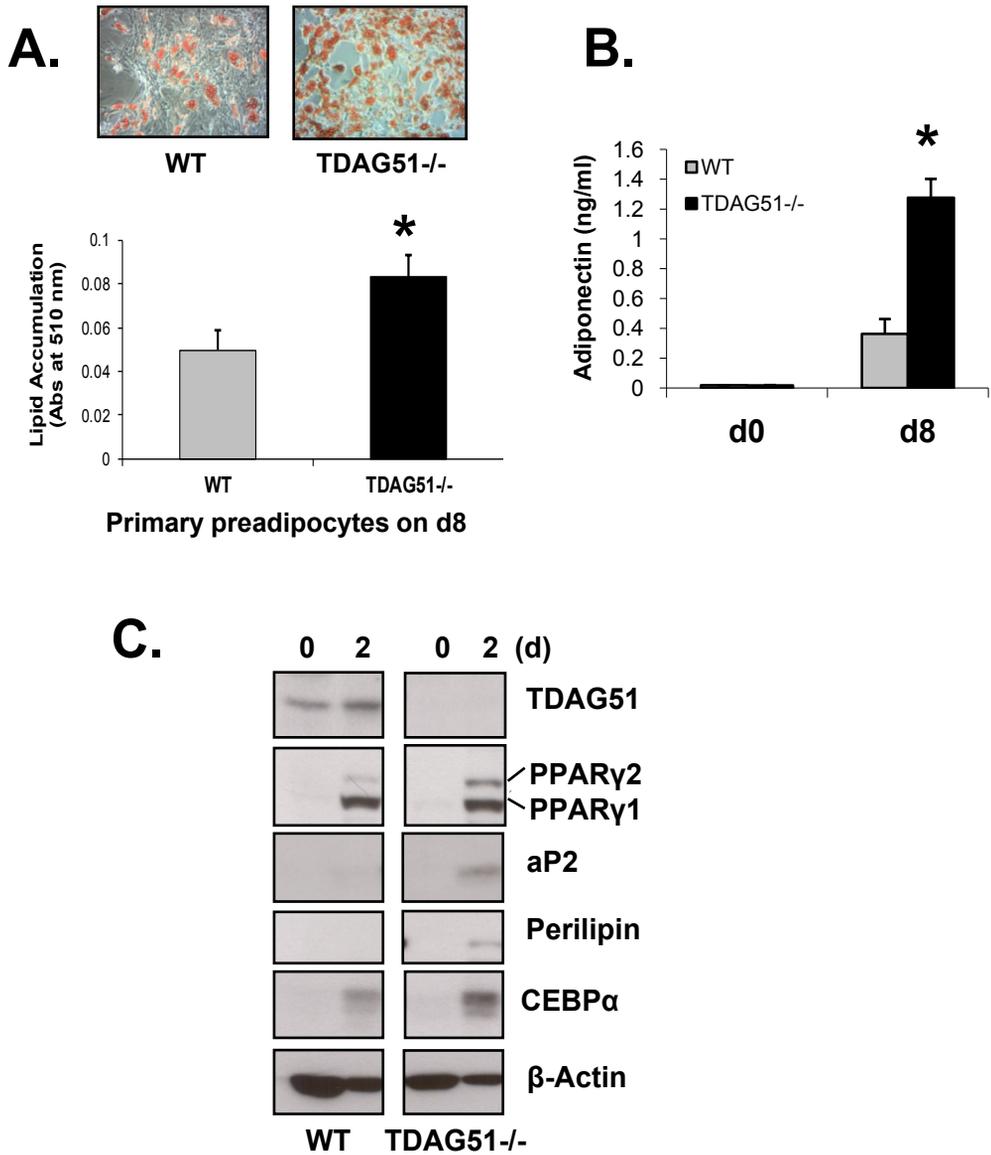


Figure 3. Absence of TDAG51 accelerates adipogenesis. (A) The SVC fraction containing primary preadipocytes were induced to differentiate and stained with Oil red O on day 8 (top panel). The staining was quantified (lower panel). Data are means \pm SD, n=3, * $P < 0.05$. (B) Adiponectin secretion from primary preadipocytes. Data represent means \pm SD, n=5, * $P < 0.05$. (C) Immunoblotting for adipogenic markers on day 0 and 2 of WT or *TDAG51*^{-/-} primary preadipocyte differentiation.

Figure 4.

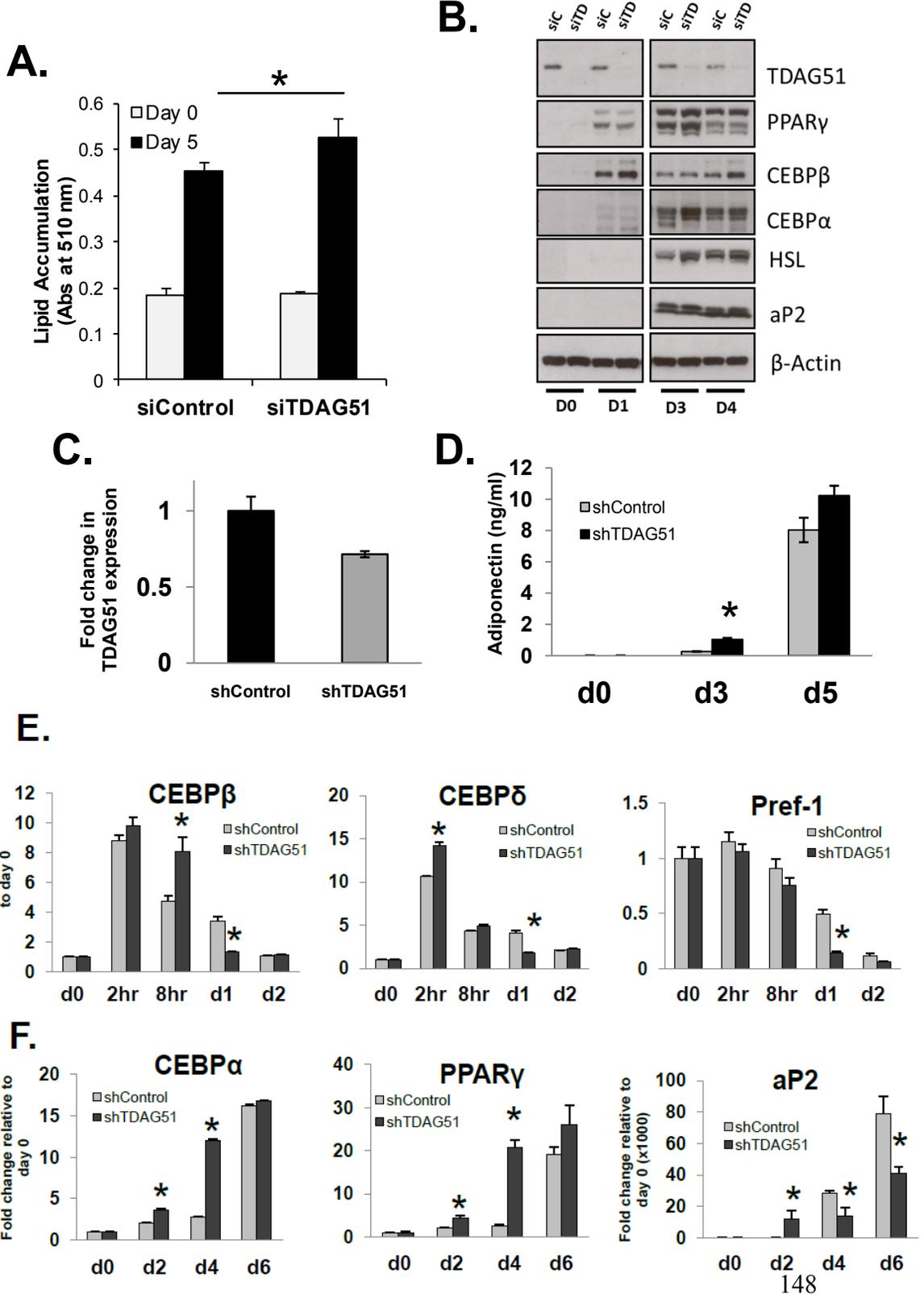


Figure 4. TDAG51 plays a role in early adipogenic events. (A) Small interference RNA (siRNA) was used to knock-down TDAG51 (siTDAG51) in 3T3-L1 preadipocytes and cells were induced to differentiate. Oil red O staining was quantified on day 5 of differentiation (n=6, * $P < 0.05$). (B) Protein lysates were collected at various time points during differentiation of 3T3-L1 transfected with either siControl or siTDAG51. Western blotting was performed to assess expression of adipogenic markers on day 0, 1, 3 and 4. (C) 3T3-L1 preadipocytes were transfected with short hairpin RNA targeting TDAG51 (shTDAG51) or non-targeting shRNA (shControl) to generate 3T3-L1 cells stably down-regulating TDAG51 expression. RNA was isolated at various time points during differentiation and RT-qPCR was performed to determine extent of TDAG51 knockdown. (D) Adiponectin secretion into the media was quantified on day 0, 3 and 5 of differentiation in shControl and shTDAG51 3T3-L1 cells (n=5, * $P < 0.01$ when comparing shControl to shTDAG51). (E) RT-qPCR examining expression of early adipogenic markers and preadipocyte marker Pref-1. Data represent means \pm SD, * $P < 0.05$ when comparing expression between shControl and shTDAG51 at a given time point. (F) RT-qPCR examining expression of late or terminal adipogenic markers. Data represent means \pm SD, * $P < 0.05$ when comparing expression between shControl and shTDAG51 at a given time point.

Figure 5.

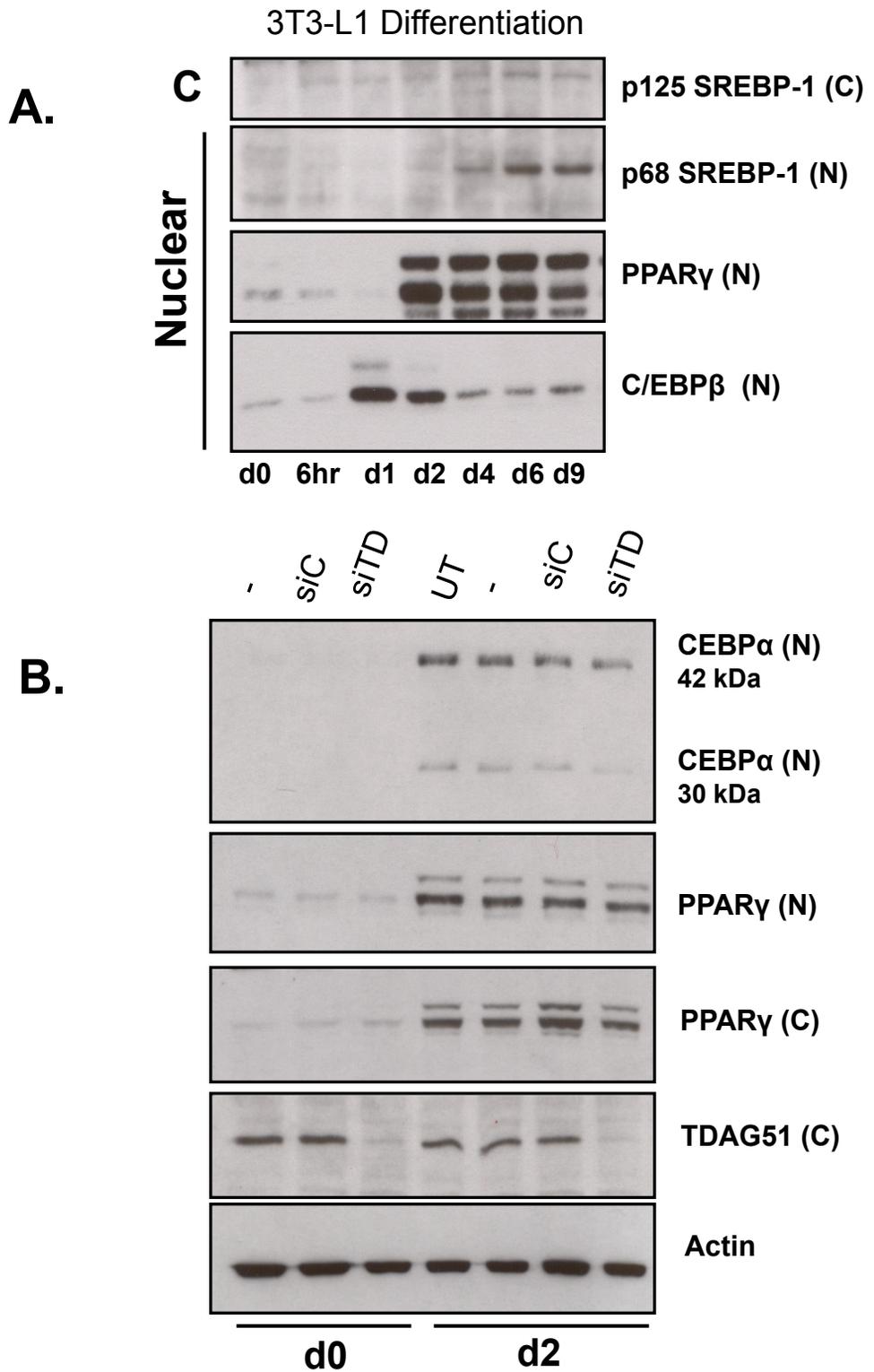


Figure 5. PPAR γ localization is not affected by TDAG51 knock-down. (A) Nuclear and cytoplasmic protein extracts were collected at various time points during differentiation of 3T3-L1 cells and immunoblotting was performed (C- cytoplasmic, N- nuclear). (B) Small interference RNA (siRNA) was used to knock-down TDAG51 (siTDAG51) in 3T3-L1 preadipocytes and nuclear and cytoplasmic protein extracts were collected on day 2 of differentiation. Immunoblotting was performed to confirm TDAG51 knock-down and examine PPAR γ expression in both the nuclear (N) and cytoplasmic (C) fractions. (- indicates transfection reagent control, siC indicates siControl transfected cells, and UT indicates untransfected cells)

SUPPLEMENTAL FIGURES

Supplemental Figure S1.

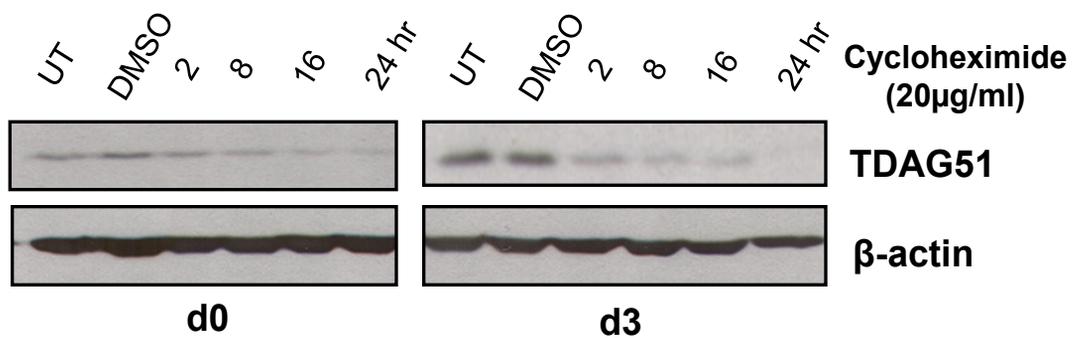


Figure S1. TDAG51 protein stability declines during differentiation. 3T3-L1 cells on day 0 (undifferentiated) or day 3 were treated with cycloheximide or DMSO control and protein extracts were isolated at the indicated time points (UT indicates untreated cells). Immunoblotting was performed to examine TDAG51 expression over time.

Supplemental Figure S2.

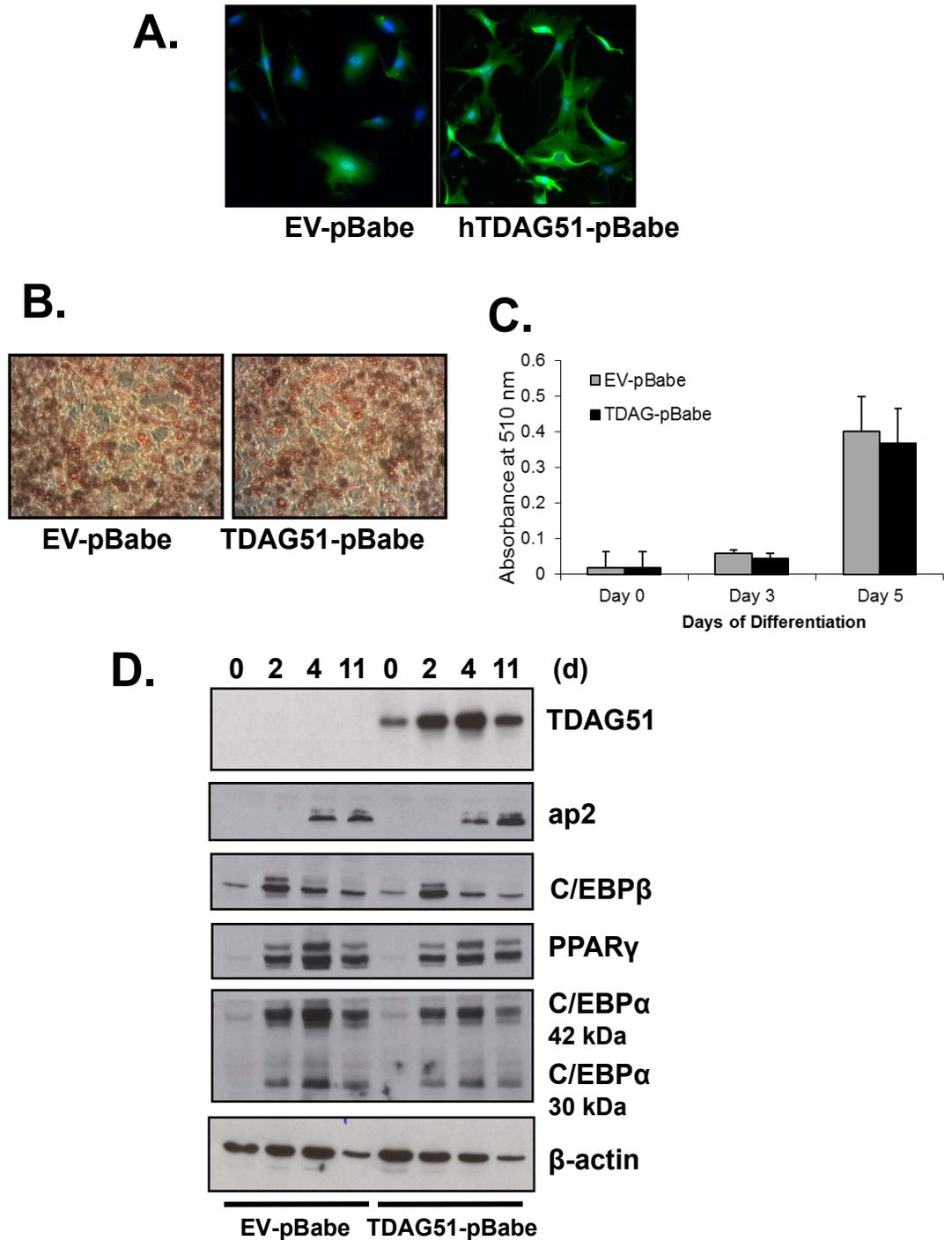


Figure S2. Stable over-expression of TDAG51 has no effect on adipogenesis. (A) 3T3-L1 cells were infected with human TDAG51-pBabe or empty vector (EV)-pBabe retroviral constructs. Stable cells were generated and immunofluorescence was performed to examine TDAG51 protein expression. (B) Oil red O staining was done performed on day 10 of differentiation to examine extent of lipid accumulation and representative pictures are shown. (C) Oil red O staining was done on day 0, 3 and 5 of differentiation and the staining in EV-pBabe or TDAG-pBabe expressing 3T3-L1 cells was quantified and graphically illustrated (n=6, NS). (D) Protein lysates were collected at various time points during differentiation to examine adipogenic markers through immunoblotting.

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CHAPTER 4

Loss of TDAG51 accelerates lipogenesis resulting in obesity, hepatic steatosis and insulin resistance

Preamble

The contents of this chapter were submitted for publication and are currently under review.

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Authors' Contribution

S. Basseri designed the experiments, performed Western blotting, ELISA, qRT-PCR analysis, cell culture studies, data analysis, and coordinated, planned, and assisted in all the animal studies and analyses. S. Lhotak, M.D. Fullerton, G.R. Steinberg and R.C. Austin contributed to designing of studies and assisted in implementation of experiments. M.D. Fullerton performed the hepatocyte isolation and performed the *de novo* lipogenesis experiment. R. Palanivel assisted with animal CT and PET data analysis and performed the metabolic cage data analysis. S. Basseri, S. Lhotak, M.D. Fullerton and R. Palanivel assisted with animal studies and tissue collection. S. Lhotak assisted with the *in vivo* diet study, maintenance of the mouse breeding colony, and performed histology and immunohistochemistry experiments, and adipocyte cell sizing. H. Jiang and K.N. Maclean provided plasma TBARS and ALT data, carbon tetrachloride liver Western blotting data, as well as liver 4-HNE stained images. S. Basseri compiled the data and

wrote the manuscript. G.R. Steinberg critically reviewed the manuscript. K.N. Maclean, G.R. Steinberg, and R.C. Austin provided intellectual input. All authors edited and reviewed the manuscript and approved the final submission. We acknowledge the help of Edward Lynn in obtaining body weight data from *TDAG51^{+/-}* mice.

Loss of TDAG51 accelerates lipogenesis resulting in obesity, hepatic steatosis and insulin resistance

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Abstract

Regulation of energy metabolism is critical for the prevention of obesity, diabetes and hepatic steatosis. Here, we report an important role for the pleckstrin-homology-related domain family member, T-cell death associated gene 51 (TDAG51), in the regulation of glucose and lipid metabolism. *TDAG51*^{-/-} mice on a standard chow diet display mature-onset insulin resistance, exhibit greater body and white adipose mass with larger adipocytes, and are predisposed to hepatic steatosis. Twenty weeks of 42% high fat diet feeding further exacerbated the metabolic symptoms in *TDAG51*^{-/-} mice and led to gross hepatic steatosis. *TDAG51*^{-/-} mice have increased hepatic triglyceride content through enhanced expression of SREBP-1 target genes. TDAG51 is inversely correlated with lipid accumulation in multiple forms of hepatic steatosis. Our data demonstrate that TDAG51 functions as a regulator of energy homeostasis at least in part through its control of adipogenesis and lipogenesis, suggesting that TDAG51 may be a therapeutic target for the treatment of obesity and insulin resistance.

Introduction

With a rise in the prevalence of obesity and diabetes worldwide, there is a pressing need to better understand the genes involved in the metabolic processes underlying these conditions. We recently observed that T-cell death associated gene 51 (TDAG51), a member of the pleckstrin homology (PH)-like domain family (1) is expressed in white adipose tissue (WAT) and its expression is modulated during adipogenesis. Furthermore, TDAG51 knock-out (*TDAG51^{-/-}*) preadipocytes exhibited increased adipogenic potential as compared to WT cells (Basseri S *et al.*, unpublished data). TDAG51 was first demonstrated to be induced upon T cell activation mediated apoptosis in culture (2), however *in vivo* absence of TDAG51 had no apparent effect on T cell apoptosis, number or function (3). In addition to its PH-like domain, TDAG51 possesses distinct C-terminal proline-glutamine and proline-histidine repeats (4), which may be involved in its pro-apoptotic function in some cell types (5). *In vitro* studies have demonstrated that TDAG51 is expressed in the cytoplasm and nucleus and inhibits protein synthesis possibly through regulation of ribosome biogenesis and/or translation (6). We have previously shown that TDAG51 is expressed in atherosclerotic lesions of mice with hyperhomocysteinemia (7), however the physiological role of TDAG51 in modulating atherogenesis is not clear.

While investigating the pro-apoptotic function of TDAG51 in the context of cardiovascular disease using TDAG51-deficient (*TDAG51^{-/-}*) mice, we noted marked fatty liver and an abundance of white adipose tissue (WAT) in various depots of *TDAG51^{-/-}* mice compared to wild-type (WT) controls. Despite the high expression of

TDAG51 in liver and WAT, among other tissues, the role of TDAG51 in lipid and carbohydrate metabolism has not been examined. Thus, in this study *TDAG51*^{-/-} mice were examined in terms of their adiposity and metabolic phenotype. *TDAG51*^{-/-} mice displayed an age-related increase in whole body adiposity and an overall metabolic profile resembling the metabolic syndrome. Furthermore, TDAG51 expression was inversely correlated with fatty liver in several mouse models of hepatic steatosis. Our findings suggest a previously unknown function for TDAG51 in promoting negative energy balance by regulating adipocyte and hepatic lipogenesis, and favouring energy expenditure.

Results

TDAG51^{-/-} mice develop increased adiposity due to reduced basal energy expenditure. To study the effect of loss of TDAG51 on weight regulation *in vivo*, 8-week old male *TDAG51^{-/-}* mice were fed a chow diet *ad libitum* for 20 weeks. Age and sex-matched WT C57BL/6 mice were used as controls. Body weight was not significantly different between WT and *TDAG51^{-/-}* mice at 8 weeks of age, however, at 28 weeks of age, a significant increase in body weight was observed in the *TDAG51^{-/-}* mice fed a standard chow diet (**Figure 1A**). Mice heterozygous for the TDAG51 allele (*TDAG51^{+/-}*) exhibited a greater rate of body weight gain but it did not reach statistical significance when compared to WT (**Supplemental Figure 1**). There was no significant difference in tissue weights (**Supplemental Figure 2A and B**), except in epididymal WAT (eWAT) where a significant increase was observed in *TDAG51^{-/-}* mice (**Figure 1B**). Computed tomography (CT) scans in 28 week-old mice showed that *TDAG51^{-/-}* mice have a 5-fold increase in total body WAT (**Figure 1C**), with fat deposition in both the visceral and subcutaneous depots (**Figure 1C top panel**). A significant increase in adiposity was also observed in the bone marrow of *TDAG51^{-/-}* mice (**Supplemental Figure 3A and B**). These findings demonstrate that absence of TDAG51 enhances adipogenic potential and WAT accumulation leading to increased body mass in *TDAG51^{-/-}* mice. Histological examination of the eWAT revealed that adipocytes in the *TDAG51^{-/-}* mice were significantly enlarged compared to WT controls (**Figure 1D**). Consistent with increased adiposity, *TDAG51^{-/-}* mice had increased levels of systemic oxidative stress (TBARS) (**Table 1**) reaching levels similar to diet-induced obese mice (**Supplemental Table 1**), as

well as increased blood leptin and reduced adiponectin concentrations as compared to WT mice (**Table 1**). Plasma inflammatory markers (TNF α , MCP1, IL12p70) tended to be higher in *TDAG51*^{-/-} mice (**Supplemental Figure 4A**). Furthermore, staining of eWAT sections to detect macrophages revealed an increase in the number of crown-like structures in comparison to WT mice (**Supplemental Figure 4B**). These findings are consistent with the notion that increased adiposity leads to adipose macrophage infiltration.

To determine whether adipogenesis, increased fat synthesis, or decreased lipolysis contribute to the increased fat deposition in *TDAG51*^{-/-} mice, WAT from overnight fasted mice were assessed for expression of proteins involved in adipogenesis and lipid metabolism. Pref-1, a marker of committed preadipocytes was 1.5-fold higher in *TDAG51*^{-/-} WAT, but expression of p-HSL and ATGL, enzymes involved in fasting-induced lipolysis were significantly reduced by 3- and 1.7-fold, respectively. Markers of *de novo* lipogenesis such as Scd-1 and FAS were not affected (**Supplemental Figure 5**).

Energy expenditure, locomotor activity, and food intake were measured using metabolic cages. Food consumption in WT and *TDAG51*^{-/-} mice was not significantly different (**Figure 1E**), suggesting that a net decrease in energy expenditure exists. *TDAG51*^{-/-} mice exhibited a decrease in metabolic rate as indicated by a significant reduction in oxygen consumption (VO₂) during both day and night hours (**Figure 1F**). Core body temperature was significantly lower in *TDAG51*^{-/-} mice during the night which corresponded with their lower VO₂ (**Figure 1G**). Furthermore, since locomotor activity

was not significantly different between genotypes (**Figure 1H**), changes in activity levels could not account for reductions in VO_2 .

Given the importance of brown adipose tissue (BAT) in energy expenditure, we assessed interscapular BAT morphology which showed that the brown adipocytes of *TDAG51^{-/-}* mice had larger lipid droplets as compared to WT brown adipocytes (**Figure 1D**). These findings suggest that loss of TDAG51 results in a decrease in whole body basal energy expenditure possibly due to increased lipid deposition in BAT converting brown adipocytes into inert white-like adipocytes. Biochemical analyses of BAT protein extracts did not suggest any differences in mitochondrial protein expression, such as UCP1, or complexes of the electron transport chain between the WT and *TDAG51^{-/-}* mice (**Supplemental Figure 6A**). Electron microscopy was performed on fixed BAT tissue to examine mitochondria shape and number which did not reveal any abnormalities (**Supplemental Figure 6B**). Furthermore, no differences were seen in the gross morphology of skeletal muscles or muscle mitochondrial activity (data not shown). Overall, these findings suggest that loss of TDAG51 results in a decrease in whole body energy expenditure which may be independent of mitochondrial activity or number.

TDAG51^{-/-} mice develop age-associated insulin resistance. Fasting plasma glucose and insulin levels were significantly elevated in the *TDAG51^{-/-}* mice at 28 weeks of age (**Figure 2A and B**). At 8 weeks of age when whole body, as well as liver and WAT weights were indistinguishable (**Figure 2A and Supplemental Figure 7A**), glucose and insulin tolerance tests revealed no differences between genotypes (**Supplemental Figure**

Figure 7B and C). However, at 28 weeks of age when *TDAG51*^{-/-} mice exhibited a significant increase in WAT and body weight, they were glucose intolerant (**Figure 2C**) and insulin resistant (**Figure 2D**) in comparison to WT mice. The efficiency of glucose uptake in various tissues was examined by the intravenous injection of fasted mice with 2-(¹⁸F) fluoro-2-deoxyglucose (2-FDG). Positron emission tomography (PET) scanning revealed a significant decrease in glucose uptake in skeletal muscles and WAT of *TDAG51*^{-/-} mice (**Figure 2E**). WAT from *TDAG51*^{-/-} mice also showed a decreased insulin response as indicated by reduced tyrosine (Y608) phosphorylation of the insulin receptor substrate (IRS)-1 and increased phosphorylation on serine (S612), which inhibits insulin receptor signalling (**Figure 2F**). Consistent with the presence of decreased glucose disposal and insulin resistance (IR), plasma free fatty acids (FFA) were significantly elevated in *TDAG51*^{-/-} mice (**Table 1**).

To investigate the cause of hyperinsulinemia and insulin resistance (IR) in *TDAG51*^{-/-} mice, pancreatic islets were histologically assessed using insulin and glucagon staining. No discernible differences in islet size or shape were observed between genotypes (**Figure 2G**). Overall, these findings demonstrate that *TDAG51*^{-/-} mice display an age-related impairment in insulin sensitivity as adiposity increases.

TDAG51 is negatively correlated with fatty liver and absence of TDAG51 leads to lipogenesis and hepatic steatosis. Given the high TDAG51 expression in WT livers, we sought to determine if TDAG51 expression is altered in fatty livers by examining three mouse models of non-alcoholic fatty liver disease (NAFLD). As shown in **Figure 3A**,

dietary, genetic and chemically-induced steatosis correlated with dramatically reduced TDAG51 protein expression. Interestingly, absence of TDAG51 increased hepatic lipid accumulation and steatosis in 28 week-old mice as evidenced by histological staining and biochemical measurement of liver TG (**Figure 3B and C**), a phenotype that was exacerbated in *TDAG51^{-/-}* mice fed a 42% high fat diet for 20 weeks compared to WT mice on the same diet (**Figure 3B**). For the sake of clarity and consistency with Figure 1 and 2, only standard chow diet-fed mice were further examined. *TDAG51^{-/-}* livers had increased staining for proteins covalently adducted with 4-HNE, a marker of lipid peroxidation (**Figure 3D**), and increased numbers of granulomas indicative of inflammation (**Figure 3E**), both of which are consistent with greater steatosis. However, there were no signs of hepatocellular injury as measured by plasma ALT (**Table 1**), and hepatic cholesterol content was not affected (data not shown). In agreement with increased hepatic TG in *TDAG51^{-/-}* livers, there was a marked increase in insulin-stimulated SREBP-1 activation, a master regulator of hepatic lipogenesis (8-10), as indicated by reduced expression of the precursor form of SREBP-1 (p125) and enhanced expression of the mature form (p68) (**Figure 3F**). Insulin-injected *TDAG51^{-/-}* livers also had reduced phosphorylation of Akt on serine 473, suggestive of hepatic IR (**Figure 3F**). Under basal fasting conditions, expression of mature SREBP-1 was not different between WT and *TDAG51^{-/-}* livers (**Supplemental Figure 8A**). However, expression of lipogenic enzymes and SREBP-1 target genes fatty acid synthase (FAS) and stearoyl-CoA desaturase (Scd)-1, were induced in livers from *TDAG51^{-/-}* mice under basal fasting conditions (**Figure 3G**).

To determine whether these results were secondary to the insulin resistant phenotype or intrinsic to the loss of TDAG51, primary hepatocytes from 6-week old WT and *TDAG51*^{-/-} mice, which display no obvious metabolic differences, were examined. *TDAG51*^{-/-} hepatocytes responded normally to insulin (**Supplemental Figure 8B**), but had enhanced lipogenesis (rate of newly synthesized fatty acid incorporation into TG) both basally and upon insulin stimulation (**Figure 3H**). Furthermore, primary hepatocytes from *TDAG51*^{-/-} mice expressed higher levels of the SREBP-1c target genes *glucokinase* (GK) and *acetyl CoA carboxylase* (ACC) (**Figure 3I**). No differences in fatty acid incorporation into diacylglycerol (DAG) or in palmitate oxidation were observed in cultured hepatocytes (data not shown). Expression of PPAR α , a transcription factor involved in fatty acid catabolism, was not affected by the absence of TDAG51 in liver tissue (**Supplemental Figure 8A**). Therefore, consistent with the observation in adipocytes, the deletion of TDAG51 promotes lipogenesis in hepatocytes.

Discussion

Dysregulation of carbohydrate and lipid metabolism pathways disrupts energy homeostasis leading to obesity, hepatic steatosis, and diabetes. Thus, identification of novel genes that regulate these pathways is essential. Here, we present evidence that TDAG51 plays a significant role in the regulation of energy metabolism. First, liver and WAT express abundant amounts of TDAG51 protein, and TDAG51 expression is negatively correlated with hepatic fat accumulation in three models of NAFLD. TDAG51 is highly expressed in preadipocytes and is absent in mature adipocytes in both mice and humans. Second, deletion of TDAG51 accelerates fatty acid synthesis and increases triglyceride storage in primary hepatocytes, and enhances lipid accumulation and adipogenesis in preadipocytes. Third, absence of TDAG51 leads to an age-associated increase in whole body adiposity and hepatic TG accumulation, and decreases insulin sensitivity in standard chow diet-fed mice. Notably, the phenotype observed in *TDAG51*^{-/-} mice resembles several aspects of high-fat diet fed mice, including increased adiposity and adipocyte size, hyperleptinemia, hypoadiponectinemia, insulin resistance, fatty liver, systemic oxidative stress, and increased lipid accumulation in BAT.

The age-dependent increase in adiposity, TG accumulation and IR in *TDAG51*^{-/-} mice are suggestive of a chronic and cumulative effect. Insulin signaling results in anabolic lipid synthesis, through activation of hepatic SREBP-1 leading to *de novo* lipogenesis in the liver (8, 9, 11). Thus, liver-specific Akt2 null mice, which exhibit defective insulin signaling, are protected from hepatic steatosis (12, 13). On the other

hand, insulin hypersensitivity due to absence of insulin signaling negative feedback loops, also leads to decreased lipogenesis and resistance to weight gain in several mouse models. For instance, deletion of protein tyrosine phosphatase 1B, a negative regulator of insulin signaling, resulted in mice that were insulin sensitive and resistant to weight gain (14). Similarly, mice deficient in S6 kinase, an effector of mTOR and inhibitor of IRS1, were protected from age- and diet- induced obesity due to loss of an insulin signaling negative feedback loop (15). Given these findings, it is unlikely that TDAG51 acts as a negative regulator of insulin signaling since we observe age-dependent IR and weight gain in *TDAG51^{-/-}* mice.

Excessive accumulation of TG can culminate in expansion of WAT, ectopic lipid accumulation and eventually IR in combination with chronic inflammation and oxidative stress (16, 17). The phenotype observed in older *TDAG51^{-/-}* mice is consistent with these metabolic disturbances. Moreover, when cells become insulin resistant, not all pathways are blunted as lipogenic pathways remain active, a concept known as selective IR (11). The reduced Akt phosphorylation in combination with increased SREBP-1 activation seen in livers from insulin-injected *TDAG51^{-/-}* mice is consistent with selective IR. Absence of TDAG51 in mice culminated in increased TG deposition in the liver. Given that SREBP-1 is a master regulator of hepatic *de novo* lipogenesis (8-10) and an increase in SREBP-1 target gene expression was observed in the livers of *TDAG51^{-/-}* mice, absence of TDAG51 likely leads to hepatic steatosis through increased SREBP-1 activity. Consistent with this result, primary *TDAG51^{-/-}* hepatocytes exhibited a significant enhancement in the rate of lipogenesis, which correlates with the strong association

between TDAG51 reduction and fatty livers. Taken together, these data suggest a role for TDAG51 in the negative regulation of SREBP-mediated lipogenesis and prevention of fatty liver.

The functions of proteins are often dictated by functional domains they possess. TDAG51 has several important domains including a PH-like domain, which contains a putative nuclear export sequence (NES) and nuclear localization sequence (NLS), as well as proline-glutamine and proline-histidine repeats near the carboxy terminus. Of these domains, the PH-like domain of TDAG51 appears to be a likely candidate of functional significance in the context of cell signaling and metabolism. PH domains play an important role in recruiting target proteins to cell membranes through their binding interactions with phospholipids such as phosphatidylinositol (3,4,5)-triphosphate (PIP3) and phosphatidylinositol (4,5)-bisphosphate (PIP2) (18, 19). Interestingly, there is a high stringency PIP3-binding site in the PH-like domain of TDAG51 as predicted by ScanSite (20). The PH-like domain of TDAG51 bears a high degree of homology to that of PI3K enhancer (PIKE) based on Protein Homology/Analog Y Recognition Engine (Phyre2) a software used for recognition of protein structure/folding (21). PIKE has recently been implicated to play a role in adipogenesis, WAT regulation, and insulin sensitivity and *PIKE*^{-/-} mice are protected from diet-induced weight gain and insulin resistance (22). These findings raise the possibility that TDAG51 may be competing with PIKE under physiological conditions. We are currently exploring whether the PH-like domain of TDAG51 is indeed mediating its effects on the regulation of lipogenesis and whole body energy metabolism and whether any potential interactions with PIKE may exist.

Collectively, the results of our study illustrate that TDAG51 plays an important role as a regulator of carbohydrate and lipid metabolism which may contribute to the phenotypes observed with the metabolic syndrome. Our findings suggest the possibility that enhancing hepatic TDAG51 expression in steatotic livers may reverse hepatic steatosis, but further investigation is required to determine if TDAG51 has potential as a therapeutic target for the treatment of fatty liver disease. Understanding this newly identified player in lipid metabolism and adipogenesis, as well as its precise regulation under normal and pathophysiological conditions will be crucial for development of effective treatments against diabetes and obesity.

Methods

Mice. *TDAG51*^{-/-} mice were back-crossed onto the C57BL/6 background for at least 9 generations. C57BL/6 or *TDAG51*^{-/-} mice were maintained under 12-hour light/12-hour dark cycles with unlimited access to standard rodent chow and water. At 8 weeks of age, male wild-type or *TDAG51*^{-/-} mice (n=8) were either continued on the regular chow diet or placed on a high fat diet (42% of kcal from fat, Harlan Teklad). At 28 weeks of age, mice were fasted for 14 hours prior to sacrifice by cervical dislocation under anaesthesia. Blood was drawn from the heart, and tissues were immediately collected for histology or biochemical assessment. Only male mice were used in this study. Unless stated otherwise, only chow diet fed mice were examined. All protocols for animal use and euthanasia were approved by the McMaster University Animal Research Ethics Board.

Genotyping. Following digestion of ear or tail tissue, DNA was isolated and used for polymerase chain reaction (PCR) to confirm mouse genotypes. The presence of wildtype and/or a disrupted *TDAG51* allele was determined using the following primers: WT1 (5'-CCG CAG CAC CTC CAA CTC TGC CTG-3'), WT2 (5'-GTC TTC AAA TAC AAT GAA AGA GTC G-3'), *TDAG51* KO1 (5'-AAA TGG AAG TAG CAC GTC CCA GTC-3'), *TDAG51* KO2 (5'-AGA GCA GCC GAT TGT CTG TTG TGC CCA GTC-3'). The settings used for the PCR were one denaturation cycle at 94°C for 2 minutes followed by 30 cycles of: 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 1 minute. A 1 kb band was obtained for wild-type *TDAG51* PCR amplified gene products and a 400 base pair band for the disrupted *TDAG51* gene.

mRNA Quantification by Real-Time PCR. Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's protocol. Using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) total RNA was reverse transcribed to obtain cDNA. qPCR was performed using Fast SYBR Green PCR master mix (Applied Biosystems) in the AB7900 HT Fast Real Time PCR System. Primer sequences were as follows: *I8S*, forward, AGT CCC TGC CCT TTG TAC ACA, reverse, CGA TCC GAG GGC CTC ACT A, *Acc1*, forward, CTT CCT GAC AAA CGA GTC TGG, reverse, CTG CCG AAA CAT CTC TGG GA, *Gck*, forward, TCT CTG ACT TCC TGG ACA AGC, reverse, CGT GTC ATT CAC CAT TGC C.

Computed tomography and Positron Emission Tomography. WT and *TDAG51^{-/-}* mice (n=5) were fasted overnight and given an intravenous injection of 2-(¹⁸F)fluoro-2-deoxyglucose (2-FDG). The mice were kept under heat lamps for 30 mins, then were anaesthetized using isoflurane and scanned on a small animal PET scanner for visualization of glucose uptake. While still under anaesthesia the mice were placed in a SPECT/CT scanner for determination of body fat content which was determined as described (23).

Metabolic Studies. For glucose tolerance tests (GTT), mice were fasted overnight and given an i.p. injection of glucose (1.5 mg/g body weight). For insulin tolerance tests (ITT), mice were fasted for 6 hours and i.p. injected with 0.75 U/kg of insulin. For *in vivo* insulin signaling experiments, tissues were collected 15 minutes after an i.p. injection of insulin (0.75 U/kg) in fasted mice as previously done (24). Metabolic studies using

indirect calorimetry were performed using the Columbus Laboratory Animal Monitoring System as described previously (25). Core body temperature was determined using subcutaneous minimeter probes.

Biochemical Analysis. Plasma adiponectin (R&D), leptin (Linco), and insulin (Chemicon) concentrations were measured using mouse ELISA kits. Plasma glucose, triglycerides (TG), cholesterol esters, and free fatty acids were measured using colorimetric assays from Wako Diagnostics. Plasma TBARS assay was carried out using an OXI-TEK TBARS Kit (Alexis Biochemicals) and plasma ALT levels were analyzed using a kit (ThermoDMA).

Cytometric Inflammatory Bead Array. Plasma cytokines were measured using a bead cytometric array (BD) as per manufacturer's protocol.

Hepatic TG measurement. Approximately equivalent weights of frozen liver tissue from mice were homogenized in PBS and triglycerides were extracted using the Folch extraction method as previously described (26). TG concentrations were quantified using the Wako TG kit.

Primary cell culture. Primary hepatocytes isolated from the liver were plated on collagen coated 6-well plates (BD) and allowed to attach for 2-3 h in Williams' medium E (Invitrogen), after which media was replaced with Williams' medium E containing 10% FBS. Isolated hepatocytes were incubated overnight at 37 °C, and the experiments were performed the following day.

Hepatocyte ³H-Acetate and ¹⁴C-palmitate labelling. Palmitate oxidation was determined as described previously (27). Sodium palmitate (0.5 mM) and ¹⁴C-palmitate (Perkin Elmer) (0.5 µCi/ml) was added to Williams' medium E containing 2% BSA and allowed to conjugate for 2-3 h. Primary hepatocytes were then incubated with palmitate media for 4 h, in the presence or absence of 10 nM insulin. Media was removed from the cells and radiolabeled CO₂ was liberated by adding equal volume of 1 M acetic acid. The ¹⁴CO₂ was trapped by benzethonium hydroxide (1 M) in a 0.5 ml microcentrifuge tube over 90 min. The microcentrifuge tube containing trapped ¹⁴CO₂ was placed in a scintillation vial and counted. For determination of *de novo* lipogenesis, hepatocytes were incubated with media containing sodium acetate (5 mM) and ³H-acetate (Perkin Elmer) (1 µCi/ml) for 1 or 4 h in the presence or absence of 10 nM insulin. Cells were washed twice with PBS and lipids were extracted by the method of Bligh and Dyer (28). Neutral lipids were quantified as previously described (29). Lipid bands were visualized and scraped into counting vials for measuring radioactivity.

Immunoblotting. Cells and tissues were homogenized in lysis buffer and protein lysates were subjected to immunoblotting as previously described (30). Antibodies were used to detect the following proteins: C/EBP α , PPAR α , SREBP-1 (H160 for detection of precursor p125, K10 for detection of mature p68), TDAG51 (Santa Cruz Biotechnology), Akt, pS473-Akt, aP2, C/EBP β , FAS, GAPDH, HSL, pS612-IRS1, perilipin, PPAR γ , Scd-1 (Cell Signaling), total IRS1, pY608-IRS1 (Invitrogen), and β -actin (Sigma).

Double immunofluorescence. Paraffin sections of mouse pancreas were deparaffinized and blocked with 5% normal goat serum (Vector Laboratories). Subsequently, sections were incubated with the mouse anti-insulin antibody cocktail (NeoMarkers MS-1379) diluted 1/200, followed by the rabbit anti-glucagon antibody (CLSG36938, Cedarlane) diluted 1/5, for 1 hour each. A mix of goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594 (Molecular Probes, Eugene, OR), diluted 1/200, was applied for 30 minutes. Slides were mounted with Permafluor (Fisher Scientific, Nepean, ON) and viewed in a Zeiss Axioplan fluorescence microscope.

Immunohistochemistry. Excised tissues were fixed in buffered formalin overnight. Paraffin sections of tissues were cut at 4 μm thickness. After deparaffinizing and blocking the endogenous peroxidase with 0.5% hydrogen peroxide in methanol, the antigen retrieval in citric buffer, pH 6.0, was performed using a rice steamer. For macrophage staining, sections of eWAT were blocked with 5% normal rabbit serum for 10 minutes, and incubated in the rat anti-Mac-3 (PharMingen) antibody diluted 1/500 in the blocking serum for 1 hour followed by biotinylated rabbit anti-rat antibody (mouse adsorbed, Vector Laboratories) diluted 1/50 in 0.05 M Tris buffer, pH 7.5, for 30 minutes. For perilipin staining, sections were blocked with 5% normal donkey serum, followed by incubation in guinea pig anti-perilipin antibody (Progen Biotechnik) diluted 1/200 in the blocking serum for 1 hour, and biotinylated donkey anti-guinea pig (Jackson ImmunoResearch) diluted 1/500 in Tris buffer, for 30 minutes. Sections were incubated in streptavidin-peroxidase (Zymed Laboratories) diluted 1/20 in Tris buffer for 10 minutes followed by development in the peroxidase substrate Nova Red (Vector

Laboratories) according to manufacturer's instructions. After counterstaining with hematoxylin, sections were dehydrated and mounted in Permount (Fisher Scientific). For detection of 4-HNE, paraffin-sectioned slides were stained with an antibody (at a 1/7500 dilution) which recognizes covalent adducts between 4-HNE and protein. The secondary antibody was biotinylated anti-rabbit from an ABC vector kit (Vector Laboratories).

Adipocyte sizing. A macro was developed in the ImagePro software to measure adipocyte size. To highlight adipocytes, paraffin sections of epididymal fat pads were immunostained with perilipin as described above. An RGB image was taken at 20x magnification and the green channel was extracted. The image was segmented into a binary image where adipocyte rims (cytoplasm) were white. Dark objects within the rims (adipocytes) were counted and the area of each measured. Manual editing allowed for closing of incompletely outlined cells and discarding objects that were not adipocytes. Objects smaller than $200 \mu\text{m}^2$ were excluded. Five microscope fields were measured per animal, 7 animals per group. Approximately a total of 1000 adipocytes were measured in each mouse.

Statistical Analysis. Results are provided as mean \pm SEM unless otherwise indicated. Statistical analyses were performed using unpaired 2-tailed Student's *t* test and significance was defined as $P < 0.05$.

Supplemental Results

Supplemental Information includes eight figures and one table.

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We thank Dr. Y. Choi (University of Pennsylvania) for kindly providing us with *TDAG51^{+/-}* mice. We acknowledge the help of K. Karimi with the BD cytometric bead array. We are grateful to C. Saab and R. Rhem for their technical assistance with the animal imaging. R.C.A. is supported by grants from the Heart and Stroke Foundation of Ontario (NA-6024) and the Canadian Institutes of Health Research (CIHR) (MOP-74477; MOP-67116). Financial support from St. Joseph's Healthcare Hamilton is also acknowledged. K.N.M. gratefully acknowledges support from NIDDK grant RO1DK074487-01. S.B. is supported by a Heart and Stroke Foundation of Canada Doctoral Award. M.D.F. is a CIHR Banting Postdoctoral Fellow and G.R.S. is a Canadian Research Chair in Metabolism and Obesity.

FIGURES

Figure 1.

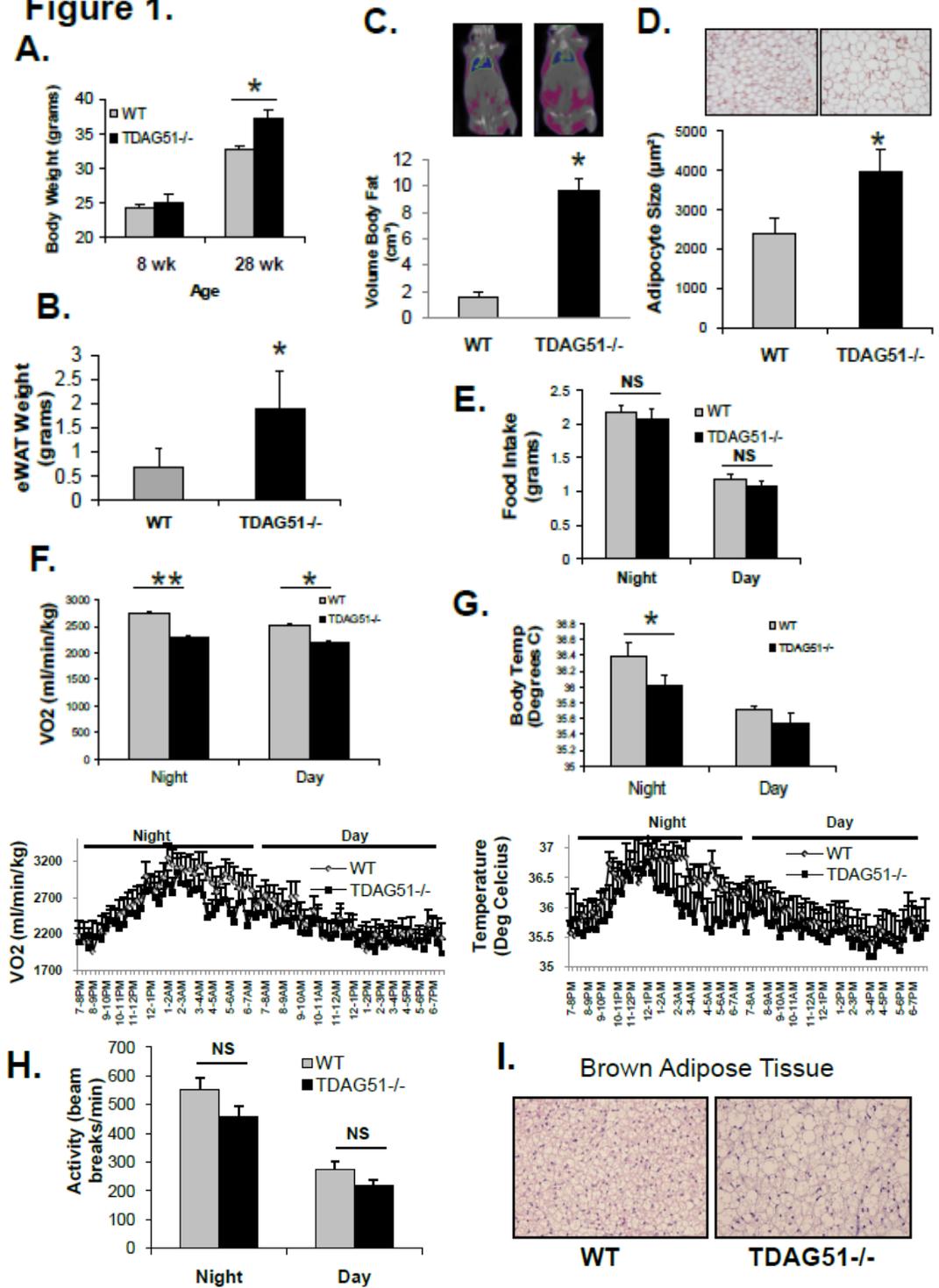
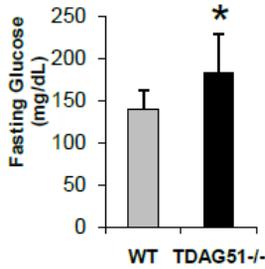


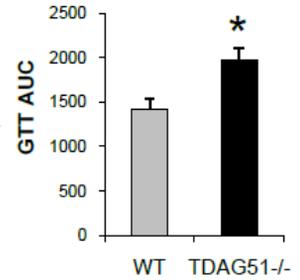
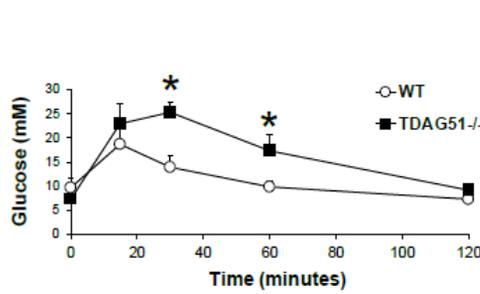
Figure 1. *TDAG51*^{-/-} mice develop increased adiposity due to reduced basal energy expenditure. (A) Body weights at 8 and 28 weeks of age in male WT or *TDAG51*^{-/-} mice. Data are expressed as means \pm SD, n=7-8, **P* < 0.05. (B) WAT weights at 28 weeks of age (n=7, **P* < 0.05). (C) CT scans in 28 week old WT and *TDAG51*^{-/-} mice. Representative images are shown where the pink areas depict the quantified WAT areas (n=5, **P* < 0.001). (D) Perilipin-stained paraffin-embedded eWAT are shown in the top panel (original size, x20). Adipocyte size in μm^2 (n=7, **P* < 0.05). (E) Food intake at 28 weeks of age (n=7, NS). (F) VO_2 (oxygen uptake) in 20 week-old mice (n=7, **P* < 0.05, ***P* < 0.01). (G) Core body temperature in 20 week-old mice (n=7, **P* < 0.05). (H) Average locomotor activity in 20 week old-mice during day and night hours. (n=7, NS). (I) H&E stained BAT from 28 week-old mice (original magnification, x40).

Figure 2.

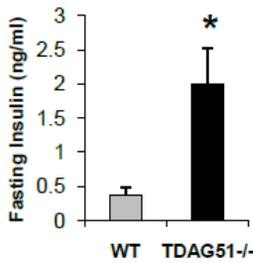
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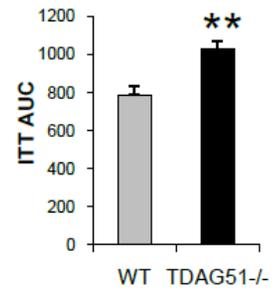
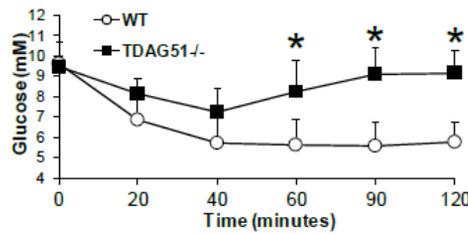
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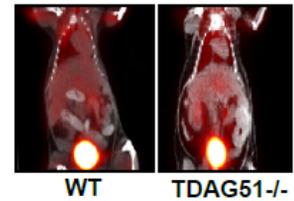
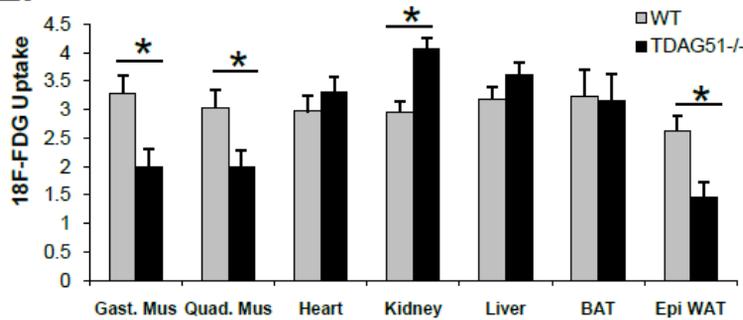
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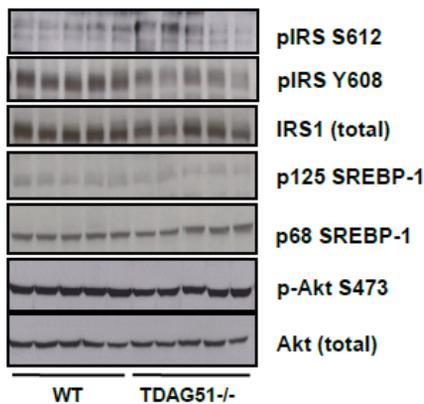
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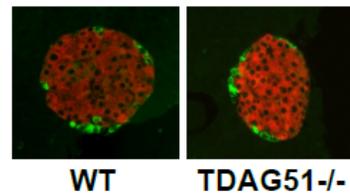


Figure 2. *TDAG51*^{-/-} mice develop age-associated insulin resistance. (A) Fasting glucose concentrations in 28 week old male mice. (B) Fasting insulin concentrations. (C) GTT after an overnight fast and the area under the curve (AUC) in the right panel (n=4, **P* < 0.05). (D) ITT after a 6-hr fast and the AUC (right panel) (n=6-7, **P* < 0.05). (E) Intravenous injection of 2-FDG followed by a PET scan. Quantification of 2-FDG in various tissues (n=5, **P* < 0.05). PET scans from representative mice are shown in the right panel. (F) Mice were injected with insulin following a 6-hour fast and epididymal WAT was collected 15 minutes later for Western blotting examining insulin receptor signaling (n=5 mice). (G) Pancreatic islets, where red staining represents insulin and green depicts glucagon (original size, x40).

Figure 3.

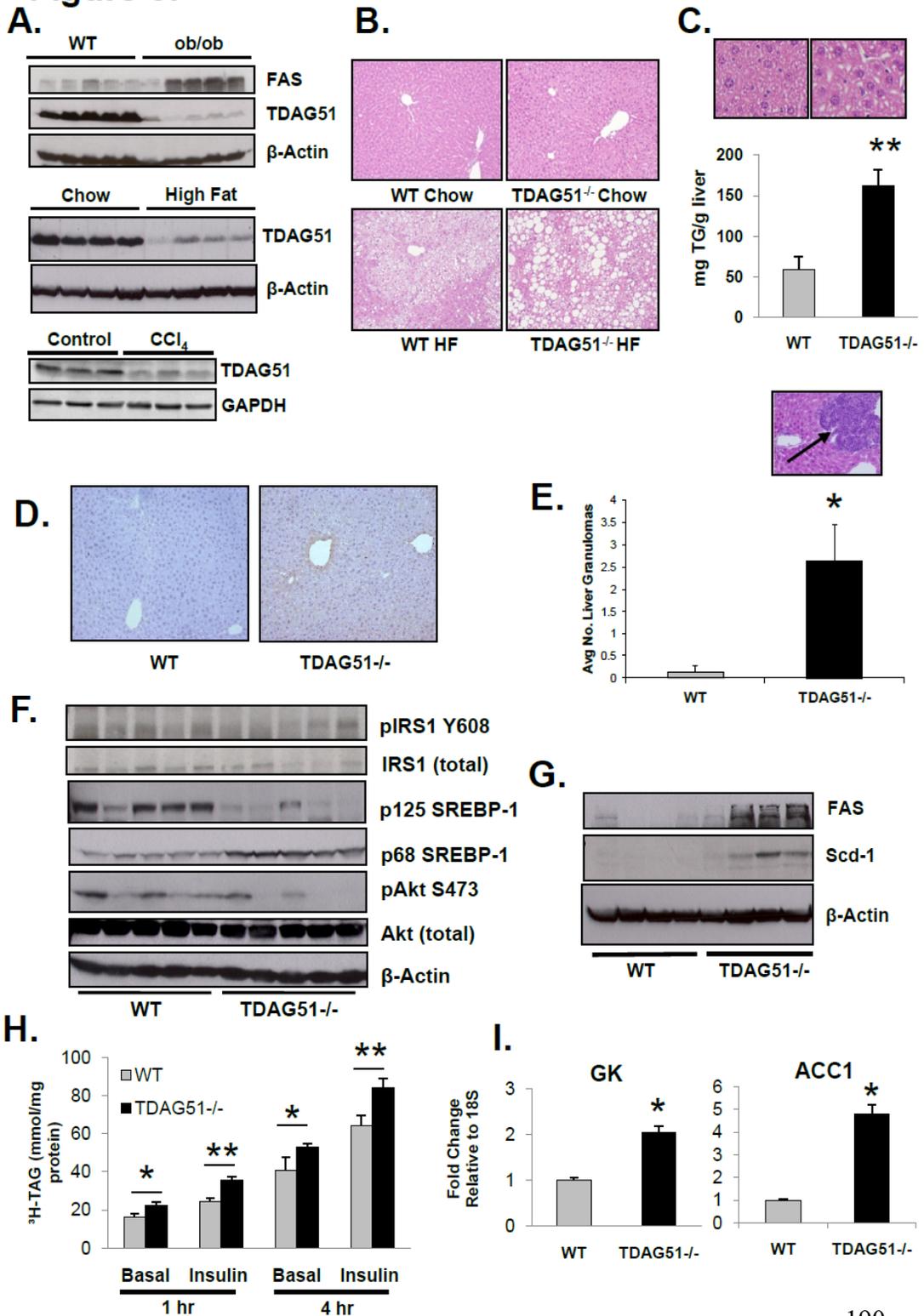


Figure 3. TDAG51 is negatively correlated with fatty liver and absence of TDAG51 leads to lipogenesis and hepatic steatosis. (A) TDAG51 expression in liver protein lysates from age-matched WT and ob/ob mice (top, n=5), chow diet-fed and high fat diet-fed (middle, n=4), as well as control and carbon tetrachloride (CCl₄)-treated mice (bottom, n=3). (B) H&E stained livers from 28 week-old WT and *TDAG51*^{-/-} mice. Mice were either fed a standard chow diet (top images) or a 42% high fat (HF) diet for 20 weeks (bottom images). (original magnification, x10) (C) Liver TG content was quantified. Data represent means ± SD, n=5, ***P* < 0.01. Pictures depict representative higher magnification images of H&E stained livers (original magnification, x60). (D) Detection of 4-hydroxynonenal (4-HNE) in liver sections (original magnification, x10). (E) Quantification of inflammatory cell infiltrations (granulomas) in H&E stained livers (n=5, **P* < 0.01). (F) Immunoblotting for insulin receptor signaling and SREBP-1 activation in liver lysates from mice 15 minutes post-insulin injection (n=5). (G) Immunoblotting of liver lysates from fasted mice (n=4). (H) Basal and insulin stimulated ³H-acetate incorporation into TG in hepatocytes from 6-week old mice. Data represent means ± SD, n=3, **P* < 0.05, ***P* < 0.01. (I) qRT-PCR analysis examining mRNA expression of *GK* and *ACCI* in cultured primary hepatocytes (values are means ± SD, n=3, **P* < 0.01).

Table 1. Plasma metabolic parameters in fasted 28 week-old mice.

Plasma	ALT (U/L)	TBARS (μ M)	Chol-E (mg/dl)	FFA (mM)	TG (mg/dl)	AdipoQ (μ g/ml)	Leptin (ng/ml)
WT	33.3 \pm 3.5	0.15 \pm 0.1	92.3 \pm 5.7	0.65 \pm 0.03	48.5 \pm 5.4	11.9 \pm 0.8	1.9 \pm 0.5
<i>TDAG51</i>^{-/-}	32.3 \pm 3.8	3.75 \pm 1.3*	94.8 \pm 2.6	0.81 \pm 0.06*	49.0 \pm 2.2	6.3 \pm 0.6**	14.7 \pm 2.6**

ALT (n=6-7); TBARS (n=6-7); Cholesterol-E (n=7-8); FFA (n=7-8); Triglycerides (n=7-8); Adiponectin (n=7-8); Leptin (n=7-8).

Data represent means \pm SEM (* P < 0.05, ** P < 0.01 between WT and *TDAG51*^{-/-}).

SUPPLEMENTAL FIGURES

Supplemental Figure S1.

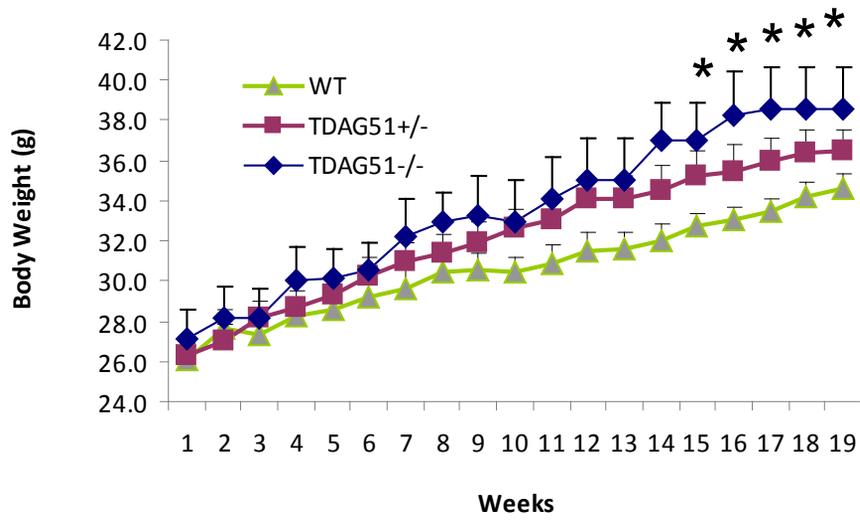


Figure S1. Weight gain comparison between WT, *TDAG51*^{+/-} and *TDAG51*^{-/-} mice.

Body weight gain in male mice on a standard chow diet, was monitored weekly for 19 weeks starting at 10 weeks of age (n=6-8, means \pm SEM, **P* < 0.05 when comparing WT to *TDAG51*^{-/-} mice).

Supplemental Figure S2.

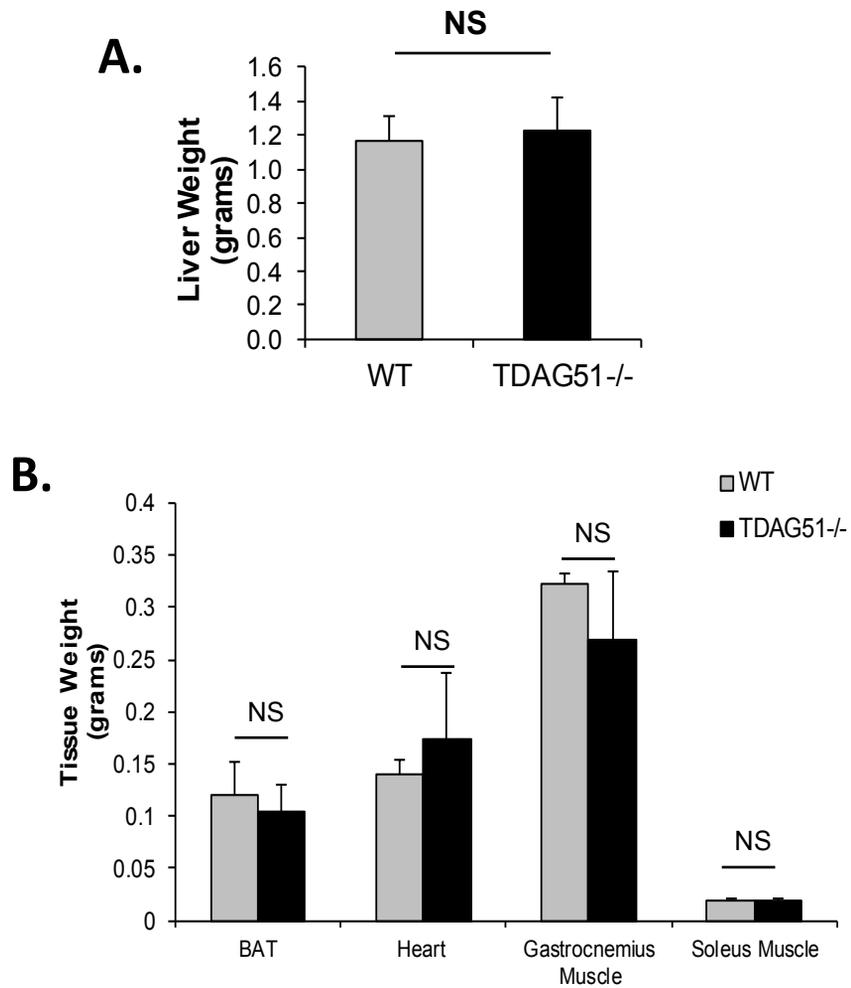


Figure S2. Tissue weight comparisons in WT and *TDAG51*^{-/-} mice. (A) Livers from 28 week-old mice were weighed (n=7, no significant difference). (B) Interscapular BAT, heart, gastrocnemius and soleus muscles were excised and weighed (n=7, no significant difference).

Supplemental Figure S3.

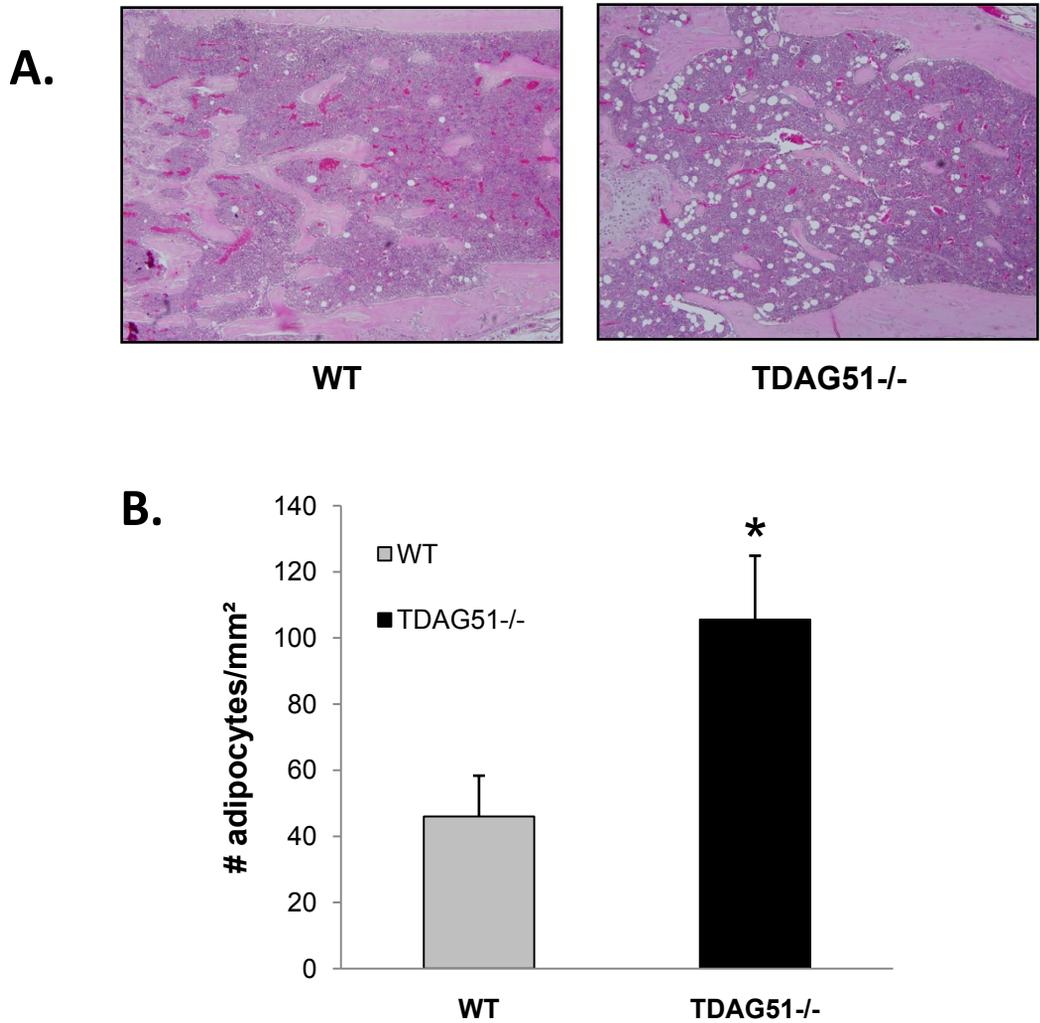


Figure S3. Infiltration of adipocytes in bone marrow. (A) De-calcified, paraffin-embedded distal femurs from 8-14 week-old female WT and *TDAG51*^{-/-} mice were sectioned and stained with H&E. Representative images are shown (original magnification, 10x). (B) Area of bone marrow was measured and the number of adipocytes were counted within that area (n=4-5, means \pm SEM, **P* < 0.05 when comparing WT to *TDAG51*^{-/-} mice).

Supplemental Figure S4.

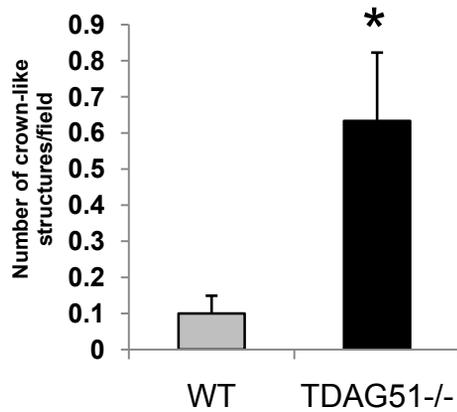
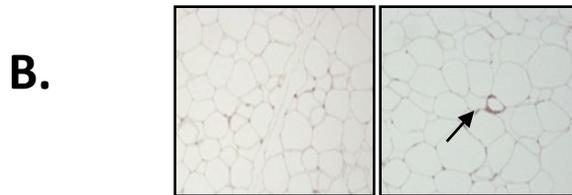
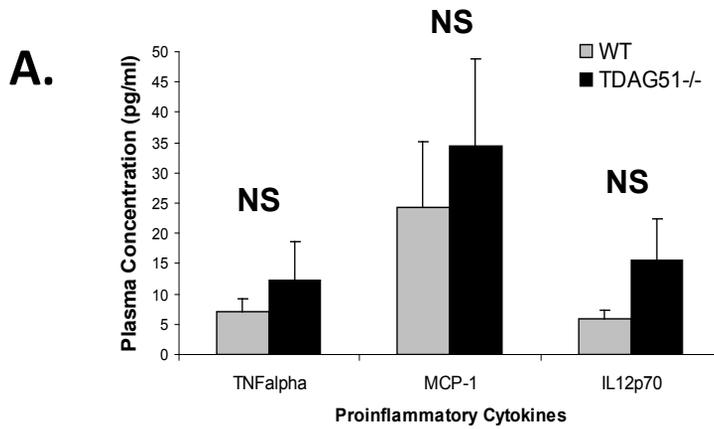


Figure S4. Plasma inflammatory cytokines and WAT macrophage infiltration. (A) Bead cytometric array using plasma to measure TNF α , MCP1, and IL12p70 in 28 week-old mice (n=5, no significant difference). (B) eWAT sections were stained with an antibody against Mac-3 to detect macrophage infiltration and crown-like structures. The number of crown-like structures were quantified and represented per field (original magnification, x20) (n=6, * $P < 0.05$).

Supplemental Figure S5.

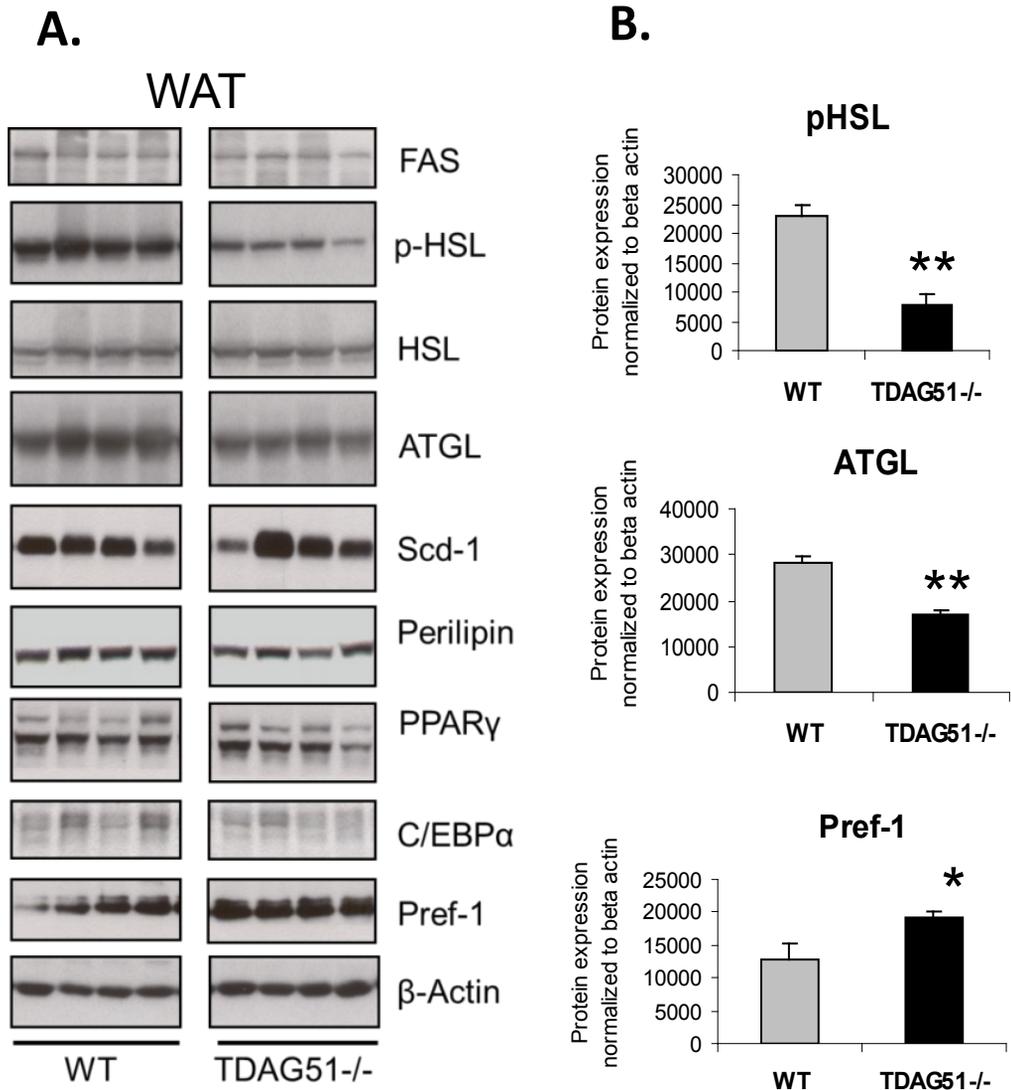
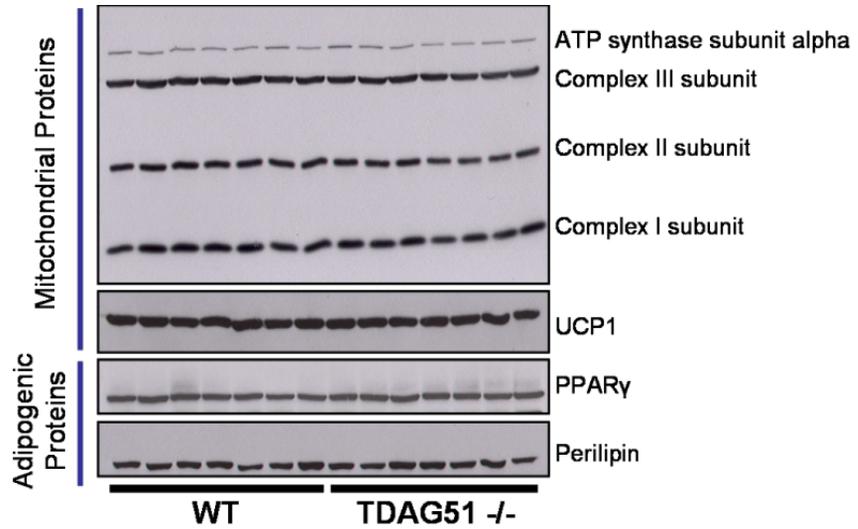


Figure S5. *TDAG51*^{-/-} mice have a significant reduction in fasting-induced lipolysis.

(A) Western blotting was performed using equal concentrations of whole WAT protein lysates from WT and *TDAG51*^{-/-} mice at 28 weeks of age that were euthanized after an overnight fast. (B) Band intensities were quantified and normalized to beta actin for the corresponding mouse sample. Graphs represent means \pm SEM, n=6, **P* < 0.05, ***P* < 0.01.

Supplemental Figure S6.

A.



B.

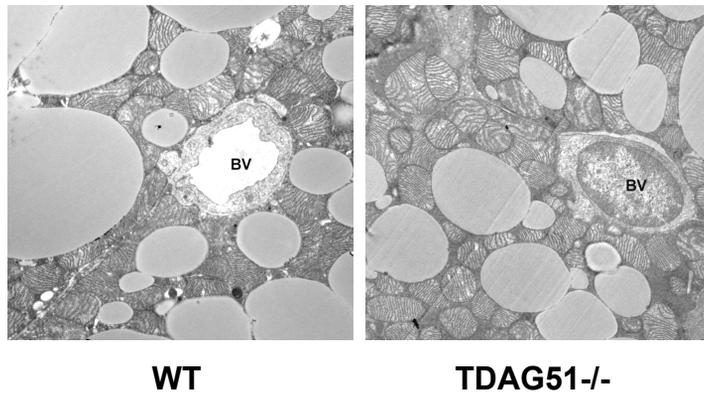


Figure S6. No differences in BAT mitochondrial components or morphology were observed. (A) Whole BAT protein lysates from WT and *TDAG51*^{-/-} mice were immunoblotted against components of the oxidative phosphorylation machinery and electron transport chain, as well as UCP1, PPAR γ , or perilipin (n=7). (B) Electron microscopy (EM) was performed to examine BAT mitochondrial morphology in 28 week-old mice. Images are representative of each genotype.

Supplemental Figure S7.

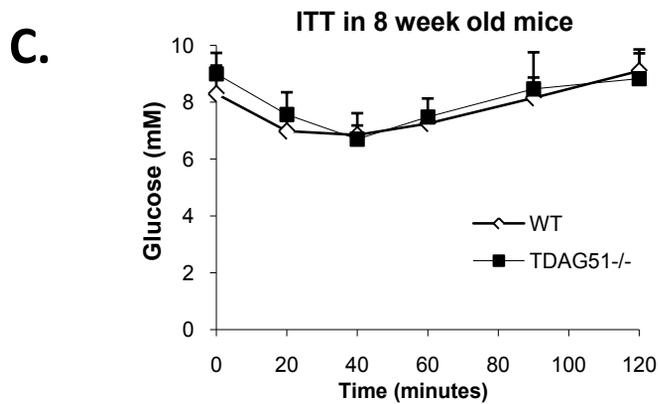
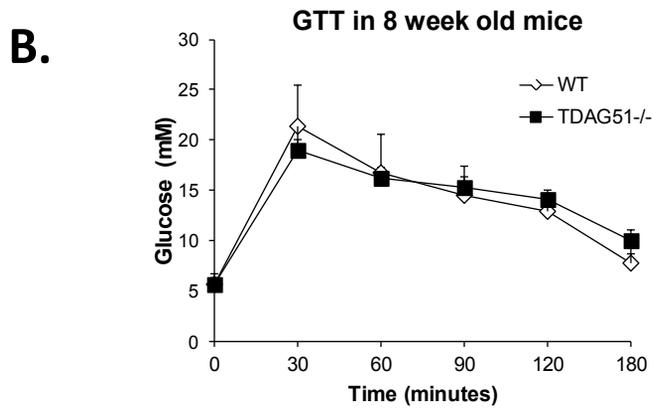
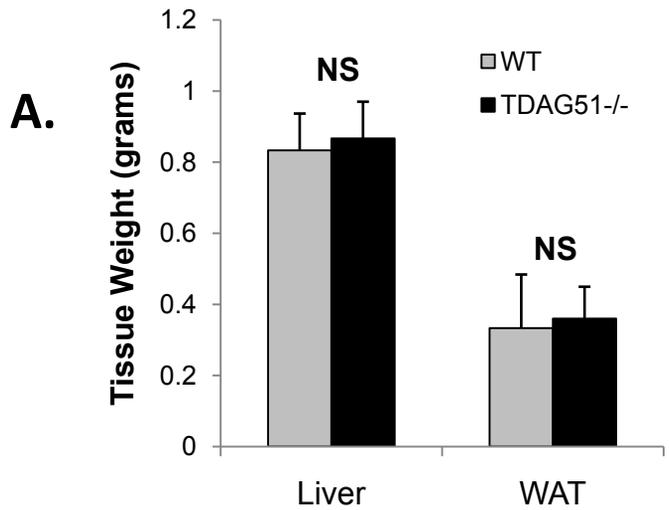
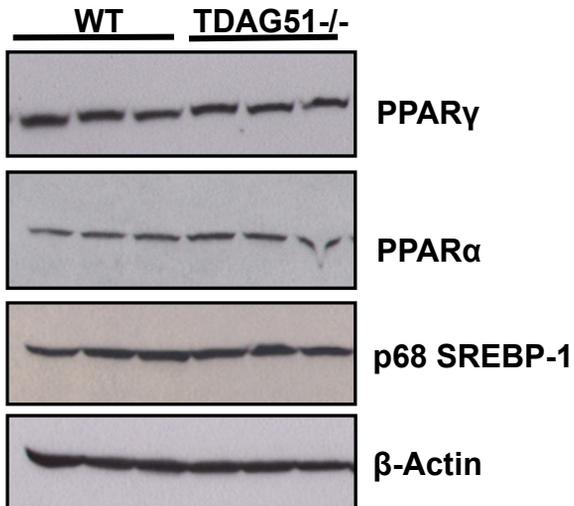


Figure S7. At 8 weeks of age there is no phenotypic difference between WT and *TDAG51*^{-/-} mice. (A) WAT and Liver weights (n=6-7, NS). (B-C) Glucose and insulin tolerance tests were performed after an overnight and 6 hour fast respectively (n=6-7, NS).

Supplemental Figure S8.

A. Livers – Chow Diet



B.

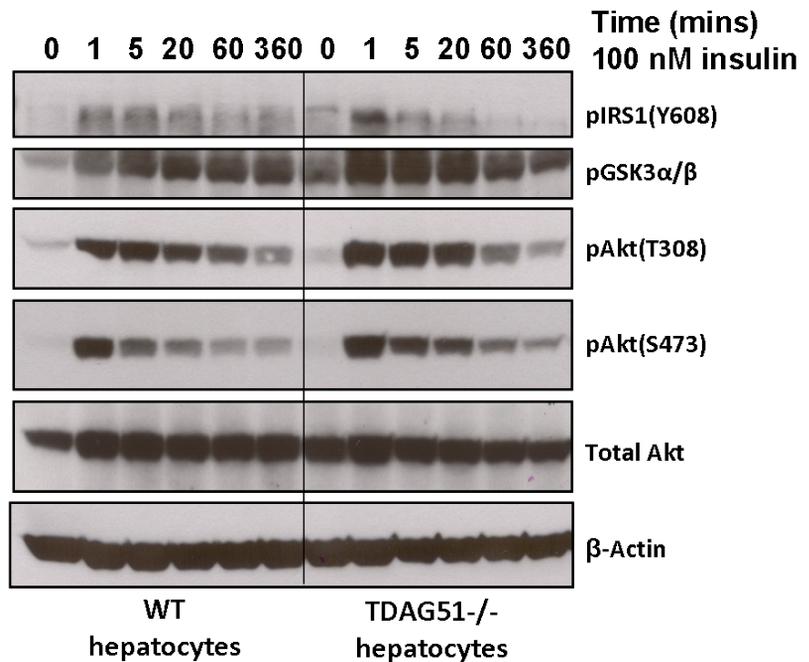


Figure S8. Hepatic PPAR α , PPAR γ and mature SREBP-1 expression in chow-fed mice, and insulin response in primary hepatocytes. (A) Comparison of PPAR γ , PPAR α and mature (active) SREBP-1 levels (p68) in protein lysates from livers of fasted mice at 28 weeks of age (chow diet mice). (B) Primary hepatocytes from 5-6 week old mice were serum-starved and then stimulated with 100 nM insulin. Western blotting was done to examine insulin receptor signaling *in vitro*.

Supplemental Table S1. Comparison of plasma metabolic parameters in fasted 28 week-old mice fed a high fat diet for 20 weeks.

Plasma	WT	<i>TDAG51</i> ^{-/-}
Glucose (mg/dl)	187.8±12.1	263.4±15.2**
Insulin (ng/ml)	6.16±2.02	7.61±1.33
ALT (U/L)	111.8±30.6	140.9±48.3
TBARS (µM)	3.54±1.19	4.63±1.69
Chol-E (mg/dl)	109.7±9.81	157.5±17.3*
FFA (mM)	0.68±0.07	0.79±0.08
TG (mg/dl)	47.3±2.4	54.1±3.1
Adiponectin (µg/ml)	2.52±0.9	1.41±0.5
Leptin (ng/ml)	22.7±2.3	38.1±3.3*

Glucose (n=6-8); Insulin (n=4-5); ALT (n=4-7); TBARS (n=4-5); Cholesterol-E (n=6-8); FFA (n=6-8); Triglycerides (n=6-8); Adiponectin (n=4); Leptin (n=5-6).

Data represent means ± SEM (**P* < 0.05, ** *P* < 0.01 between WT and *TDAG51*^{-/-}).

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CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

Preamble

Parts of this chapter have been adapted from a review article and a book chapter which were written by the author of this thesis. The details of these publications are listed here.

Publication 1 (Review Article)

Sana Basseri and Richard C. Austin. (2012) Endoplasmic Reticulum stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13. doi: 10.1155/2012/841362

Authors' Contribution: S. Basseri conducted the literature review, compiled and summarized the research in the field, and wrote the review article in its entirety. R.C. Austin edited the review article, provided intellectual input and acts as the corresponding author.

Publication 2 (Book Chapter)

Sana Basseri and Richard C. Austin. (2012) Chapter 10: Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In "ER stress in Health and Disease" to be published by *Springer* in July 2012.

Authors' Contribution: S. Basseri conducted the literature review, compiled and summarized the research in the field, and wrote the book chapter in its entirety. R.C. Austin edited the chapter, provided intellectual input and acts as the corresponding author.

5.0. DISCUSSION

When I began this thesis project, a number of diseases had already been linked to ER function and/or activation of ER stress/UPR pathways, such as neurodegenerative diseases (Dimcheff et al., 2003; Hetz et al., 2003; Kitao et al., 2004; Koo et al., 1999; Mattson et al., 2001; Nishitoh et al., 2002; Paschen, 2003; Paschen and Doutheil, 1999), IR and diabetes (Harding et al., 2001; Nakatani et al., 2005; Oyadomari et al., 2002a; Oyadomari et al., 2002b; Ozawa et al., 2005), and atherosclerosis (Devries-Seimon et al., 2005; Dickhout et al., 2005; Zhou et al., 2005). The idea for this project was sparked by a publication in *Science* (Ozcan et al., 2004), showing for the first time the presence of ER stress in liver and adipose tissues of obese mice, suggesting that ER stress may be the link between obesity and insulin action and T2D. Furthermore, in a subsequent publication it was demonstrated that alleviating ER stress in obese mice with small molecular weight chemical chaperones improved glucose tolerance and insulin sensitivity, and resolved fatty liver disease (Ozcan et al., 2006). However, at the time there was little mechanistic insight into ER stress/UPR signalling in the context of lipogenesis and whole body energy metabolism, and virtually nothing was known about the role of UPR pathways in adipogenesis and adipocyte function. Given that pre- and mature adipocytes are central to the control and regulation of adiposity and whole body energy homeostasis, this thesis project aimed to first address the question of whether ER stress/UPR activation plays a role in the formation and differentiation of adipocytes from their precursor fibroblast-like preadipocytes. Activation of ER stress/UPR pathways during cellular differentiation had previously been implicated in plasma B cells (Gass et

al., 2002; Iwakoshi et al., 2003) and in monocyte to macrophage differentiation which we published in subsequent years (Dickhout et al., 2011). Through this thesis project (**chapter 2**), I have demonstrated that activation of the UPR occurs during adipocyte differentiation and illustrated that treatment of preadipocytes with 4-PBA, an inhibitor of ER stress/UPR activation, inhibited lipid accumulation and adipogenesis (Basseri et al., 2009).

Since the publication of the work contained in **chapter 2** of this thesis, subsequent studies have extended my findings and provided evidence for the importance of all three arms of the UPR: PERK, IRE1 and ATF6 in adipocyte differentiation (Bobrovnikova-Marjon et al., 2008; Lowe et al., 2011; Sha et al., 2009). In **chapter 3** and **4**, TDAG51, a previously identified ER stress/UPR regulated gene, was illustrated to be an important player in adipogenesis, lipid metabolism and whole body energy homeostasis. Links between UPR signalling components and regulation of lipogenic and adipogenic transcriptional activation are beginning to emerge, uncovering a novel role for UPR target genes in the control of lipid metabolism, which will be discussed in detail in section **5.1**. Still, the precise molecular mechanisms are not entirely understood. Future *in vivo* studies are required to better understand the impact of ER stress and activation of UPR pathways on development and progression of metabolic diseases.

Attempts at examining the role of UPR genes specifically during adipogenesis *in vivo* have been difficult due to the lack of ideal mouse models for studying adipogenesis (Cristancho and Lazar, 2011). Development of WAT-specific knockout mice is

dependent on expression of adipocyte-specific genes such as PPAR γ , aP2 (FABP4), or adiponectin (Cristancho and Lazar, 2011), however, these genes generally begin to be expressed in the mature and differentiated adipocyte, creating a major limitation to the study of adipogenesis from multipotent stem cells. Therefore, to study the role of UPR genes in early adipogenic events and adipocyte commitment stages *in vivo*, development of models with targeted expression or deletion of genes specific to multipotent cells and committed preadipocytes will be necessary.

Nonetheless, in recent years significant advances in the field of energy homeostasis, lipogenesis, and obesity have been made using mice with conditional deletions in the liver or germline deletions where a specific UPR gene has been targeted. Given that fatty liver disease and hepatic steatosis are often associated with the metabolic syndrome, IR and obesity, such mouse models serve as useful tools to better understand the molecular pathways linking UPR signalling to lipid metabolism. The contribution of this thesis to the field and new insights gained from these recent animal studies are discussed below.

5.1. The link between ER stress and Adipogenesis, Lipid Metabolism and Obesity¹

5.1.1. Adipogenesis and UPR activation

We and others hypothesized that UPR activation during adipogenesis would allow the cells to cope with the increased ER stress which may be present due to the elevated protein load and lipid biosynthesis (Basseri et al., 2009; Gregor and Hotamisligil, 2007). Adipogenesis, which results in dramatic morphological changes and formation of a highly secretory endocrine cell, must rely on the ability of the ER to adapt to the increased protein secretory load. For example, differentiation of B cells into antibody producing plasma cells, as well as monocyte to macrophage differentiation, have been associated with activation of UPR pathways (Dickhout et al., 2011; Gass et al., 2002; Iwakoshi et al., 2003). The lipid droplets in adipocytes are surrounded by a number of proteins including the ER proteins GRP78 and calnexin, suggesting the importance of the ER in adipocyte TG-rich lipid droplet formation and lipid trafficking (Prattes et al., 2000). **Chapter 2** of this thesis demonstrated that differentiation of the 3T3-L1 preadipocytes depends upon activation of UPR pathways and increased expression of ER chaperones such as GRP78 (Basseri et al., 2009). Furthermore, treatment of 3T3-L1 cells with the ER stress inhibitor 4-PBA, blocked adipogenesis and lipid accumulation (Basseri

¹ Contents of section 5.1 have been adapted from the following book chapter with kind permission of Springer Science and Business Media: **Basseri S** and Austin RC. (2012) Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In “*ER stress in Health and Disease*”. In press at *Springer*.

et al., 2009). In support of my findings, the IRE1 α -XBP1 pathway was demonstrated to be required for adipogenesis by directly regulating C/EBP α expression (Sha et al., 2009). The PERK arm of the UPR was also shown to be required for adipogenesis by altering lipogenic gene expression and SREBP-1 activity (Bobrovnikova-Marjon et al., 2008). Most recently, ATF6 α deficiency in C3H10T1/2 cells, an adipogenic cell line model, resulted in reduced adipogenic gene expression and blunted lipid accumulation (Lowe et al., 2011). Taken together, these studies indicate that all three arms of the UPR are required for adipogenesis to occur, but clearly further investigation into the molecular mechanisms is required.

While induction of UPR pathways and the requirement of UPR target genes such as XBP1 during adipogenesis represent a physiological form of UPR activation, acute and persistent ER stress is a potent repressor of adipogenesis. This was illustrated by treatment of 3T3-L1 cells with ER stress-inducing agents such as Tm or thapsigargin, which led to a reduction in *Glut4* and *Cebpa* mRNA expression (Miller et al., 2007). Furthermore, other compounds and media conditions that induce ER stress have also been shown to inhibit adipocyte differentiation, leading to decreased lipid accumulation and reduced expression of adipogenic markers (Batchvarova et al., 1995; Shimada et al., 2007). Such acute and unresolved ER stress leads to induction of CHOP, which by heterodimerizing with the C/EBP family of transcription factors, represses their activity. Indeed, ectopic expression of CHOP in 3T3-L1 cells inhibited adipogenesis (Batchvarova et al., 1995). **Figure 5.1.** illustrates the distinct roles ER stress and UPR activation play in adipogenesis and adipose tissue function. These results are consistent with the recent

notion that UPR activation can lead to differential outcomes on metabolic regulation depending on the nature of the ER stress conditions, acute/unresolved versus adaptive ER stress, as in the hepatic phenotypes observed in livers with defective UPR. The findings of studies examining the role of various UPR genes in adipogenesis are summarized in **Table 5.1.**

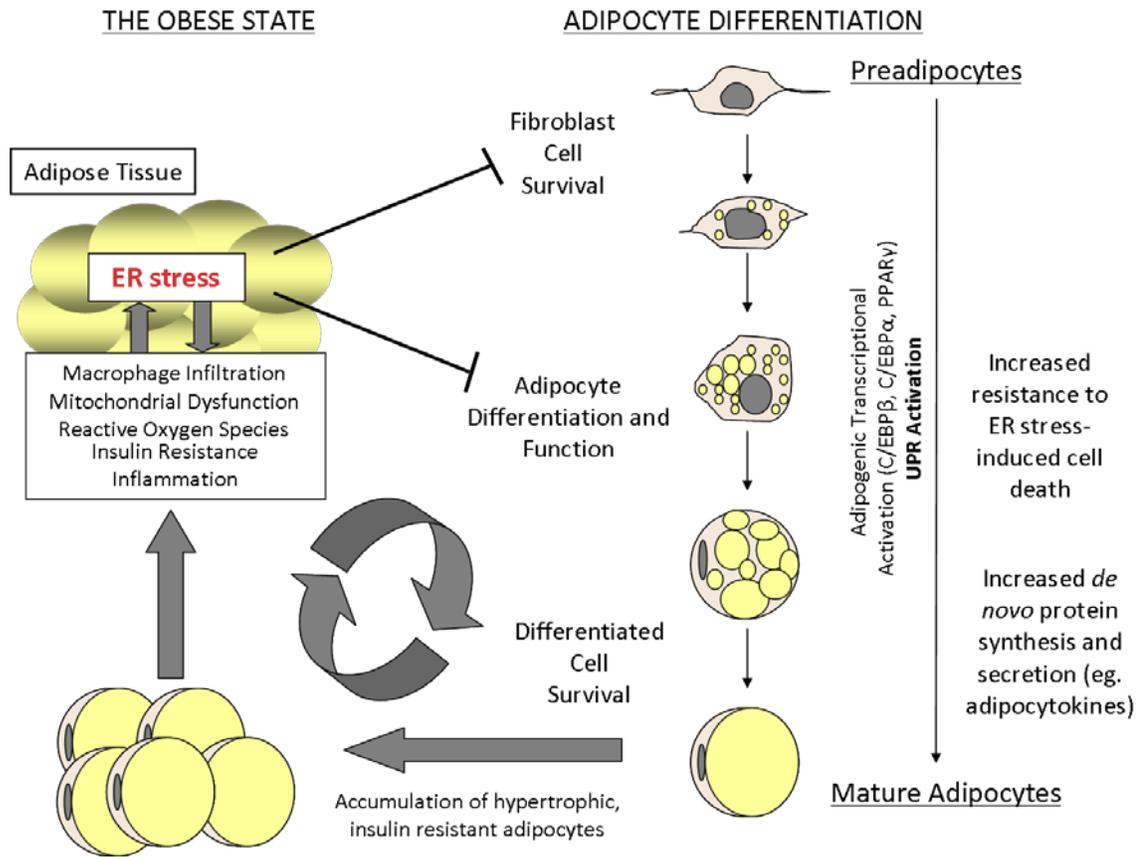


Figure 5.1. Proposed model depicting the differential effects of ER stress and UPR activation on adipogenesis and adipose tissue function.

Figure 5.1. Proposed model depicting the differential effects of ER stress and UPR activation on adipogenesis and adipose tissue function. Based on the findings in this thesis and the current knowledge in the field the following model was proposed. As preadipocytes begin to differentiate into adipocytes, they activate the UPR pathways. UPR activation allows the differentiating adipocyte to cope with increased *de novo* protein synthesis, as well as the increased demand for protein folding and secretion. Up-regulation of ER chaperones such as GRP78, PDI, and calreticulin in differentiating adipocytes enhances their survival and primes the cells to resist ER stress caused by hypoxia, inflammation and oxidative stress, which are often present in obese insulin resistant adipose tissue. As the adipose tissue expands, more adipocytes become hypertrophic and insulin resistant, promoting the recruitment of macrophages, thereby further exacerbating the ER stress conditions within the tissue. Since these conditions are highly unfavourable and can lead to ER stress-mediated cell death, preadipocytes which have low basal levels of ER chaperones and foldases may undergo apoptosis. Acute ER stress is also a potent inhibitor of adipocyte differentiation by up-regulating CHOP, which can interfere with the transcriptional control of adipogenesis, and by increasing JNK-1 activity leading to disruption in insulin receptor signalling. Hence, there is a vicious cycle whereby ER stress leads to more hypertrophied adipocytes as adipogenesis is blunted and sensitive preadipocytes are more likely to undergo ER stress-mediated apoptosis. Thus, with an increase in large insulin-resistant, dysfunctional adipocytes, unfavourable conditions increase within the adipose tissue providing an environment in which hypertrophic adipocytes are more likely to thrive.

5.1.2. Role of ER stress/UPR pathways in hepatic lipogenesis

In addition to adipogenesis, recent studies have shown a strong link between ER stress/UPR activation and lipogenesis. Many of these studies have examined this link by focusing on the liver, which have profoundly enhanced our understanding of the role of UPR pathways in metabolism. The liver is rich in ER as it is one of the most important secretory organs in the body (Cnop et al., 2011). Changes in nutritional status impact the liver UPR, as fasted and re-fed mice exhibit signs of physiological ER stress and enhanced p-eIF2 α expression, which resolves within hours, and this response is more pronounced with high fat diet (HFD) feeding (Cnop et al., 2011). A recent study indicates that IRE1 α signalling is rhythmically activated by a circadian clock, which is closely linked to the circadian regulation of hepatic lipid metabolism (Cretenet et al., 2010). However, chronic unresolved ER stress has been linked to development of hepatic steatosis and IR. A study performed in our laboratory demonstrated that homocysteine-induced ER stress results in dysregulation of TG and cholesterol biosynthetic pathways and culminates in hepatic steatosis in hyperhomocysteinemic mice (Werstuck et al., 2001). Similarly, injection of lean mice with Tm, increased ER stress and led to development of hepatic steatosis (Yang et al., 2007). Genetically obese *ob/ob* mice and HFD-fed obese mice, which exhibit hepatic steatosis and IR, also show signs of ER stress (Ozcan et al., 2004; Yang et al., 2007). Based on these findings, we sought to determine if alleviating ER stress through treatment of HFD-fed mice with 4-PBA would affect weight gain, adipose tissue mass and liver lipid content (**chapter 2**). Treatment of mice with 4-PBA led to a significant decrease in GRP78 expression in WAT and decreased

adipocyte size and WAT weight. However, a reduction in liver ER stress markers or hepatic TG content was not observed in our study (**chapter 2**).

Hepatic over-expression of GRP78, a chaperone that is protective against ER stress (Morris et al., 1997), reduced expression of ER stress markers, and suppressed SREBP-1c cleavage and expression of its target genes in obese *ob/ob* mice (Kammoun et al., 2009). Furthermore, livers from GRP78 over-expressing mice exhibited diminished hepatic steatosis and enhanced insulin sensitivity (Kammoun et al., 2009). Liver-specific GRP78 knockout (LGKO) mice on the other hand, are prone to hepatic fat accumulation and IR, and are sensitized to a spectrum of liver diseases induced by HFD-feeding, alcohol, drugs, and toxins (Ji et al., 2011). Loss of this master regulator of the UPR, directly disturbs ER homeostasis (increased p-IRE, p-PERK, p-eIF2 α , and *Xbp1s* in the LGKO liver), and thereby sensitizes the liver to acute and chronic liver diseases (Ji et al., 2011). Furthermore, Ji *et al.* showed that 4-PBA treatment was effective at alleviating these disorders in LGKO mice.

Since beginning this PhD thesis project, numerous studies have provided evidence linking ER stress sensors PERK, IRE1 α , and ATF6 α , and their downstream signalling to hepatic lipid metabolism. In fact, the majority of studies examining these links were published after I began my studies and thus will be discussed in great detail here as they build upon my research findings. As summarized in **Table 5.1.**, knock-out and transgenic mouse models have been utilized to examine specific arms of the UPR, thereby gaining a better understanding of their role in regulating hepatic lipogenesis.

Table 5.1. The role of UPR genes in obesity, hepatic steatosis, lipogenesis and adipogenesis: Summary of phenotypes in mice and cells with genetic alterations of essential UPR genes

UPR Gene	Alteration	Phenotype	Ref.
Hspa5 (GRP78)	Heterozygous mutation	Resistance to HFD-induced obesity, diabetes, and hepatic steatosis, due to enhanced adaptive UPR response	(Ye et al., 2010)
	Conditional deletion in liver	Susceptible to liver disease including fatty liver	(Ji et al., 2011)
	Over-expression in liver	Protected from hepatic steatosis due to decreased SREBP-1c activity	(Kammoun et al., 2009)
Ern1 (IRE1α)	Germline deletion embryo fibroblasts	Protected from Tm-induced inhibition of insulin receptor signalling	(Ozcan et al., 2004)
	Conditional deletion in liver	Enhanced lipogenesis and hepatic steatosis upon Tm treatment	(Rutkowski et al., 2008; Zhang et al., 2011)
Ern2 (IRE1β)	Germline deletion	Hyperlipidemia when fed a high fat and high cholesterol diet	(Iqbal et al., 2008)
Xbp1	Germline deletion embryo fibroblasts	Attenuated adipogenic differentiation due to reduced C/EBP α expression	(Basseri et al., 2009; Sha et al., 2009)
	Conditional deletion in liver	Hypolipidemia, and reduced hepatic lipogenesis, in particular with high carbohydrate diet feeding	(Lee et al., 2008)
	Heterozygous mutation	Increased body weight on a HFD, impaired insulin signalling, and hyperinsulinemia	(Ozcan et al., 2004)
Eif2k3 (PERK)	Conditional deletion in mammary gland	Reduced mammary gland triglycerides due to reduced SREBP-1c activity	(Bobrovnikova-Marjon et al., 2008)
	Germline deletion embryo fibroblasts	Attenuated adipogenesis and lipid accumulation	(Bobrovnikova-Marjon et al., 2008)

Eif2a (eIF2α)	Heterozygous S51A mutation	Obese and diabetic with HFD feeding, increased hepatic steatosis, severe glucose intolerance due to reduced pancreatic insulin secretion	(Scheuner et al., 2005)
	Homozygous S51A mutation in liver	Development of fatty liver upon Tm challenge	(Rutkowski et al., 2008)
Ppp1r15a (GADD34)	Conditional enforced expression in liver	Protected from HFD-induced glucose intolerance and hepatic steatosis due to decreased eIF2 α signalling	(Oyadomari et al., 2008)
Atf4	Germline deletion	Decreased adiposity and lipogenic gene expression, enhanced fatty acid oxidation	(Wang et al., 2010; Yoshizawa et al., 2009)
Ddit3 (CHOP)	Over-expression in preadipocytes	Inhibited adipogenesis by interfering with C/EBPs	(Batchvarova et al., 1995)
	Germline deletion	Increased adiposity only in females, hepatic lipid deposition, decreased WAT and liver inflammation, but no alterations in glucose or insulin sensitivity	(Ariyama et al., 2007; Maris et al., 2012)
Atf6 (ATF6α)	Germline deletion	Normal, but increased liver steatosis upon Tm challenge due to liver dysfunction	(Rutkowski et al., 2008; Yamamoto et al., 2010a)
	Knock-down in C3H10T1/2 cells	Impaired adipogenic gene expression and reduced lipid accumulation	(Lowe et al., 2011)

Table modified from reference (Lee and Glimcher, 2009)

(Table has been reproduced from the following book chapter with a minor modification with kind permission of Springer Science and Business Media: Basseri S and Austin RC. (2012) Chapter 10: Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In "ER stress in Health and Disease". In press at Springer.)

5.1.2.1. *PERK pathway*

Lipid-induced ER stress in liver cells leads to enhanced degradation of apoB100 through proteasomal and non-proteasomal pathways (Ota et al., 2008). Similarly, glucosamine-induced ER stress increased apoB100 degradation and attenuated its biosynthesis through a PERK-eIF2 α mechanism (Qiu et al., 2009). The importance of p-eIF2 α signalling in the early stages of hepatic steatosis was illustrated when three days of HFD-feeding induced phosphorylation of eIF2 α , hepatic lipid accumulation, and IR, without differences in mRNA expression of ER stress markers *Xbp1s*, *Grp78* or *Chop* (Birkenfeld et al., 2011).

Several studies have specifically examined the PERK arm of the UPR and its function in lipogenesis and hepatic lipid metabolism. PERK deficiency in mammary epithelial cells and adipocytes attenuated lipogenesis at least in part through suppression of SREBP-1 activity (Bobrovnikova-Marjon et al., 2008). This study showed that translation of *Insig1* decreased in a PERK-eIF2 α -dependent manner, allowing for SREBP-1 activation and *de novo* lipogenesis (Bobrovnikova-Marjon et al., 2008). To elucidate the role of the PERK-eIF2 α pathway in hepatic steatosis and glucose homeostasis, transgenic mice with enforced expression of an active C-terminal fragment of GADD34 under the control of the albumin promoter (Alb:GC Tg mice), were generated (Oyadomari et al., 2008). GADD34 in association with protein phosphatase 1 (PP1) targets p-eIF2 α for dephosphorylation, and as a result, these mice exhibited attenuated eIF2 α signalling in the liver. Alb:GC Tg mice were more insulin sensitive and had fasting hypoglycemia and reduced glycogen stores. Under the dietary stress of HFD-

feeding, the transgenic mice had sustained insulin sensitivity and reduced hepatosteatosis with decreased PPAR γ and its target gene expression. Furthermore, the expression of lipogenic transcription factors C/EBP β and C/EBP α , was also reduced in the livers of transgenic mice (Oyadomari et al., 2008). Further analysis of Alb:GC Tg mice indicated that the fasting hypoglycemia was due to reduced hepatic gluconeogenesis and that there was cross-talk between liver and peripheral glucose metabolism, rendering tissues such as muscle and adipose tissue insulin resistant (Birkenfeld et al., 2011).

In contrast to the hepatic phenotype of Alb:GC Tg mice, a liver-specific homozygous S51A mutation in eIF2 α , which prevents eIF2 α from becoming phosphorylated, promoted the development of fatty liver after a Tm challenge in mice harbouring this mutation (Rutkowski et al., 2008). These contrasting outcomes in response to different forms of ER stress, HFD feeding (adaptive) and Tm (acute and persistent), suggest that the source and severity of ER stress play an important role in the regulation of hepatic steatosis. Together, these studies suggest that translational control through eIF2 α phosphorylation serves as an important regulatory mechanism in hepatic lipogenesis, which can lead to differential outcomes on gene expression under physiological and severe stress conditions. Furthermore, hepatic ER stress pathways can influence other tissues and thereby control peripheral insulin sensitivity and glucose uptake.

5.1.2.2. *IRE1 pathway*

Since *Irel1* α -null mice are not viable, hepatocyte-specific *Irel1* α -null (*Irel1* $\alpha^{\text{Hep}^{\text{fl}}/-}$) mice were generated to examine the role of IRE1 α in the liver. Treatment of these mice with the ER stress inducer Tm, led to expression of pro-apoptotic transcription factors ATF4, CHOP and ATF3, and increased the number of apoptotic cells in comparison to control mice (Zhang et al., 2011). *Irel1* $\alpha^{\text{Hep}^{\text{fl}}/-}$ mice exhibited greater TG accumulation and hepatic steatosis due to increased expression of lipogenic transcription factors (PPAR γ 1, ChREBP, C/EBP β , C/EBP δ , and LXR α), other lipogenic regulators (ADRP and FITs), and genes encoding lipogenic enzymes (SCD1, DGAT1, DGAT2 and ACC1) in the liver, particularly in Tm-injected mice. Plasma lipids on the other hand, were decreased in Tm-injected *Irel1* $\alpha^{\text{Hep}^{\text{fl}}/-}$ mice as compared to control mice, due to suppression of apoB secretion. The results indicate that IRE1 α is required for protection against steatosis under ER stress conditions, and suggest that absence of hepatic IRE1 α causes defective adaptation to ER stress (Zhang et al., 2011).

XBP1s, the downstream target of IRE1 α , was dramatically induced in livers of mice fed a high carbohydrate diet, but expression of ER stress markers GRP78 or CHOP was not affected (Lee et al., 2008). As expected, high carbohydrate diet feeding markedly increased expression of lipogenic genes (*Fasn*, *Scd1*, *Acc1* and *Acc2*). Given the absence of ER stress under the same conditions, it suggests that XBP1 regulates hepatic lipogenesis independently of an ER stress response. Indeed, Tm-induced steatosis

occurred in the presence and absence of XBP1, suggesting that it does not regulate ER stress-induced hepatic steatosis (Rutkowski et al., 2008).

Hepatic deletion of XBP1 (*Xbp1* Δ liver), reduced expression of lipogenic genes (*Scd1*, *Dgat2*, and *Acc2*) (Lee et al., 2008). Unlike wild-type livers, a high carbohydrate diet did not induce expression of lipogenic genes in *Xbp1* Δ livers. Interestingly, liver nuclear extracts from high carbohydrate-fed mice indicated that XBP1 directly binds to the promoter of *Dgat2*, *Scd1* and *Acc2*. These findings demonstrate that XBP1 is required for hepatic *de novo* lipogenesis. Loss of XBP1 resulted in hypodyslipidemia with reduced hepatic TG secretion, but normal apoB protein content, stability, and secretion (Lee et al., 2008). Liver XBP1 deficiency itself did not induce ER stress and no obvious abnormalities were observed in the mice, however, there was constitutive activation of its upstream activator IRE1 α (Lee et al., 2008). Such a negative feedback mechanism may be compensating for the absence of XBP1. Given that IRE1 α is required for repressing lipogenic gene expression (albeit more so under ER stress) (Zhang et al., 2011), its increased activation in XBP1-deficient hepatocytes may partly explain the contrasting effects of IRE1 α -deficiency and XBP1-deficiency on hepatic lipogenesis.

5.1.2.3. *ATF6 pathway*

ER stress causes cleavage and activation of both ATF6 and SREBP-2 (Colgan et al., 2007; Haze et al., 1999). The active nuclear form of ATF6 was shown to interact with nuclear SREBP-2 and inhibit the activity of SREBP-2 in liver and kidney cells (Zeng et al., 2004). A number of recent studies have explored the role of ATF6 in fatty liver

disease and lipogenesis *in vivo* (Rutkowski et al., 2008; Wu et al., 2007; Yamamoto et al., 2010a). Under non-stressed conditions and similar to *Irel1 α ^{Hepfe/-}* mice, *Atf6 α* -knockout (*Atf6 α ^{-/-}*) mice appeared phenotypically normal (Wu et al., 2007; Yamamoto et al., 2007), however, Tm injection resulted in liver dysfunction and revealed a defect in the liver's ability to recover (Wu et al., 2007; Yamamoto et al., 2010a). Livers of Tm-injected *Atf6 α ^{-/-}* mice had enhanced hepatic lipid droplet formation (increased ADRP expression), as compared to *Atf6 α ^{+/+}* mice, and resulted in steatosis and much greater hepatic TG and cholesterol levels, collectively due to reduced FA β -oxidation (reduced PPAR α), and decreased VLDL formation (Yamamoto et al., 2010a). *Atf6 α ^{-/-}* livers exhibited increased numbers of apoptotic cells, but greatly reduced expression of ER chaperones after Tm injection. *Atf6 α ^{-/-}* mouse livers had a more sustained induction of CHOP following Tm-injection as compared to wild-type mice, indicating reduced adaptation and slow recovery from ER stress. Furthermore, since CHOP is a dominant negative regulator of the C/EBP family of proteins, it can impact on metabolic gene expression and activity of SREBP-1 and PPAR α (Yamamoto et al., 2010a). Unlike *Irel1 α ^{Hepfe/-}* mice, Tm-injected *Atf6 α ^{-/-}* mice had suppressed expression of lipogenic genes, suggesting that ATF6 α does not protect from increased *de novo* lipogenesis under ER stress conditions (Rutkowski et al., 2008). *Atf6 α ^{-/-}* mice fail to up-regulate ER quality control proteins in response to Tm-induced ER stress, and were defective in apoB100 protein folding contributing to hepatic lipid accumulation (Yamamoto et al., 2010a). Furthermore, upon closer examination, Tm-injected *Atf6 α ^{-/-}* mice became severely resistant to exogenous insulin, similar to the observation in Alb:GC Tg mice with defective hepatic eIF2 α signalling (Rutkowski et al.,

2008). These findings support the notion that unresolved ER stress can lead to IR (Ozcan et al., 2004), and highlight the importance of ATF6 in ER quality control under ER stress conditions.

5.1.3. Link between ER stress and obesity

5.1.3.1. Insight from animal studies

Both HFD-fed and genetically obese *ob/ob* mice exhibited increased expression of ER stress markers in liver and WAT tissue as compared to regular diet-fed or lean controls, respectively (Ozcan et al., 2004), a finding which paved the way for this PhD thesis project and many other studies. Given the presence of WAT inflammation, hypoxia, mechanical stress, excess lipid accumulation in hypertrophied adipocytes, and abnormal energy fluxes in obesity (Cinti et al., 2005; Fu et al., 2011; Gregor and Hotamisligil, 2007; Guo et al., 2007; Hosogai et al., 2007; Ozcan et al., 2004), these conditions in obesity may be chronic stimuli causing ER stress. Furthermore, ER stress activation was found to have profound effects on insulin action in liver cells. Specifically IRE1 α and expression of XBP1s, play an important role in JNK activation by modulating insulin receptor substrate-1 (IRS-1) phosphorylation status (in particular under ER stress conditions), thereby altering insulin signalling (Ozcan et al., 2004). *Xbp1* heterozygous (*Xbp1*^{+/-}) mice fed a HFD exhibited a small but significant increase in body weight. In addition, they exhibited progressive hyperinsulinemia due to increased ER stress, and impaired insulin receptor signalling in the liver and WAT, as compared to wild-type mice

(Ozcan et al., 2004). These experiments clearly showed a link between ER stress, peripheral IR, and obesity.

In association with ER stress and IR in obesity, hepatic gluconeogenesis and glucose output are also increased, contributing to fasting hyperglycemia (Ozcan et al., 2004). A recent study has identified cross-talk between ER stress and hepatic gluconeogenesis, which can explain why hepatic glucose production is increased in obesity. CREB regulated transcription co-activator 2 (CRTC2) was identified as a dual sensor for ER stress and fasting gluconeogenesis (Wang et al., 2009). During ER stress conditions, CRTC2 is dephosphorylated and translocates to the nucleus where it associates with nuclear ATF6 α , and promotes expression of ER quality control genes by binding to the *Xbp1* promoter. However, in the nucleus CRTC2 also associates with CREB to induce gluconeogenic gene expression. Thus, ATF6 α competes with CREB for binding to CRTC2. In obesity, there is reciprocal down-regulation of hepatic ATF6 α protein and enhanced CREB expression, leading to gluconeogenic gene expression and increased blood glucose concentrations (Wang et al., 2009). Furthermore, over-expression of hepatic ATF6 α reversed CRTC2 effects on gluconeogenesis. This study has identified CRTC2 as the molecular link between chronic ER stress and hyperglycemia in obesity.

HFD-feeding induces ER stress in liver and WAT, including increased phosphorylation of eIF2 α (p-eIF2 α), which leads to attenuation of global protein translation (Lefterova et al., 2009; Ozcan et al., 2004; Ye et al., 2010). WAT secretory

proteins play an important role in whole body energy and glucose homeostasis, however, ER stress has been associated with reduced translation of secretory proteins (Harding et al., 2000a; Harding et al., 2000b; Harding et al., 1999), which may contribute to the pathogenesis of obesity. There is evidence for ER stress mediated translational defects in adiponectin assembly and secretion, caused by suppression of adiponectin-interacting protein DsbA-L, which plays a role in the oligomerization and stabilization of adiponectin (Zhou et al., 2010a). ER stress in adipocytes results in decreased secretion of leptin and adiponectin (Xu et al., 2010), and decreased expression of resistin at the transcriptional level, which closely corresponded to resistin protein levels (Lefterova et al., 2009). Similarly, hypoxia-induced ER stress down-regulates adipose tissue adiponectin mRNA levels (Hosogai et al., 2007), suggesting ER stress mediated transcriptional changes in adipocytes. Indeed, repression of adipogenic genes such as PPAR γ and C/EBP α , and induction of CHOP, a negative regulator of adipogenesis, under ER stress conditions have been linked to the decreased transcription of these adipokines (Hosogai et al., 2007; Lefterova et al., 2009). Furthermore, ER stress induces leptin resistance in the brain, contributing to weight gain and obesity (Hosoi et al., 2008; Ozcan et al., 2009).

Mice heterozygous for the S51A mutation in eIF2 α appear normal, but are more prone to development of obesity, hepatic steatosis, and diabetes when fed a HFD, as compared to wild-type mice (Scheuner et al., 2005). These mice also exhibited profound glucose intolerance due to reduced insulin secretion from the pancreas (Scheuner et al., 2005), indicating the importance of eIF2 α in pancreatic insulin production, but also in

whole body metabolic homeostasis. One of the transcripts that escape translational attenuation by p-eIF2 α signalling is ATF4 (Harding et al., 2000a). ATF4 expression leads to CHOP induction, followed by GADD34 expression, which forms a negative feedback loop by de-phosphorylating eIF2 α . *Atf4*-knockout mice (*Atf4*^{-/-}), exhibit smaller WAT relative to body weight (Yoshizawa et al., 2009), with decreased expression of lipogenic genes, but increased FA β -oxidation (Wang et al., 2010). In contrast, CHOP-deficient (*Chop*^{-/-}) female mice exhibit greater adiposity, with enlarged visceral adipocytes, but no changes in glucose tolerance (Ariyama et al., 2007). A subsequent study confirmed these findings and further demonstrated that *Chop*^{-/-} mice remain insulin sensitive, exhibit increased hepatic lipid deposition, but reduced pro-inflammatory cytokine expression and macrophage infiltration into WAT and liver (Maris et al., 2012). Expression of C/EBP β , and the 30 kDa isoform of C/EBP α , were enhanced in *Chop*^{-/-} mice as compared to wild-type controls, suggestive of accelerated adipogenesis (Ariyama et al., 2007). Differences in body weight and WAT mass for most depots that were measured did not reach statistical significance in male mice (Ariyama et al., 2007). Overall, these findings suggest that CHOP may be an important link between increased lipid deposition and inflammation-mediated insulin resistance.

Similar to the phenotype observed in 4-PBA treated mice from my study, HFD-fed mice heterozygous for the *Grp78* gene (*Grp78*^{+/-}), which showed a 40% decrease in p-eIF2 α , were resistant to diet-induced obesity and diabetes (Ye et al., 2010). GRP78 is protective against ER stress (Kammoun et al., 2009; Morris et al., 1997), and although ER stress activates inflammation and represses insulin signalling leading to IR (Gregor

and Hotamisligil, 2007; Ozcan et al., 2004; Xu et al., 2010), *Grp78*^{+/-} mice are protected from obesity and IR. Analysis of WAT from *Grp78*^{+/-} mice indicated that a reduction in GRP78 may lead to low levels of chronic ER stress activating an adaptive UPR response, characterized by enhanced protein translation, up-regulation of ER chaperones, and ERAD, thus enhancing ER folding capacity (Ye et al., 2010). This type of UPR response is similar to the physiological UPR activation I observed during differentiation of adipocytes (Basseri et al., 2009), which also occurs in differentiating macrophages (Dickhout et al., 2011), and B cell to plasma cell differentiation (Gass et al., 2002), allowing the cells to cope with the extra demand for protein synthesis and trafficking in the ER. A summary of the phenotypes in mice, and cells derived from mice with genetic alterations in essential UPR genes is provided in **Table 5.1**.

Alleviation of ER stress/UPR activation has been linked to improved metabolic outcomes. Treatment of mice with chemical chaperones 4-PBA or TUDCA have been successful at reducing ER stress, and improving fatty liver, glucose and leptin tolerance, and insulin sensitivity (Ota et al., 2008; Ozcan et al., 2009; Ozcan et al., 2006). Oral administration of TUDCA has been effective at reducing hepatic steatosis in *ob/ob* mice by reducing expression of genes involved in *de novo* lipogenesis (Yang et al., 2010), while 4-PBA has been shown to reduce lipid-induced ER stress, thereby enhancing apoB100 secretion from liver cells (Ota et al., 2008). By lowering hypothalamic ER stress, 4-PBA and TUDCA enhance the sensitivity of neurons to leptin, alleviating leptin resistance in obesity (Hosoi et al., 2008; Ozcan et al., 2009). Furthermore, **chapter 2** of this thesis demonstrated that 4-PBA supplementation of mice fed a HFD is also effective

at reducing weight gain, decreasing adipocyte size, and reducing plasma glucose and TG levels over 17 weeks (Basseri et al., 2009). The therapeutic potential of chemical chaperones for the treatment of obesity and its co-morbidities is discussed in greater detail in section **5.3**.

5.1.3.2. Human studies linking ER stress/UPR activation to obesity

Consistent with the observations in mice, obese, insulin resistant individuals express markers of ER stress in their subcutaneous WAT including increased expression of UPR related proteins, and splicing of *Xbp1* mRNA (Boden et al., 2008). An increase in phosphorylation and activity of JNK-1, an IRE1 α target, was also observed. Given that JNK-1 inhibits IRS-1 activity (Gregor and Hotamisligil, 2007; Ozcan et al., 2004), these findings suggest that ER stress is one of the mediators of defective insulin receptor signalling and inflammation leading to IR in obesity (Boden et al., 2008). In another study, obese, non-diabetic subjects showed increased expression of ER stress markers, including p-eIF2 α , and enhanced ER chaperone expression, which significantly correlated with increased body mass index (BMI) and percent fat. JNK-1 activity was not affected in obese non-diabetic subjects and only a modest correlation between insulin sensitivity and ER stress was observed (Sharma et al., 2008). In a study comparing *Grp78* and *Xbp1* mRNA expression in subcutaneous and visceral WAT of lean, overweight, obese and severely obese individuals, a significant increase in these ER stress markers was observed in visceral fat, which was even more pronounced in severe obesity (Vendrell et al., 2010). However, the presence of diabetes did not significantly affect the

expression of these particular ER stress markers (Vendrell et al., 2010). These studies suggest a strong correlation between obesity and ER stress markers, but the direct relationship between ER stress, IR and T2D requires further examination. Inhibition of ER stress through oral administration of TUDCA, enhanced insulin sensitivity in the liver and muscle by 30%, but did not alter ER stress or insulin sensitivity in the WAT of obese subjects (Kars et al., 2010).

Weight loss through gastric bypass surgery in obese subjects was effective at improving WAT, liver and skeletal muscle insulin sensitivity (Gregor et al., 2009). Subcutaneous WAT from these subjects one year after surgery exhibited reduced phosphorylation of JNK-1 and eIF2 α , and lower *Grp78* and *Xbp1s* mRNA expression as compared to pre-surgery. Furthermore, there was reduced GRP78 and p-eIF2 α expression in livers post-surgery. These results indicate that metabolic improvement through weight loss is associated with a reduction in ER stress in WAT and liver tissues (Gregor et al., 2009). These data are consistent with the findings of a study that illustrated a reduction in macrophage infiltration and inflammation in subcutaneous WAT three months after bypass surgery-mediated weight loss in morbidly obese subjects (Cancello et al., 2005).

5.2. The impact of lipids on ER stress²

The relationship between ER stress and lipid metabolism is bi-directional. While activation of ER stress pathways can result in lipogenesis and altered lipid homeostasis, lipids and aberrant lipid metabolism can also cause ER stress (Fu et al., 2011; Gentile et al., 2011; Guo et al., 2007; Wei et al., 2006). Saturated fatty acids such as palmitate and stearate are known inducers of ER stress in various cell types and can modulate survival and apoptotic signals in the cell (Guo et al., 2007; Wei et al., 2006). A recent study carried out comparative proteomic and lipidomic analysis of fractionated ER from lean and obese liver tissues (Fu et al., 2011). The results suggested enrichment of metabolic enzymes involved in lipid metabolism and a down-regulation of ER-associated protein synthesis genes in the obese ER proteome. These findings implied that the ER in obese liver cells shifts from being the major site of protein synthesis to carrying out lipid synthesis and lipid metabolism functions (Fu et al., 2011). Furthermore, the analysis revealed that there is a greater proportion of *de novo* synthesized saturated fatty acids incorporated into hepatic ER lipids than dietary polyunsaturated fatty acids. Another interesting finding was the increased proportion of phosphatidylcholine (PC) in comparison to phosphatidylethanolamine (PE), both abundant ER membrane phospholipids, in the liver ER from obese mice (Fu et al., 2011). The increased PC/PE

² This section has been adapted from: Basseri S and Austin RC. (2012) Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13.

ratio led to perturbation in the calcium transport activity of the SERCA pump resulting in impaired ER calcium retention. Since ER calcium is important for ER homeostasis and chaperone function, such changes in calcium concentrations would lead to protein misfolding and ER stress. This appears to be a plausible mechanism for hepatic ER stress in obesity (Fu et al., 2011). Hepatic ER stress can promote *de novo* lipogenesis and IR as described above which then in turn may lead to further exacerbation of the ER stress situation, creating a vicious cycle.

5.3. Therapeutic potential targeting ER stress in dyslipidemia and obesity³

ER stress and UPR activation have been implicated in the pathogenesis of a number of diseases such as diabetes, obesity, cancer, renal, cardiovascular and neurodegenerative diseases as well as fatty liver disease (Austin, 2009; Dickhout and Krepinsky, 2009; Hosoi and Ozawa, 2010; Kaplowitz et al., 2007; Minamino and Kitakaze, 2010). As such, potential ways of attenuating ER stress and UPR activation would provide opportunities in pharmacological intervention in a wide array of diseases. A recent study revealed for the first time in humans that obese insulin-resistant subjects express markers of ER stress in their WAT (Boden et al., 2008). Similarly, an association between ER stress and obesity was also found in obese non-diabetic subjects (Sharma et

³ Sections 5.3 and 5.3.2. were adapted from: Basseri S and Austin RC. (2012) Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012: 841362. Epub 2011 Dec 13.

al., 2008). Gastric bypass surgery-mediated weight loss in obese patients was effective at reducing ER stress in adipose and liver tissues and improved insulin sensitivity (Gregor et al., 2009). Furthermore, when ER stress was reduced by hepatic over-expression of GRP78 in *ob/ob* mice, hepatic TG and cholesterol content was reduced and insulin sensitivity improved (Kammoun et al., 2009). These findings together with data in rodents indicating the presence of ER stress in tissues of obese animals (Ozcan et al., 2009; Ozcan et al., 2004) suggest a strong association between ER stress and obesity. Therefore, the ER serves as an important and new target organelle for development of treatments to combat obesity and its metabolic complications.

The use of small molecules called chemical chaperones have been examined in a number of disease models as potential tools for lowering ER stress and preventing the activation of UPR pathways. These chaperones similar to molecular chaperones non-selectively stabilize mutant proteins and assist in their folding and translocation across membranes (Engin and Hotamisligil, 2010). Most chemical chaperones are osmolytes and equilibrate cellular osmotic pressure. These can be categorized into 3 classes: carbohydrates (such as glycerol and sorbitol), amino acids (such as glycine and taurine), and methylamines (such as betaine) (Engin and Hotamisligil, 2010; Welch and Brown, 1996). The drawback to the use of most chemical chaperones is their non-specificity and high dose requirement for effective protein folding properties. However, two such chemical chaperones, 4-PBA and TUDCA have been approved by the US Food and Drug Administration (FDA) and are used in humans. Currently, 4-PBA is approved for use in children with urea-cycle disorders as an ammonia scavenger, while TUDCA is being

tested for its liver-protecting properties in cholestatic liver disease in humans (Engin and Hotamisligil, 2010).

The low molecular weight fatty acid 4-PBA has been tested in a number of disease models for its ability to facilitate protein folding and trafficking, ultimately relieving ER stress (Basseri et al., 2009; Kuang et al., 2010; Mizukami et al., 2010; Ozcan et al., 2009; Ozcan et al., 2006; Qi et al., 2004; Rahman et al., 2009; Vilatoba et al., 2005; Wiley et al., 2011; Xiao et al., 2011; Zode et al., 2011). The chaperoning property of 4-PBA was first identified when investigating its effect on the translocation and trafficking of a mutant cystic fibrosis transmembrane conductance regulator protein (CFTR). Addition of 4-PBA to the cells allowed for stabilization of the mutant CFTR protein and facilitated their translocation to the cell membrane (Lim et al., 2004). In addition to its chaperone properties, 4-PBA also possesses HDAC inhibitor activity and is under investigation as an anti-cancer drug (Appelskog et al., 2004; Jung, 2001; Ricobaraza et al., 2010).

Another effective reagent that has been shown to have chaperone properties is TUDCA, which can be classified as a hydrophilic endogenous bile acid (Engin and Hotamisligil, 2010). TUDCA has anti-apoptotic properties by reducing calcium efflux and blocking ER stress mediated caspase-12 activation (Sola et al., 2003). Furthermore, TUDCA also activates cell survival pathways such as PI3K signalling, thereby inhibiting cell death (Schoemaker et al., 2004). Apart from these signalling properties, TUDCA can interact with the mineralocorticoid receptor and promotes its dissociation from cytosolic

chaperones thereby preventing its translocation to the nucleus for transcriptional activity. In the case of primary neurons, addition of TUDCA was effective at preventing amyloid beta-peptide induced apoptosis through its chaperoning properties (Sola et al., 2006).

In recent years, several studies have identified beneficial effects of 4-PBA and TUDCA supplementation in treatment of IR, obesity and diabetes. Oral administration of 4-PBA and TUDCA to obese and insulin resistant *ob/ob* mice normalized hyperglycemia, restored insulin sensitivity in the liver, muscle and white adipose and diminished fatty liver disease (Ozcan et al., 2006). Part of this thesis examined the effect of 4-PBA supplementation on diet-induced obesity (**chapter 2**). For this purpose C57BL/6 mice were placed on a HFD, which has previously been reported to induce ER stress in WAT and liver tissue, and concomitantly supplemented with or without 4-PBA in the drinking water. Mice treated with 4-PBA gained significantly less weight, exhibited lower plasma glucose, TG and leptin levels, and had smaller adipocytes as compared to mice on a HFD alone (Basseri et al., 2009). Chemical chaperones also have chaperone activity within the central nervous system (Inden et al., 2007; Ozcan et al., 2009). Leptin, an adipocyte derived hormone which acts on hypothalamic neurons to suppress appetite, is important in regulating energy expenditure and body weight (Friedman, 1998). ER stress may be one of the factors resulting in leptin resistance in the brain, as injection of Tm leading to hypothalamic ER stress, increased food consumption and weight gain despite elevated blood leptin concentrations (Ozcan et al., 2009). Both 4-PBA and TUDCA were shown to be effective at lowering hypothalamic ER stress and increasing the sensitivity of neurons to leptin, thereby reducing body weight in genetic and diet-induced obesity models

(Friedman, 1998). In the context of atherosclerosis, 4-PBA was effective at protecting macrophages against palmitate-induced ER stress and apoptosis in culture (Erbay et al., 2009). A reduction in ER stress and apoptosis was also observed in the macrophages within the atherosclerotic lesions of mice treated with 4-PBA, which were smaller in size (Erbay et al., 2009). These findings indicate that 4-PBA treatment can protect cells from the deleterious effects of lipid accumulation on disease progression.

ER stress has been linked to fatty liver disease and liver injury (Anderson et al., 2011; Wang et al., 2006). Lipid-induced ER stress inhibits apoB100 secretion in liver cells promoting the development of steatosis (Ota et al., 2008). Treatment of hepatoma cells with 4-PBA leads to the inhibition of lipid-induced ER stress and enhanced apoB100 secretion (Ota et al., 2008). Consistent with the studies on macrophages and progression of atherosclerosis, alleviating lipid-induced ER stress in hepatocytes also protects the cells from ER-associated apoptosis (Kim et al., 2010). Since hepatocellular injury and damage can lead to progression of fatty liver disease into steatohepatitis (Jou et al., 2008; Tevar et al., 2011), blocking ER stress serves as an important treatment strategy (Vilatoba et al., 2005). A recent study examined the effects of oral administration of TUDCA on hepatic steatosis and hepatic gene expression in *ob/ob* mice (Yang et al., 2010). Yang *et al.* found a significant decrease in liver fat content and reduced expression of genes involved in *de novo* lipogenesis with TUDCA treatment (Yang et al., 2010). However, they did not find any differences in body weight or insulin sensitivity over the three week duration of the study. Examination of the effects of orally administered TUDCA on insulin sensitivity in obese human subjects revealed a 30% improvement in

insulin sensitivity in muscle and liver tissues but no alterations in hepatic TG content was observed (Kars et al., 2010). The differences in the mechanism of action between oral treatment and intraperitoneally injected TUDCA may explain some of these contrasting outcomes (Yang et al., 2010).

5.3.1. Clinical Trials

The effectiveness of chemical chaperones such as 4-PBA and TUDCA as a treatment strategy for dyslipidemia, cardiovascular disease, diabetes and obesity require further study in human subjects. Both 4-PBA and TUDCA have additional functions which may be directly or indirectly alleviating ER stress conditions. Furthermore, 4-PBA and TUDCA are not currently approved by the Food and Drug Administration for the treatment of dyslipidemia or IR. Interestingly, a recent clinical trial study examined the effect of buphenyl (trade name for 4-PBA) on fatty acid-induced impairment of glucose-stimulated insulin secretion, to determine if 4-PBA can prevent the effects of elevated plasma FFA and hyperglycemia-mediated ER stress on beta cell function and insulin sensitivity (Xiao et al., 2011). This was a randomized, single-blind, interventional study, which took place at the Toronto General Hospital in Canada, where male participants between the ages of 35-60 with a BMI >27 kg/m² were recruited. The inclusion criteria were: fasting TG 2-5 mM, waist circumference >90 cm, fasting blood glucose <7 mM, and hemoglobin >130 g/L. 4-PBA was administered orally at a dose of 7.5 g/day for two weeks prior to pancreatic function tests (ClinicalTrials, 2010; Xiao et al., 2011). The

findings showed that 4-PBA was effective at reducing lipid-induced beta cell dysfunction and partially alleviating insulin resistance in humans (Xiao et al., 2011).

Another clinical trial is currently recruiting participants at Washington University School of Medicine, to study the effectiveness of TUDCA/PBA in obese subjects with IR and hyperlipidemia, in order to elucidate the impact of ER stress on metabolic function (ClinicalTrials, 2011). This is also a randomized, single-blind interventional study aiming to determine the effect of 1750 mg/day of TUDCA or 20 g/day of 4-PBA over the course of 4 weeks, in both sedentary males and females between the ages of 18-65 with a BMI of 30-45. This trial will examine the following outcome measures: body fat distribution (intrahepatic, intramyocellular and intra-abdominal), *in vivo* insulin sensitivity in adipose, liver and skeletal muscle tissue, hepatic VLDL-TG and VLDL-apoB100 secretion rates, as well as skeletal muscle and adipose tissue insulin signalling and inflammation.

These clinical trials offer hope that years of data generated from laboratory experiments using cultured cells and animal models can be taken to the next step, namely to advance the medical field and treat human disease. Importantly, the outcomes of these trials and any future trials will ultimately enhance the pharmacological tools available for the treatment of obesity, diabetes and dyslipidemia, diseases that have reached epidemic proportions worldwide.

5.3.2. Summary

Discovery of new chemical and biological approaches to enhance ER function and protein trafficking would be useful for treating a number of ER stress-related diseases and thus present significant therapeutic opportunities. Furthermore, ways of targeting specific UPR components would allow for enhanced specificity in targeting ER stress/UPR pathways in various disease states (Engin and Hotamisligil, 2010). Currently, small molecules that can target IRE1 α and alter its endonuclease activity offer hope for further study. These kinase-inhibiting RNase attenuators can also selectively enhance XBP1 mRNA splicing and lead to prevention of apoptotic cell death, while attenuating IRE1 α -mediated decay of mRNA such as those encoding ER chaperones (Han et al., 2009). The recent finding that unfolded peptides can directly bind to IRE1 and promote its oligomerization and activation, suggests that compounds that can target its peptide binding groove and oligomerization interface may be effective at regulating IRE1 activity (Gardner and Walter, 2011). Finally, given the challenges with directly measuring ER stress, assay systems which can assess actual cellular ER stress will prove to be useful (Merksamer et al., 2008).

5.4. TDAG51 as a novel player in adipogenesis and metabolic regulation

5.4.1. ER stress and TDAG51 expression in preadipocytes

While previous studies had clearly shown TDAG51 to be up-regulated by ER stress-inducing agents such as homocysteine and DTT (Hossain et al., 2003), as well as farnesol

(Joo et al., 2007), 3T3-L1 preadipocytes treated with ER stress-inducing agents exhibited suppressed TDAG51 protein expression. Though this finding appears contradictory, it supports the conclusion made in **chapter 3** and **4** that TDAG51 must have differential regulation and/or function in various cell types. Given that some tissues express much higher levels of TDAG51 relative to other tissues, there must be tissue or cell type-specific transcriptional or translational control of TDAG51 that have not yet been fully explored. It is not entirely clear why acute ER stress decreased TDAG51 expression, but one potential mechanism could be through PERK-eIF2 α mediated attenuation of TDAG51 protein translation under severe or persistent ER stress conditions. Along these lines, it is also conceivable that TDAG51 may directly affect protein translation given that TDAG51 over-expression inhibits protein biosynthesis both *in vitro* and *in vivo* (Hinz et al., 2001). Therefore, it is possible that ER stress-mediated TDAG51 repression may allow for translation of proteins required for enhancing ER function/folding capacity. Future experiments are required to address this question.

Given the known pro-apoptotic function of TDAG51 in many cell types (Gomes et al., 1999; Hossain et al., 2003; Nagai et al., 2007; Neef et al., 2002; Park et al., 1996), it is also possible that its expression is down-regulated as an adaptive mechanism to prevent ER stress-mediated apoptosis. On the other hand, TDAG51 has also been implicated in cell survival in fibroblasts (Toyoshima et al., 2004), therefore raising the possibility that ER stress-mediated down-regulation of TDAG51 in 3T3-L1 cells may be a signal promoting cell death under such acute and persistent stress conditions.

5.4.2. The role of TDAG51 in adipocyte differentiation and metabolic regulation

The findings presented in **chapter 3** of this thesis show for the first time that WAT expresses relatively high levels of TDAG51 and that it is a novel player in adipogenesis. Relative to undifferentiated preadipocytes, mature or differentiated mouse and human adipocytes do not express TDAG51. Interestingly, lipid-laden fatty livers from leptin-deficient *ob/ob* mice, diet-induced obese mice, or carbon tetrachloride-treated mice also exhibit suppressed TDAG51 expression (**chapter 4**). These findings suggested that 1) TDAG51 may regulate lipid metabolism pathways by attenuating lipogenesis, or 2) cellular lipid levels may modulate TDAG51 expression or stability. In support of the former, primary hepatocytes isolated from young *TDAG51^{-/-}* mice had a greater rate of FA incorporation into TG molecules both basally and upon insulin stimulation. In comparison to WT mice, liver tissue from *TDAG51^{-/-}* mice exhibited increased mRNA expression of several SREBP target genes which are involved in *de novo* lipogenesis and lipid metabolism pathways (**Appendix 2**). Furthermore, absence or knockdown of TDAG51 in primary preadipocytes and 3T3-L1 cells, respectively, accelerated adipogenesis and lipid accumulation. Although a definitive mechanism for the inverse correlation between TDAG51 expression and adipogenesis and/or lipid accumulation was not established, several mechanisms were alluded to in **chapter 3** and **4** of this thesis. The available literature on TDAG51 in the context of metabolism is limited and the majority of studies to date identified and described TDAG51 in the context of cancer biology. Nonetheless, based on the available information and the results of this thesis

project, several mechanisms for the function of TDAG51 in adipogenesis and lipid metabolism can be proposed:

First, TDAG51 expression may be down-regulated in fatty livers and lipid-laden adipocytes in response to lipid-induced ER stress. Treatment of cells with ER stress-inducing agents down-regulated TDAG51 protein expression (**chapter 3**). As described in section **5.2.**, recent studies have shown that lipids are a potent inducer of ER stress (Fu et al., 2011; Gentile et al., 2011). Furthermore, ER stress/UPR pathways are activated in both fatty livers (Kammoun et al., 2009; Ozcan et al., 2004) and differentiating and/or hypertrophied adipocytes (Basseri et al., 2009; Gregor and Hotamisligil, 2007). Therefore, it appears plausible that TDAG51 down-regulation occurs in response to lipid-induced ER stress in hepatocytes and differentiating adipocytes.

Second, TDAG51 may be involved in growth factor-mediated signalling that occurs during early adipogenesis and precedes lipogenic gene expression. It is well established that IGF-1 signalling is critical in adipogenesis (Smith et al., 1988), and TDAG51 is a target of IGF-1 in fibroblasts (Toyoshima et al., 2004) and is up-regulated in response to IGF-1 and insulin in preadipocytes, particularly when cells are serum-starved (**Appendix 1A**). Furthermore, Toyoshima *et al.* demonstrated that p38 MAPK was essential for the induction of TDAG51 expression, while PI3K, ERK or JNK pathways were not involved in IGF-1-mediated regulation of TDAG51 expression in NIH-3T3 cells (Toyoshima et al., 2004). Insulin shares a number of signalling pathways with IGF-1 and is a well-known activator of SREBP-1, leading to *de novo* lipogenesis

(Hegarty et al., 2005; Shimomura et al., 1999). SREBP-1 cleavage and activity may be regulated by PI3K as well as Akt, ERK, PKC and other signalling molecules (Kotzka et al., 1998; Kotzka et al., 2000; Leavens et al., 2009; Matsumoto et al., 2003; Mora et al., 2005; Taniguchi et al., 2006; Yamamoto et al., 2010b).

Given that absence or knockdown of TDAG51 increases lipid accumulation in hepatocytes and adipocytes, it suggests that TDAG51 may play a role as a negative regulator of SREBP-1 activity either directly by interacting with SREBP-1, SCAP or Insig, or alternatively, it may do so indirectly by modulating the activity of upstream kinases leading to SREBP-1 activation. For example, Insig1 protein has been shown to be down-regulated by the PERK-eIF2 α pathway leading to SREBP activation (Bobrovnikova-Marjon et al., 2008). Given that TDAG51 was also down-regulated by ER stress (possibly through translational attenuation as discussed in section **5.4.1.**), involvement of the ER stress/PERK pathway in TDAG51 down-regulation and SREBP activation appears to be a plausible mechanism to explore. Furthermore, increased TDAG51 expression in response to IGF-1 and/or insulin signalling may serve as an inhibitory signal to prevent excessive lipid accumulation under anabolic conditions. It is noteworthy that absence of TDAG51 did not alter the secretion of TG-rich VLDL particles from the liver suggesting that development of fatty liver in the *TDAG51*^{-/-} mice is unlikely to be due to decreased TG secretion (**Appendix 3**).

Third, the function of TDAG51 is likely mediated through its pleckstrin homology (PH)-like domain. As already discussed in **chapters 1, 3 and 4**, TDAG51

possesses a PH-like domain, a structural motif commonly found in many signalling molecules. Interestingly, a recent study showed that PHLDB1, a PH domain-containing protein was involved in adipocyte insulin signalling and GLUT4 translocation (Zhou et al., 2010b). The PH-like domain of TDAG51 contains a high stringency PI-3,4,5-P₃ binding site as predicted by ScanSite. Given that PI-3,4,5-P₃ are important intracellular messengers that bind to PH domains of many proteins allowing for activation of downstream effectors such as Akt, it is quite possible that TDAG51 regulates growth factor-mediated signalling through this functional domain. Preliminary studies using siRNA knockdown of TDAG51 in 3T3-L1 cells or over-expression of mouse TDAG51 in 3T3-L1 cells did not identify any striking differences in insulin signalling responses with alterations in TDAG51 expression in preadipocytes (**Appendix 1B**). However, for future studies it would perhaps be more relevant to examine insulin signalling responses in differentiating adipocytes which express higher levels of insulin receptors and are dependent on insulin signalling to carry out their biological function.

It is worth noting that the PH domain also plays a role in cytoskeletal remodelling (Lemmon and Ferguson, 2000). In support of this, previous studies in the Austin laboratory have illustrated the co-localization of TDAG51 to focal adhesion kinases (Hossain *et al.* unpublished data). Furthermore, we and others have demonstrated that TDAG51 plays a role in cell migration (Johnson et al., 2011). These findings strengthen the hypothesis that the PH domain of TDAG51 likely contributes to its function.

Fourth, given the high degree of homology between the PH-like domain of TDAG51 and that of PI3K enhancers (PIKE), it is possible that TDAG51 and PIKE may be interacting and competing for the same binding partners. There are three PIKE isoforms, which result from alternative splicing or differential transcription initiation of the same gene (Chan and Ye 2007). PIKE-S and PIKE-L bind to PI3K and enhance its activity, while PIKE-A interacts with its downstream effector and enhances the activity of Akt (Chan and Ye, 2007). Both PIKE-S and PIKE-L are brain-specific, while PIKE-A expression was found in the brain as well as liver, lung, skeletal muscle, thymus, spleen, small intestine and blood leukocytes (Chan and Ye, 2007). Recently, PIKE-A was shown to be important for adipogenesis and its absence protected HFD-fed mice from development of obesity and IR (Chan et al., 2010). Consistent with the predicted PI-3,4,5-P₃ binding of TDAG51, PIKE-A also strongly binds to phosphoinositides. Interestingly, PIKE-A and TDAG51 appear to have opposite functions: 1) While PIKE-A is a proto-oncogene and is up-regulated in cancers (Liu et al., 2007), TDAG51 is down-regulated in several cancers and has been described to have pro-apoptotic properties in most studies. 2) PIKE-A deficiency inhibited adipogenic gene expression and lipogenesis (Chan et al., 2010), while loss of TDAG51 accelerated adipogenesis in our study. 3) While PIKE-A deficient mice were protected from development of obesity and IR, *TDAG51*^{-/-} mice exhibited fatty liver, IR and increased adiposity even when fed a standard chow diet. These opposing phenotypes, despite the high degree of homology between the PH domains of TDAG51 and PIKE-A, suggest that they may have opposing functions, perhaps by competing for the same target enzyme. It is also worth noting that

PIKE is a GTP-binding protein and its effects on PI3K and Akt activity are mediated by its GTPase activity (Yan et al., 2008). On the other hand, no GTPase activity has been reported for TDAG51.

Fifth, TDAG51 may be targeted for degradation under lipogenic conditions. Similar to PIKE-A which can be phosphorylated (preventing its degradation), recently TDAG51 was also shown to have potential phosphorylation sites (Johnson et al., 2011), however this led to targeting of TDAG51 for degradation. Johnson *et al.* demonstrated that TDAG51 is a negative regulator and effector of Aurora A kinase, a serine/threonine kinase important for cell proliferation. It is not clear whether Aurora kinases play a role in adipogenesis or lipogenesis. Furthermore, it is currently unknown whether TDAG51 has other phosphorylation sites that are critical for its stability or that may alter its function. However, given the results presented in **chapter 3**, it is possible that in addition to repressed gene expression, a post-translational mechanism may be in place whereby TDAG51 is targeted for degradation to allow for activation of lipogenic gene expression or promote lipid droplet formation. Furthermore, given its pro-apoptotic characteristics, TDAG51 down-regulation may act as a cell survival mechanism during nutritional and mechanical stress conditions as lipid droplets begin to form. This would be consistent with the described hepato-protective effects of hepatic triglyceride droplet formation in obese insulin-resistant individuals (Choi and Diehl, 2008), which would allow for safe storage and degradation of toxic lipids like cholesterol and fatty acids, preventing their accumulation in the ER membrane (Hapala et al., 2011).

5.4.3. Future Directions

Much remains unclear about the function of TDAG51, its transcriptional, translational, and post-translational regulation in different cell types, its cellular localization and potential binding partners. Therefore, future studies are needed to address several questions pertaining to the results presented herein through the following objectives:

- 1) *To define how ER stress leads to repression of TDAG51 expression and whether it promotes cell adaptation/survival.* To determine which arm of the UPR may be involved in TDAG51 regulation under ER stress conditions, MEFs deficient in UPR components can be utilized. MEFs, similar to 3T3-L1 cells, are fibroblast-like cells capable of adipogenic differentiation. Given the availability of MEFs deficient in *Ire1*, *Xbp1*, *Atf4*, *Perk*, *Chop*, *Atf6*, and MEFs bearing an eIF2 α S51A mutation, each arm of the UPR can be individually assessed. MEFs can be treated with ER stress inducing agents, Tm, thapsigargin, DTT, as well as physiological ER stressors such as palmitate or varying concentrations of glucose, to examine the effect on TDAG51 expression and thereby identify potential regulators of ER stress-mediated TDAG51 repression. Although 4-PBA on its own did not affect TDAG51 expression in 3T3-L1 cells (**chapter 3**), it would be worth examining its effect in the presence of an ER stress-inducing agent. Cells can be pre- or co-treated with 4-PBA or TUDCA to determine if alleviating ER stress conditions can prevent the effects of ER stress on TDAG51.

2) *To evaluate the role of TDAG51 in growth factor-mediated signalling during adipogenesis and in hepatocytes.* Primary preadipocytes and hepatocytes isolated from WT or *TDAG51*^{-/-} mice can be subjected to insulin, IGF-1, and other growth factors in a time and dose-dependent manner. Through immunoblotting, the phosphorylation status of proteins in the PI3K, p38 MAPK, ERK signalling pathways can then be examined, since these kinases play an important role in adipogenesis (Aouadi et al., 2007; Bost et al., 2005a; Bost et al., 2005b; Engelman et al., 1998; Sakaue et al., 1998), particularly in the early stages when TDAG51 is expressed. Furthermore, insulin is a potent activator of SREBP-1 in hepatocytes. Microarray analysis of livers from mice fed a chow diet indicated up-regulation of more than ten SREBP target genes in *TDAG51*^{-/-} livers (**Appendix 2**). Furthermore, given the results shown in **chapter 4** indicating increased expression of SREBP-1 target genes in *TDAG51*^{-/-} hepatocytes, while hepatic TG secretion is not affected by absence of TDAG51 (**Appendix 3**), it can be hypothesized that TDAG51 plays a role in regulating lipogenic pathways.

As indicated in **chapter 1 (Figure 1.3.)**, TDAG51 has a putative nuclear export and nuclear localization signal (NES and NLS, respectively), in the PH-like domain, suggesting that it may be able to shuttle in and out of the nucleus. Therefore, the TDAG51 construct with a GFP tag (TDAG51-GFP) which is currently available in the Austin laboratory, can be utilized to examine the localization of TDAG51 under different treatment conditions, and during adipocyte differentiation. An *in vitro* binding assay or co-immunoprecipitation

studies can be performed to determine whether TDAG51 binds to activated kinases during growth factor signalling thereby exerting its effects on metabolism.

3) *To determine whether the increased lipid accumulation in TDAG51^{-/-} adipocytes and hepatocytes occurs through a SREBP-1 mediated mechanism.* Given the increased expression of SREBP target genes and TG accumulation in TDAG51^{-/-} cells, the role of SREBP-1 (the isoform primarily involved in TG synthesis pathways) in mediating the effects of loss of TDAG51 on lipogenesis would be worth examining. Compounds such as fatostatin or betulin can be utilized to block SREBP activation (Kamisuki et al., 2009; Tang et al., 2011), in order to determine whether increased TG synthesis in TDAG51^{-/-} hepatocytes or adipocytes is mediated through an SREBP-dependent pathway. Furthermore, SREBP activity can be determined by transfecting cells with the SRE-GFP construct and determining extent of green fluorescence in WT versus TDAG51^{-/-} cells. Finally, siRNA targeting SREBP-1 can be utilized to specifically down-regulate SREBP-1 expression and determine if TG synthesis and accumulation can be reduced in TDAG51^{-/-} cells.

4) *To further define the functional domains in TDAG51 which mediate its effects on adipogenesis and lipogenesis.* Given that TDAG51 possesses a PH-like domain and other distinct regions within its open reading frame, generation of a series of mutants would help identify the functional domain in TDAG51. Based on the available literature and the results presented in this thesis, the PH-like domain

which is often involved in cell signalling and cytoskeletal remodelling, would likely be important for TDAG51 function. A construct containing the PH-like domain of TDAG51 with a GFP tag can be utilized to perform the experiments described above in order to determine if the TDAG51-PH mutant has similar effects on growth factor signalling as the WT full-length TDAG51. Alternatively, generation of a mutant deficient in the PH-like domain can be constructed to determine if it has any activity. Currently, constructs with mutations in the NLS, NES, and PQ regions of TDAG51 are available in our laboratory to identify the region of TDAG51 that is important for mediating its function and/or localization.

- 5) *To examine potential phosphorylation sites and their effects on protein stability, localization or function.* A recent study has indicated that PHLDA1 can be phosphorylated by Aurora A, leading to its degradation (Johnson et al., 2011). Therefore, there is a possibility that phosphorylation of TDAG51 can impart new functions on this protein that have yet to be examined. It would therefore be imperative to identify any post-translational modifications to TDAG51 and potential upstream post-translational regulators of TDAG51 that may affect its function and localization.
- 6) *To determine if TDAG51 can serve as a novel therapeutic target for the treatment of obesity, diabetes, fatty liver and dyslipidemia.* Firstly, it would be useful to evaluate the effects of nutritional status on TDAG51 expression, ie. whether fasting and re-feeding or the presence of hyperglycemia or hyperlipidemia can

influence its expression in various tissues/cell types. For instance, serum starvation of 3T3-L1 preadipocytes markedly reduced TDAG51 expression (**Appendix 1A**). Secondly, given that TDAG51 expression declines in fatty livers, an adenoviral vector containing TDAG51 can be injected intravenously into *ob/ob* mice (which develop hepatic steatosis) or wild-type mice prior to HFD feeding, to determine if hepatic over-expression of TDAG51 can prevent lipid accumulation. Thirdly, generation of mice with a tissue-specific knockout of TDAG51 in WAT, liver, skeletal muscles and other tissues, would greatly enhance our understanding of TDAG51 and its function *in vivo*. Finally, a high throughput screening approach can be utilized to identify molecules that can specifically induce TDAG51 expression. Once tested on multiple cell types in culture and its appropriate dose and extent of toxicity determined, a candidate compound can be tested in *TDAG51^{-/-}* mice for its effects on reversing the metabolic phenotype observed in these mice. Given that we have shown reduced glucose uptake in WAT and skeletal muscles and a blunted insulin response in liver tissue from *TDAG51^{-/-}* mice, identification of potential compounds that effectively induce TDAG51 expression in most insulin-sensitive cell types would be useful.

5.5. Concluding Remarks⁴

While the function of UPR pathways in ER homeostasis and protein folding are well-defined, recent studies including those performed as part of this PhD thesis, have uncovered novel and unexpected roles for the ER sensors, IRE1, PERK, and ATF6, and their downstream targets in lipid metabolism and metabolic regulation. There is a growing body of evidence indicating links between ER stress and obesity, IR, T2D, and fatty liver. An important factor in how the UPR pathways regulate lipid metabolism, is the presence of ER stress and whether it is physiological and adaptive ER stress seen with nutritional stress, or acute and unresolved ER stress that occurs with Tm treatment. Deficiency in ATF6 α , as well as hepatic deficiency in IRE1 α and eIF2 α signalling, results in development of fatty liver in mice exposed to Tm. Hepatic deletion of XBP1 on the other hand, the downstream target of IRE1 α , causes diminished lipogenesis and its effects on *de novo* lipogenesis are independent of ER stress activation. It is important to further understand the role of the UPR and the specific arms and components of each pathway in regulating lipid metabolism under metabolic or physiological ER stress conditions. Furthermore, identification of novel genes such as TDAG51 that are involved in the regulation of the UPR pathways and the cell's response to ER stress conditions will be crucial for advancing the field and clarifying the role of each arm of the UPR in metabolic homeostasis, both in health and disease. Such knowledge will allow us to

⁴ Section 5.5 is adapted from the following book chapter with kind permission of Springer Science and Business Media: **Basseri S** and Austin RC. (2012) Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In “*ER stress in Health and Disease*”. In press at *Springer*.

manipulate the expression or activity of specific UPR mediators, potentially leading to a novel approach for reducing the risk of obesity, diabetes, hepatic steatosis, and dyslipidemia.

5.6. References

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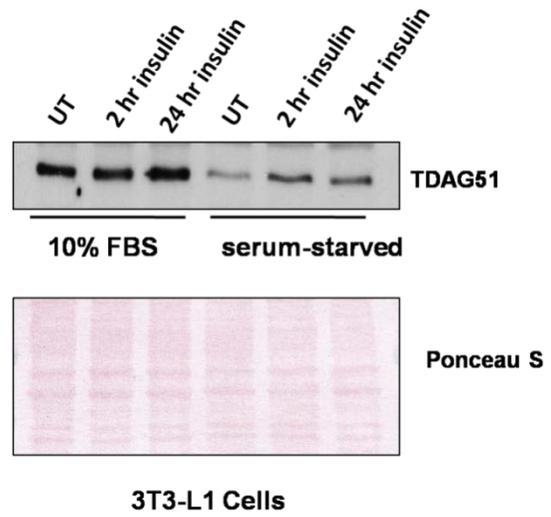
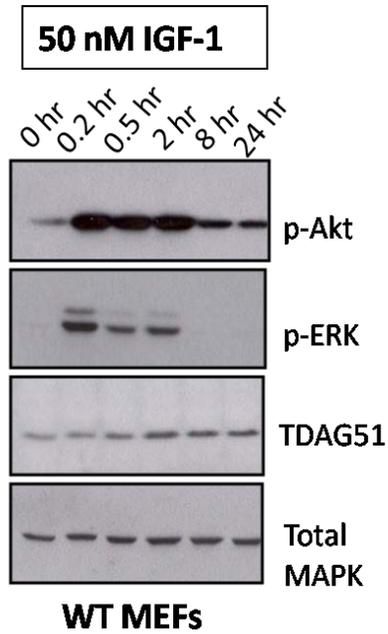
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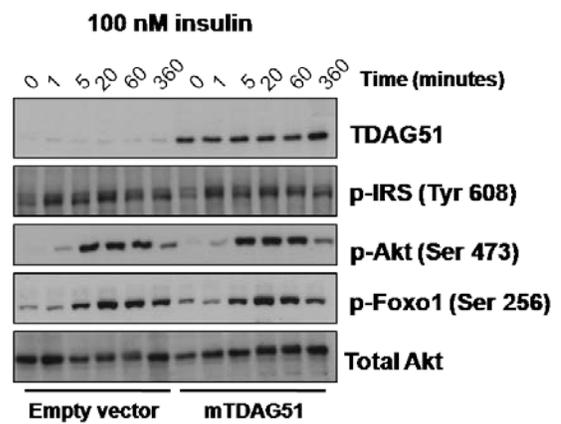
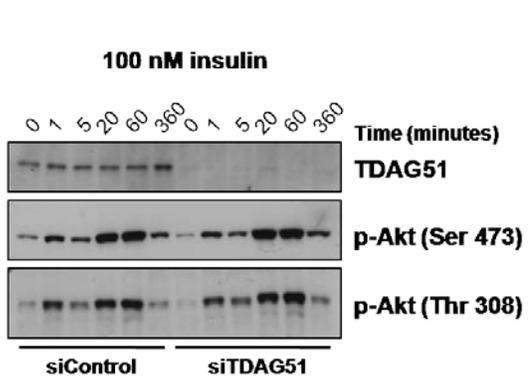
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APPENDIX 1

A.



B.



APPENDIX 1 - Figure Legend

A) TDAG51 expression in response to IGF1/insulin. (Left) Wild-type MEFs were treated with 50 nM IGF-1 in 10% FBS media and protein lysates were collected over 24 hours of treatment. (Right) 3T3-L1 cells were treated with 100 nM insulin in either serum-free (0% FBS overnight) or 10% FBS media conditions for 2 or 24 hours. Protein lysates were collected. Ponceau S staining of the nitrocellulose membrane is shown as an indicator of protein loading.

B) Knock-down or over-expression of TDAG51 in 3T3-L1 cells does not affect insulin receptor signalling. (Left) 3T3-L1 cells were transfected with siRNA targeting TDAG51 or siControl as a transfection control. Cells were treated with 100 nM insulin under serum-starvation conditions and lysates were collected over the course of 6 hours. TDAG51 expression was examined as an indicator of TDAG51 knock-down efficiency. Phosphorylation of Akt (on serine 473 and threonine 308 residues) was assessed as an indicator of insulin responsiveness. (Right) 3T3-L1 cells were transfected with a plasmid containing mouse TDAG51 using lipofectamine. pcDNA3.1 empty vector was used as a transfection control. Cells were treated with 100 nM insulin under serum-starvation conditions and protein lysates were collected over the course of 6 hours. TDAG51 expression was examined to confirm TDAG51 over-expression. Phosphorylation of Akt, Foxo-1 and IRS1 were examined to assess insulin signalling.

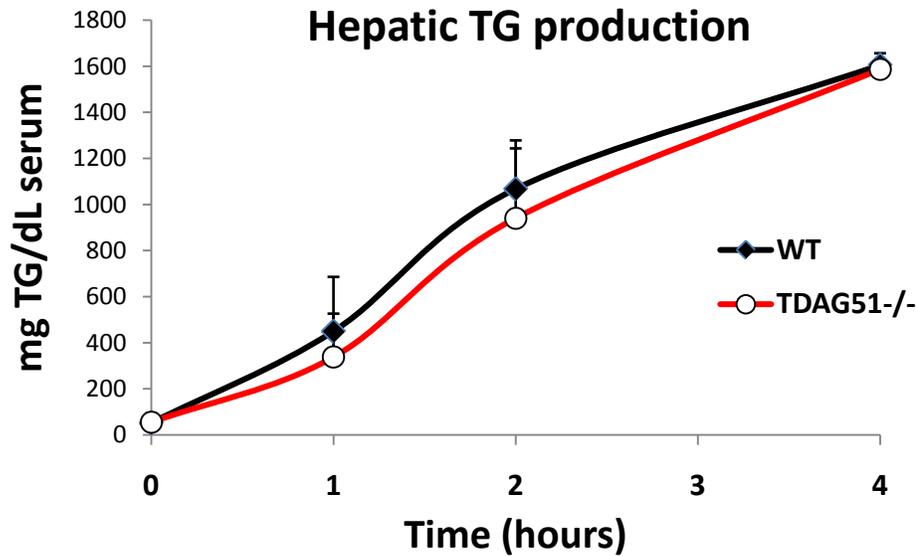
APPENDIX 2**Table 1 - Genes induced in the livers from 28-week old TDAG51^{-/-} mice as compared to WT mice fed a chow diet (* indicates SREBP target genes)***GENES ORGANIZED INTO FUNCTIONAL GROUPS*

	Symbol	Fold Induction
<u>Lipid Metabolism/Insulin Signaling</u>		
glucokinase *	Gck	11.4
ATP-binding cassette, sub-family D (ALD), member 2 *	Abcd2	5.8
CD36 antigen *	Cd36	4.3
D site albumin promoter binding protein	Dbp	3.6
stearoyl-Coenzyme A desaturase 1 *	Scd1	3.5
squalene epoxidase *	Sqle	3.2
acyl-CoA thioesterase 3	Acot3	3.1
acetoacetyl-CoA synthetase *	Aacs	2.8
apolipoprotein L 3	Apol3	2.6
progesterin and adipoQ receptor family member VII	Paqr7	2.2
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 *	Hmgcs1	2.1
hydroxyprostaglandin dehydrogenase 15 (NAD)	Hpgd	2.0
24-dehydrocholesterol reductase *	Dhcr24	2.0
fatty acid synthase *	Fasn	2.0
very low density lipoprotein receptor *	Vldlr	1.9
monoacylglycerol O-acyltransferase 1 *	Mogat1	1.9
<u>Binding/Transporter</u>		
lectin, galactose binding, soluble 1	Igals1	3.9
zinc finger and BTB domain containing 16	Zbtb16	3.4
phospholipid transfer protein	Pltp	2.9
hemoglobin alpha, adult chain 1	Hba-a1	2.9
aquaporin 8	Aqp8	2.8
major facilitator superfamily domain containing 2	Mfsd2	2.6
serine (or cysteine) peptidase inhibitor, clade A, member 6	Serpina6	2.3
solute carrier family 2 (facilitated glucose transporter), member 2	Slc2a2	2.3
solute carrier family 17 (monocarboxylic acid transporters), member 8	Slc17a8	2.1
solute carrier family 16 (monocarboxylic acid transporters), member 13	Slc16a13	2.1
solute carrier family 17 (sodium phosphate), member 3	Slc17a3	2.0
Prenylated Rab acceptor 1 domain family, member 2	Praf2	1.9
<u>Electron transport and redox</u>		
cytochrome P450, family 2, subfamily b, polypeptide 9	Cyp2b9	27.6
cytochrome P450, family 2, subfamily c, polypeptide 38	Cyp2c38	2.1
glutathione S-transferase, theta 3	Gstt3	2.1
cytochrome P450, family 2, subfamily a, polypeptide 4	Cyp2a4	2.0
protocadherin beta 11	Pcdhb11	2.0
glutathione S-transferase, alpha 3	Gsta3	2.0
MAS-related GPR, member B13	Mrgprb13	2.0
<u>Proliferation and apoptosis</u>		
EGL nine homolog 3 (C. elegans)	Egln3	2.4

vanin 1	Vnn1	2.3
cysteine rich protein 61	Cyr61	2.2
cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	Cidea	2.2
kidney expressed gene 1	Keg1	2.1
tensin 1	Tns1	2.0
<u>Regulation of Transcription and or signal transduction</u>		
nuclear receptor subfamily 1, group D, member 1	Nr1d1	2.9
regulator of G-protein signaling 16	Rgs16	2.8
dual specificity phosphatase 6	Dusp6	2.5
GLI-Kruppel family member GLI1	Gli1	2.5
metastasis suppressor 1	Mtss1	2.4
retinoic acid early transcript gamma	Raet1c	2.4
protein phosphatase 1, regulatory (inhibitor) subunit 3B	Ppp1r3b	2.4
transcription elongation factor A (SII)-like 8	Tceal8	2.2
basic transcription factor 3-like 4	Btf3l4	2.0
mitogen-activated protein kinase 4	Mapk4	1.9
<u>Steroid synthetic process</u>		
StAR-related lipid transfer (START) domain containing 4 *	Stard4	2.6
sterol-C4-methyl oxidase-like	Sc4mol	2.1
aldo-keto reductase family 1, member C20	Akr1c20	2.1
hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 3	Hsd3b3	2.0
<u>Steroid binding activity</u>		
nuclear receptor subfamily 0, group B, member 2	Nr0b2	2.6
androgen receptor	Ar	2.4
<u>Miscellaneous</u>		
carbonic anhydrase 3	car3	6.8
arrestin domain containing 3	Arrdc3	5.2
serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antitrypsin, antitrypsin), member 7	Serpina7	2.9
homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Herpud1	2.8
ubiquitin specific peptidase 2	Usp2	2.7
transmembrane and coiled-coil domains 4	Tmco4	2.7
carbonic anhydrase 5a, mitochondrial	Car5a	2.6
liver-expressed antimicrobial peptide 2	Leap2	2.5
carboxylesterase 1	Ces1	2.5
sulfotransferase family, cytosolic, 1C, member 2	Sult1c2	2.4
indolethylamine N-methyltransferase	Inmt	2.2
membrane metallo endopeptidase	Mme	2.2
cAMP-regulated phosphoprotein 19	Arpp19	2.2
dimethylarginine dimethylaminohydrolase 1	Ddah1	2.1
heat shock protein 1	Hspb1	2.0
carboxylesterase 5	Ces5	2.0
beta galactoside alpha 2,6 sialyltransferase 1	St6gal1	2.0

Table 2 - Genes down-regulated in the livers from 28 week-old TDAG51^{-/-} mice as compared to WT mice fed a chow diet*GENES ORGANIZED INTO FUNCTIONAL GROUPS*

	Symbol	Fold Reduction
<u>Lipid Metabolism/Insulin Signaling</u>		
insulin-like growth factor binding protein 1	Igfbp1	4.4
preimplantation protein 4	Prei4	3.6
glutamate oxaloacetate transaminase 1, soluble	Got1	3.4
insulin receptor substrate 2	Irs2	2.3
insulin-like growth factor binding protein 2	Igfbp2	2.2
<u>Metal Ion Binding/Transporter</u>		
monooxygenase, DBH-like 1	Moxd1	35.1
metallothionein 2	Mt2	9.9
asparagine synthetase	Asns	8.6
metallothionein 1	Mt1	4.9
SNF1-like kinase	Snf1lk	4.2
solute carrier family 22 (organic anion transporter), member 7	Slc22a7	3.9
ring finger protein 125	Rnf125	3.7
cadherin 1	Cdh1	3.6
peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Ppargc1a	3.4
solute carrier family 39 (zinc transporter), member 4	Slc39a4	3.4
<u>Cytochromes</u>		
cytochrome P450, family 2, subfamily c, polypeptide 70	Cyp2c70	17.2
cytochrome P450, family 7, subfamily b, polypeptide 1	Cyp7b1	5.3
cytochrome P450, family 17, subfamily a, member 1	Cyp17a1	2.5
<u>Acute Phase Response and Inflammation</u>		
orosomucoid 2	Orm2	47.5
lipocalin 2	Len2	12.7
serum amyloid P-component	Apcs	5.6
orosomucoid 3	Orm3	4.3
serum amyloid A 3	Saa3	3.5
orosomucoid 1	Orm1	3.5
fibrinogen-like protein 1	Fgl1	3.0
<u>Cell differentiation/Cell cycle/Cell Shape</u>		
growth arrest and DNA-damage-inducible 45 gamma	Gadd45g	4.3
SNF1-like kinase	Snf1lk	4.2
CDC42 effector protein (Rho GTPase binding) 5	Cdc42ep5	3.7
cadherin 1	Cdh1	3.6
<u>Miscellaneous</u>		
proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	Prg4	5.5
UDP glucuronosyltransferase 2 family, polypeptide B37	Ugt2b37	4.4
eukaryotic translation initiation factor 4E binding protein 3	Eif4ebp3	4.2
nicotinamide N-methyltransferase	Nnmt	3.2
trefoil factor 3, intestinal	Tff3	3.0

APPENDIX 3**APPENDIX 3 - Figure Legend**

Hepatic triglyceride (TG) production. Wild-type and *TDAG51*^{-/-} male mice at 8 weeks of age were fasted overnight. Blood was collected at time point 0 via the tail vein prior to intraperitoneal administration of 1g/kg poloxamer 407 (lipoprotein lipase inhibitor). Blood samples were also collected at 1, 2 and 4 hours following the P407 injection. Triglyceride concentration was measured in serum samples using a Wako TG assay kit and expressed as mg/dL.

APPENDIX 4

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Sana Basseri, PhD Candidate

Department of Medical Sciences, McMaster University

November 30, 2011

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APPENDIX 5 – Condensed Curriculum Vitae

Sana Basseri

Education

- 2006 - 2012 **Doctor of Philosophy**
PhD in Medical Sciences
McMaster University, Hamilton, ON
- 2002- 2006 **Honours Bachelor of Science**
BSc in Biochemistry and Biomedical Sciences
Graduation with Distinction
McMaster University, Hamilton, ON

Research Experience

Areas of expertise/interest: Obesity, diabetes, insulin resistance, adipose tissue biology, fatty liver disease, lipid and lipoprotein metabolism, glucose metabolism and homeostasis, type 2 diabetes, cardiovascular disease, cellular differentiation, cellular signalling pathways (insulin signalling, endoplasmic reticulum stress/unfolded protein response pathways), cell biology, physiology

- May 2008 - **Ph.D. Student in Medical Sciences**
2012
Supervisor: Dr. Richard Austin, Professor, McMaster University and St. Joseph's Healthcare, Hamilton, ON
- Sep 2006 – **M.Sc. Student in Medical Sciences**
May 2008
Supervisor: Dr. Richard Austin, Professor, McMaster University and Henderson Research Centre, Hamilton, ON
- Successfully transferred to the PhD program in May 2008
- Sep 2005 – **Undergraduate Biochemistry Thesis Project**
April 2006
Supervisor: Dr. Bernardo Trigatti, Associate Professor, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON
- May 2005 – **Undergraduate Summer Research Student**
Sep 2005
Supervisors: Dr. Arya Sharma, Professor of Medicine and Director of the Canadian Obesity Network & Dr. Richard Austin, Professor of Medicine, McMaster University, Henderson Research Centre, Hamilton

Leadership during graduate studies

- Sep 2010 – **Vice-chair of the Canadian Obesity Network-Students and New Professionals (CON-SNP) McMaster Chapter**
 Aug 2011
- Organizing monthly Nutrition and Metabolism Seminar Series with an emphasis on obesity research (basic science and clinical aspects)
 - Advocating for obesity research by organizing a forum for discussion and learning
 - Coordinating events (advertising, fund allocation, speaker invitation and correspondence)
 - Assisting other members with their tasks
- Sep 2009 – **Academic Representative for the Health Sciences Graduate Students Federation (HSGSF)**
 Aug 2010
- Planning academic events for all health sciences graduate students on a monthly basis
 - Locating speakers and organizing academic events (event advertising, refreshments, speaker honorarium, room booking)
- Sep 2009 - **Secretary and Financial Director of the National CON-SNP Executive Committee**
 Aug 2010
- Managing and distributing funds to university chapters across Canada
 - Monthly teleconference meetings with the CON-SNP executives, brainstorming on ideas and providing suggestions
 - Recording meeting minutes
 - Allocating funds and book-keeping
 - Planning the 2nd Canadian Obesity Student Meeting in Ottawa in June 2010
- Sep 2009 – **Vice Chair of the McMaster University Chapter of CON-SNP**
 Aug 2010
- Working closely with the Chair to advance the McMaster University Chapter and planning events and activities on campus
 - Contributing to establishment of the Nutrition and Metabolism Seminar Series at McMaster University
- Nov 2008 - **Activities Director of the McMaster University Chapter of CON-SNP**
 Sep 2009
- Working as a group on advocating for the development and establishment of the McMaster University Chapter and obtaining approval from the University and the Faculty of Health Sciences Graduate Studies
 - Organizing seminar series and invitation of guest speakers/graduate students
 - Working as a group, preparing event posters and crafting letters for departments, staff and students, raising awareness and promoting interest in nutrition and obesity research

Awards & Accomplishments

- 2011-2012 Faculty of Health Sciences Graduate Programs Excellence Award
- 2010-2011 Faculty of Health Sciences Graduate Programs Excellence Award
- 2009-2011 Heart and Stroke Foundation of Canada Doctoral Award (\$21,000/year for 2 years)
- 2009-2010 OGSST Graduate Award – Declined (\$15,000)
 - 2010 Invited oral presentation at the 2nd Canadian Obesity Student Meeting, Ottawa, ON
 - 2010 Invited oral presentation at the Annual Medical Sciences Research Day, Hamilton, ON
 - 2010 Oral Presentation Award at the McMaster University Medical Sciences Research Day
- 2009-2010 Faculty of Health Sciences Graduate Programs Excellence Award
 - 2009 Poster Presentation Award at the Canadian Oxidative Stress Conference in Winnipeg, MB
 - 2009 Invited oral presentation at the FASEB Research Conference, Saxtons River, Vermont
 - 2009 Poster Presentation Award on Faculty of Health Sciences Research Day
 - 2009 Invited oral plenary session at the Obesity Society Conference, Washington, DC
 - 2008 Invited moderated poster presentation at the Canadian Cardiovascular Congress, Toronto, ON
- 2007 - 2008 Heart and Stroke Foundation Master's Studentship Award- Renewal (\$18,000)
 - 2007 Best Poster Award at Health Research in the City Conference, Hamilton, ON (Trophy)
- 2006 - 2007 Heart and Stroke Foundation Master's Studentship Award (\$18,000)
 - 2006 Graduate Entrance Scholarship (\$5000)

Publications, Abstracts & Presentations

Published Refereed Papers (in chronological order)

- **Basseri S**, Austin RC (2012). Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International*. 2012; 841362. Epub Dec 13 2011. (Invited review article)
- Dickhout JG, Lhotak S, Hilditch BA, **Basseri S**, Colgan SM, Lynn EG, Carlisle RE, Zhou J, Sood SK, Ingram AJ, and Austin RC (2011). Induction of the unfolded protein response after monocyte to macrophage differentiation augments cell survival in early atherosclerotic lesions. *FASEB J*. 2011 Feb; 25(2):576-89.

- **Basseri S**, Lhotak S, Sharma AM, Austin RC (2009). The Chemical Chaperone 4-Phenylbutyrate Inhibits Adipogenesis By Modulating The Unfolded Protein Response. *Journal of Lipid Research*. Dec 2009; 50 (12): 2486:501.
- **Basseri S**, Austin RC (2008). ER stress and lipogenesis: a slippery slope toward hepatic steatosis. *Developmental Cell*. Dec 2008; 15: 795-796.
- Dickhout JG, **Basseri S**, Austin RC (2008). Macrophage function and its impact on atherosclerotic lesion composition, progression, and stability: the good, the bad, and the ugly. *Arterioscler Thromb Vasc Biol*. August 2008; 28(8):1413-5.

Papers Submitted/Under Review for Publication

- **Basseri S**, Lhotak S, Fullerton M, Palanivel R, Jiang H, Maclean K, Steinberg G, Austin RC. (2012) Loss of TDAG51/PHLDA1 accelerates lipogenesis resulting in obesity, fatty liver disease and insulin resistance. Submitted, currently under review at *Diabetes*.
- Al-Jarallah A, Zhang Y, Rizvi AA, Tedesco VC, Wei E, Sofowora K, **Basseri S**, Covey SD, Chen X, Kim K-W, Austin RC, Lehner R, and Trigatti BL. (2012) SR-B1 protects macrophages against free cholesterol-induced apoptosis in an HDL dependent manner. Currently in revision.

Published Book Chapters

- **Basseri S**, Austin RC (2012). Chapter 10: Endoplasmic reticulum stress and the unfolded protein response in lipid metabolism and obesity. In: Agostinis P and Samali A (eds), in *Endoplasmic Reticulum Stress in Health and Disease*. To be published by *Springer* in July 2012.
- **Basseri S**, Caldwell J, Sengupta S, Kumar R, Austin RC (2009). Chapter 14: Homocysteine as an endogenous toxin in cardiovascular disease. In: Bruce B and O'Brien P (eds), in *Endogenous Toxins*. Wiley-VCH, 2009, pg 349-378. Published online January 2010.