2011-02-10

Role of the Yeast Ste20 Protein Kinase Ortholog Map4k4 in Adipose Tissue Function: A Dissertation

Kalyani V. P. Guntur
University of Massachusetts Medical School

Role of the Yeast Ste20 Protein Kinase Ortholog Map4k4 in Adipose Tissue Function

A Dissertation Presented

By

KALYANI V. P. GUNTUR

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

FEBRUARY 10, 2011

INTERDISCIPLINARY GRADUATE PROGRAM

ROLE OF THE YEAST Ste20 PROTEIN KINASE ORTHOLOG MAP4K4 IN ADIPOSE TISSUE FUNCTION

A Dissertation Presented By

Kalyani V.P. Guntur

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Michael Czech, Ph.D., Thesis Advisor

Jongsoon Lee, Ph.D., Member of Committee

Heidi Tissenbaum, Ph.D., Member of Committee

Fumihiko Urano, M.D., Ph.D., Member of Committee

Yong-Xu Wang, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Silvia Corvera, M.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the School

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
February 10, 2011

Dedication

I Dedicate This Dissertation to All My Teachers

Acknowledgements

First I would like to express my appreciation and sincere gratitude to my mentor Dr. Michael. P. Czech for his unwavering support, encouragement, guidance, advice and allowing me to learn from my mistakes and grow as a scientist. I would also like to thank him for providing a great work environment that is conducive for team efforts and at the same time promotes independent thinking.

I would like to thank my dissertation committee members Dr. Silvia Corvera, Dr. Heidi Tissenbaum, Dr. Fumihiko Urano, Dr. Young-Xu Wang for their immense support & encouragement all through my graduate career and for teaching me to look at my data in a new perspective. I would also like to thank Dr. Jongsoon Lee for being part of my Thesis Defense committee and for his critical analysis of my work.

I would also like to thank past and present Czech lab members for their helpful discussions and for their friendship. I would like to especially thank Adilson Guilherme for teaching me how to deal with everyday frustrations and disappointments at work and for his friendship. I also would like to thank him for all the healthy arguments and discussions that we had that allowed me to think out loud and helped me learn to better communicate scientific ideas.

I also want to thank Myriam Aouadi for being so patient with me and teaching me mouse work and for her infectious enthusiasm for science. I also would like to thank Sarah Nicoloro for all her help with the mouse work and helpful discussions and her expert advice with RNA work and tissue processing. I am fortunate to have found such a great friend in her and I am grateful to her for being my family away from my family.

I also would like to thank Greg Tesz, Mengxi Wang, and Matthieu Prot for their help with the mouse work. I would like to thank Joe Virbasius, Rachel Roth and Emilie Boutet for helpful discussions and their help with writing my dissertation. I especially would like to thank Rachel Roth and Laura Danai for their friendship. I also would like to thank Sridevi Narasimhan for the helpful discussions and for her friendship. I would also like to thank Alison Burkart for

always providing differentiated adipocytes when in need and for the helpful discussions and for her friendship.

I would also like to thank all my teachers – my parents - my first teachers. All this would not have been possible without the support and encouragement of my parents. Thanks to both my mother and father for teaching me the importance of hard work and for training me to see for the silver lining on a dark cloud. I am in debt to them for all the sacrifices they made to make me reach this far.

I would like to thank Mrs. Hari Priya, my High School Mathematics teacher who saw in me that I did not know I had. She constantly challenged me that helped to bring the best out of me. Her encouragement has changed my life path. I also want to thank Dr. Sudha Ramaswamy and Dr. Padma Reddy my college professors for teaching me to always aim high. I would like to thank Dr. Aparna Dutta Gupta my professor at masters for teaching me the basic bench work and teaching the importance of paying attention to detail. I would also like to thank Dr. Robert Renthal, Professor at UT San Antonio for giving me the opportunity to learn to work independently and for his faith and trust in me.

I want to thank my daughter Srikari for putting up with my crazy schedule and for all the nights and weekends that she missed being with me. Last and most importantly I want to thank my loving husband Sudhakar without his support and encouragement I would not have been in graduate school.

Abstract

Obesity has increased globally in epidemic proportions and as have the associated disorders. Insulin resistance that could further lead to type 2 diabetes is a major obesity associated dysfunction. Studies using insulin resistant mouse models and observations from human subjects exhibiting insulin resistance provide evidence for ectopic lipid deposition in organs like liver, muscle and heart as one of the major risk factors for developing insulin resistance. These observations suggest that deregulated adipose function to sequester and store excess energy as fat, could lead to insulin resistance. Furthermore, several studies have demonstrated adipose tissue dysfunction leading to inflammation and related syndromes. Interestingly, a mouse model with transgenic expression of glucose transporter in the adipose tissue exhibited improved glucose tolerance and increased insulin sensitivity despite development of obesity, upon high fat feeding. Thus mechanisms that improve adipose function could alleviate insulin resistance and associated diseases.

Mitogen activated protein kinase kinase kinase kinase 4 (MAP4K4) was identified in our laboratory as a negative regulator of adipocyte function. Interestingly, siRNA mediated knockdown of MAP4K4 promoted PPARγ protein expression. Additionally, silencing of MAP4K4 increased adipocyte triglyceride content. Because MAP4K4 is a negative regulator of PPARγ expression and adipocyte function, understanding the mechanism by which MAP4K4 regulates PPARγ expression is of interest. Thus, for the first part of this thesis, I characterized the signaling pathways utilized by MAP4K4 to regulate PPARγ expression in cultured adipocytes. Here I show that MAP4K4 regulates PPARγ expression through regulation of its protein translation. siRNA mediated MAP4K4 gene silencing stimulated PPARγ protein

synthesis without changing its mRNA transcription or its protein degradation. This increase in PPARγ protein translation was due to an increase in the activity of mammalian target of rapamycin (mTOR). The increase in PPARγ protein expression mediated by mTOR activation was a specific effect of the 4E-BP1 phosphorylation that leads to its inactivation and was not a general increase in mTOR activity towards all of its substrates. Finally, adenovirus mediated over expression of MAP4K4 inhibited mTOR activation, and suppressed PPARγ protein translation.

For the second part of this thesis, I assessed the role of MAP4K4 in adipocytes in vivo. To accomplish this, a lentivirus mediated shRNA construct was generated to attenuate MAP4K4 expression selectively in the mouse adipose tissue. First we demonstrate that the MAP4K4 shRNA construct is able to efficiently silence the expression of MAP4K4 *in vitro* when co-expressed with Cre recombinase. Furthermore, we show that following modification of the lentiviral conditional vector that was introduced into a mouse embryo at one cell stage, and crossing the resulting founders with aP2-Cre mice, adipose tissue specific MAP4K4 gene silencing was achieved. Moreover, shRNA mediated gene silencing is a faster and an inexpensive means of achieving tissue specific gene knockdown relative to the available traditional gene knockout approaches.

Utilizing these adipose specific MAP4K4 gene knockdown mice, I reveal that MAP4K4 silencing enhanced fat mass as well as PPARγ expression significantly. This is accompanied by improved whole body insulin sensitivity. Furthermore, when challenged with high fat diet, adipose-specific MAP4K4 silenced mice exhibit enhanced adiposity with decreased lean mass. Moreover, adipocyte cell size and triglyceride content are significantly increased.

Interestingly, despite increased adiposity, hepatic insulin sensitivity is significantly improved leading to decreased glucose output. Thus MAP4K4 is an important regulator of adipocyte function that mediates whole body glucose homeostasis, through a mechanism that is yet to be identified.

Table of Contents

Approval Page……ii

Dedication……..……iii

Acknowledgements……iv

Abstract…….…..vi

List of Tables……xiv

List of Figures……...xv

List of Frequently Used Abbreviations…….xix

Copyright Information…….xxii

CHAPTER I: Introduction……..... 1

Adipogenesis…….. 2

Insulin Signaling……. 7

Insulin Activation of PI3K/Akt Pathway……...10

Insulin Activation of MAPK Pathway……...11

Role of mTOR in Metabolic Regulation……12

mTOR Binding Partners……16

TSC1/TSC2 Mediated mTOR Regulation……18

PI3K/Akt Independent mTOR Regulation……. 19

mTOR Substrates…….21

mTOR Regulation of Protein Synthesis……..24

Glucose Metabolism - Glucose Uptake……..27

Regulation of Glycogen Synthesis…….29

Gluconeogenesis……30

Regulation of Gluconeogenesis……..32

Lipid Metabolism - Lipogenesis…….33

Regulation of Lipogenesis……..34

Lipolysis and Fatty Acid Oxidation……...36

Insulin Resistance……...41

Role of Insulin……44

Role of S6K1 in Inducing Insulin Resistance……45

Role of Adipokines in Mediating Insulin Sensitivity and Resistance…46

Adiponectin……46

Leptin…….48

Role of Fatty Acids and Lipid Mediators in Promoting Insulin

Resistance……50

Inflammation……...51

Immune Cell Recruitment……...51

Cytokines……54

Role of TNF-α in Mediating Insulin Resistance……...….54

NFκB Regulation……...…55

Activator Protein-1 (AP-1) Regulation…….…....58

Role of Interleukins in Promoting Insulin Resistance - Interleukin-1…...61

Interleukin-6……...…63

Role of PPARs in Insulin Sensitivity and Resistance……...64

Regulation of PPARγ……....67

MAP4K4……...68

Specific Aims……73

CHAPTER II: Map4k4 Negatively Regulates Peroxisome Proliferator-activated Receptor (PPARγ) Protein Translation by Suppressing the Mammalian Target of Rapamycin (mTOR) Signaling Pathway in Cultured Adipocytes……74

Abstract……75

Introduction…….76

Experimental Procedures…….78

Results…….82

Map4k4 Gene Silencing Enhances PPARγ Protein Expression at a Translational Step……82

Protein Synthesis and mTOR Activation Are Up-regulated upon Map4k4 Gene Silencing…..88

Map4k4 Depletion Enhances 4E-BP1 Phosphorylation……..91

4E-BP1 and 4E-BP2 Are Required for Map4k4 Regulation of PPARγ Protein……96

Up-regulation of PPARγ Level upon Map4k4 Depletion Is Suppressed by mTOR Inhibition...100

Enhanced Cell Size upon Map4k4 Gene Silencing in 3T3-L1 Adipocytes…….102

Adenovirus-mediated Map4k4 Overexpression Decreases mTOR Phosphorylation……..104

Discussion……108

Limitations and Future Perspectives ……...……112

CHAPTER III: Adipose-Specific Knockdown of MAP4K4 Results in Obese Mice with Enhanced Insulin Sensitivity……116

Contributions……...116

Abstract……117

Introduction……..118

Experimental Procedures…….120

Results…….125

Generation and Validation of Adipose-Specific Map4k4 Knockdown Mice: shRNA-pSico vector construct……..…125

Conditional RNAi in Mice……...…128

Map4k4 Deficiency in Adipose Tissue Enhances Adiposity……...132

Contribution of Brown Adipose Tissue to the Map4k4 Knockdown Phenotype. ……..134

Map4k4 Knockdown in the Adipose Tissue Enhances PPARγ Protein Expression……...136

Adipose Specific Map4k4 Gene Silencing Regulates Whole body Insulin Sensitivity……..138

Enhanced Adiposity with Decreased Lean Mass in the Adipose Specific Map4k4 Knockdown Animals upon High Fat Feeding…….141

Map4k4 Deficient Adipose Tissue Regulates Hepatic Insulin Sensitivity……..144

Role of Adipose Cell Size in the KD Mouse Phenotype…….148

Discussion……151

Limitations and Future Perspectives……163

CHAPTER IV: Discussion……..173

Regulation of PPARγ Expression by MAP4K4 in Adipocytes……..174

Adipose MAP4K4 Depletion Regulates Hepatic Insulin Sensitivity…….177

References……..184

List of Tables

Table 2.1 Primer sequences used in Chapter II……...107

List of Figures

FIGURE 1.1 Adipogenesis is a multi step process……..3

FIGURE 1.2 A complex transcriptional cascade regulates adipogensis……..5

FIGURE 1.3 Insulin Signaling Mediated Regulation of Metabolic Pathways……9

FIGURE 1.4 Upstream and Downstream of mTORC1 Signaling in Mammalian Cells...14

FIGURE 1.5 mTOR mediated control of protein synthesis ……..25

FIGURE 1.6 Lipid Storage and Lipolysis in Adipocytes……..44

FIGURE 1.7 Regulation of Insulin Resistance – Mediators and Pathways……...48

FIGURE 1.8 Map4k4 functions in different cell types……..84

FIGURE 2.1 Gene silencing of *Map4k4* enhances PPARγ protein but not PPARγ mRNA in 3T3-L1 adipocytes…….96

FIGURE 2.2. Depletion of *Map4k4* does not prolong the half-life of PPARγ protein in 3T3-L1 adipocytes……..……98

FIGURE 2.3. Regression Analysis……..……...99

FIGURE 2.4. *Map4k4* gene silencing enhances protein synthesis and mTOR phosphorylation……102

FIGURE 2.5. *Map4k4* gene silencing in 3T3-L1 adipocytes enhances 4E-BP1 phosphorylation……105

FIGURE 2.6 Map4k4 gene silencing does not enhance p70S6K phosphorylation……106

FIGURE 2.7. Map4k4 gene silencing does not change eIF2-α phosphorylation……107

FIGURE 2.8. 4E-BP1 and 4e-BP2 silencing does not enhance PPARγ protein level….109

FIGURE 2.9. 4E-BP1 and 4e-BP2 are required for increased PPARγ protein levels upon *Map4k4* gene silencing……110

FIGURE 2.10. Rapamycin inhibits *Map4k4* regulation of 4E-BP1 phosphorylation and PPARγ expression……113

FIGURE 2.11. Depletion of Map4k4 enhances cell size in 3T3-L1 Adipocytes……...103

FIGURE 2.12. Adenovirus-mediated overexpression of *Map4k4* decreases PPARγ protein level as well as mTOR (Ser-2448) phosphorylation in 3T3-L1 adipocytes……....105

FIGURE 2.13. Model for *Map4k4*-mediated PPARγ protein regulation……106

FIGURE 2.14 Map4k4 mediated differential regulation of mTOR substrates p70S6K1

and 4E-BP1……..113

FIGURE 3.1. Generation of shRNA and gene silencing of MAP4K4 in 3T3-L1 cells..127

FIGURE 3.2. Conditional knockdown of MAP4K4 in transgenic mice…….130

FIGURE 3.3. Specificity of Map4k4 gene silencing……...131

FIGURE 3.4. MAP4K4 silencing in adipose tissue enhances adiposity…….133

FIGURE 3.5. Metabolic Parameters……135

FIGURE 3.6. MAP4K4 regulates PPARγ synthesis in adipocytes.....137

FIGURE 3.7. MAP4K4 knock-down mice exhibit enhanced whole body insulin sensitivity…….....139

FIGURE 3.8. Glucose metabolism in SD fed condition…….140

FIGURE 3.9. MAP4K4 silencing in adipose tissue increases high fat diet induced obesity……...142 & 143

FIGURE 3.10. Graphical representation of Metabolic Parameters…….146

FIGURE 3.11. MAP4K4 deficiency in adipose tissue causes increased hepatic insulin sensitivity…….147

FIGURE 3.12. MAP4K4 knockdown results in adipocyte hypertrophy……149

FIGURE 3.13. Adipose tissue and liver triglyceride content……..150

FIGURE 3.14. Circulating hormone levels……..154

FIGURE 3.15. Graphical representation of plasma cytokine levels……157

FIGURE 3.16. Enhanced Adiposity in 18 month old mice…….159

FIGURE 3.17. Map4k4 in the adipose regulates liver insulin sensitivity……...162

FIGURE 3.18 Hypothetical model for Map4k4 mediated IL-1α regulation …….165

FIGURE 3.19 Map4k4 regulates p70S6K1……167

FIGURE 3.20 Hypothetical model for Map4k4 function in the liver…….169

FIGURE 3.21 Hypothetical model for role of Map4k4 stimulated adipocyte lipolysis in regulating macrophage recruitment and thus hepatic gluconeogenesis ……..171

FIGURE 4 Role of adipocyte Map4k4 in inducing insulin resistance – a hypothetical model ……..182

List of Frequently Used Abbreviations

Abbreviation	Term
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
PPARγ	Peroxisome proliferator-activated receptor-γ
mTORC1	mammalian target of rapamycin complex-1 (mTOR)
mTORC2	mammalian target of rapamycin complex-2
4E-BP1	eIF4E-binding protein-1
eIF4-E	eukaryotic initiation factor 4-E
p70S6K1	p70 ribosomal S6 kinase-1
siRNA	small interfering RNA
shRNA	short hairpin RNA
KD	pSico-ShMap4k4-aP2Cre (knockdown)
C	pSico-ShMap4k4 (control)
Cre	cre recombinase
aP2/Fabp4	fatty acid-binding protein – 4
TAG	Triacylglycerol
FFAs	free fatty acids
IL-1α	Interleukin-1α
IRS-1/2	Insulin receptor substrate 1/2

IR	Insulin receptor
Akt	protein kinase B
PI3K	phosphatidylinositol 3-kinase
GLUT4	glucose transporter-4
MAPK	mitogen activated kinase
ATMs	adipose tissue macrophages
DIO	diet induced obesity
SD	standard diet
HFD	high fat diet
TNFα	Tumor necrosis factor-α
MCP-1	macrophage chemoattractant protein-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
PPARα	Peroxisome proliferator-activated receptor-α
PPARβ/δ	Peroxisome proliferator-activated receptor-β/δ
C/EBPα	CCAAT/enhancer-binding protein-α
C/EBPβ	CCAAT/enhancer-binding protein-β
C/EBPγ	CCAAT/enhancer-binding protein-γ
C/EBPδ	CCAAT/enhancer-binding protein-δ

ERK-1/2	extracellular signal-regulated kinases ½
JNK 1-3	Jun amino terminal kinases 1-3
PRAS40	proline rich substrate of Akt1-40
TSC1	Tuberous Sclerosis Complex-1 (Hamartin)
TSC2	Tuberous Sclerosis Complex-2 (Tuberin)
Rheb	RAS activated in brain
AMPK	AMP kinase
NFκB	nuclear factor-κB
IKKβ	inhibitory κ-B kinase β
IκBα	inhibitory κ-B α
ATGL	adipose triglyceride lipase
HSL	hormone sensitive lipase
PKA	protein kinase A
GyK	glycerol kinase
GCKs	germinal center kinases
PEPCK	phosphoenolpyruvate carboxykinase
G-6-Pase	glucose-6-phosphatase
PKC	protein kinase C
TZDs	Thiazolidinediones

Copyright Information

CHAPTER I: Introduction

Adipose tissue is a specialized connective tissue that functions to store fat in the form of triglycerides. As the major and efficient way of energy storage, fat provides a buffer for imbalances when energy intake is not equal to energy output. Great strides have been made in the field of adipose biology since Conrad Gessner a Swiss naturalist, first identified the adipose tissue in 1551. But adipose tissue continues to be an enigma. Instead of being considered a passive storage depot, adipose tissue is now recognized as an active metabolic as well as endocrine organ. Lipodystrophy, characterized by the selective loss of adipose tissue, and obesity characterized by excess accumulation of body fat, represent two extremes of adipose tissue dysfunction. Much attention has been focused on obesity, due to the global catastrophic rise in its prevalence and its associated metabolic diseases. The risks associated with obesity include and are not limited to hypertension, coronary heart disease, stroke, pulmonary abnormalities, arthritis, gall bladder disease, cancer and most importantly, type 2 Diabetes.

Insulin resistance is a hallmark of type 2 diabetes. Insulin is synthesized and secreted by the β-cells present in the islets of Langerhans of the pancreas, in response to several stimuli that include protein and glucose in the blood produced from the ingested food. In mammals, liver, muscle and adipose form the major insulin responsive tissues. Along with the key storage function, adipose tissue also communicates with the other metabolic tissues, and forms a major source of information of its own nutritional status, as well as of the whole organism. Even though numerous important discoveries made

during the last 15 years have added substantially to the vast knowledge of adipose tissue biology, much remains to be known. Better understanding of the mechanisms involved in the dysfunction of the adipose tissue, might provide potential new therapeutic strategies for the treatment of obesity associated insulin resistance.

Adipogenesis

Two types of adipocytes are known in mammals, brown and white, which have distinct physical as well as molecular and physiological properties. These adipocytes are found in specific regions of the body as adipose depots, as well as in combination with other cell types such as connective tissue. Subcutaneous and the visceral areas form the two major locations of white adipocytes that differ in their physiological and molecular functions, where increase in visceral adipose tissue correlates with elevated risk for insulin resistance and cardiovascular disease, while increase in subcutaneous adipose tissue does not [1-4].

Adipogenesis is a complex process involving a temporally regulated set of gene expression events that involve cell cycle proteins, transcriptions factors, co-activators and co-repressors. Much of the knowledge of adipogenesis is from observations made in cell culture systems, such as 3T3-L1 mouse preadipocytes. The process of adipocyte differentiation involves two phases (summarized in Figure 1.1). The commitment of a pluripotent stem cell to the adipocyte lineage [5] is the first phase, known as determination; terminal differentiation is the second phase, where the preadipocyte acquires the characteristics of mature adipocyte.

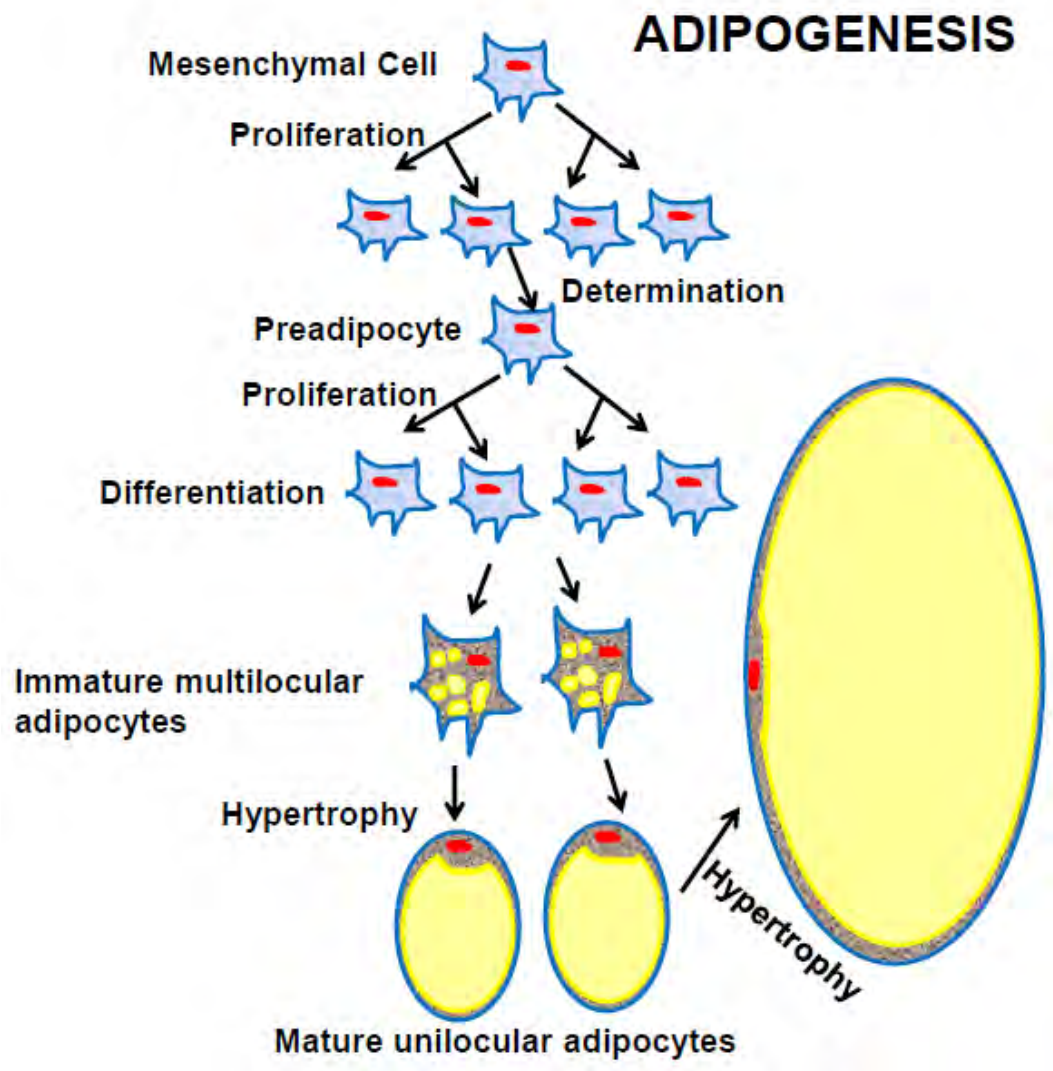


FIGURE 1.1: Adipogenesis is a multi step process. Mesenchymal cells proliferate (clonal-expansion). Some of these cells differentiate into preadipocytes. Preadipocytes proliferate at the site of adipogenesis (clonal-expansion). Preadipocytes undergo a second differentiation step and begin to accumulate lipids. Lipids in the cell appear as small droplets (multilocular cells) that later fuse into one large droplet (unilocular cells). The adipocyte can continue to enlarge by accumulating additional lipids. The average mesenchymal cell is 10-20µm in diameter, while primary adipocytes reach 100-200µm in diameter.

CCAAT/enhancer-binding proteins [6] are a family of transcription factors that include the isoforms α, β, γ, δ and CHOP (transcription factor homologous to CCAAT/enhancer-binding protein). While the isoforms α, β, δ exhibit pro-adipogenic functions [7], γ and CHOP are anti-adipogenic [8]. C/EBPβ and C/EBPδ expression is induced early during adipogenesis. Sequential expression of C/EBPβ and C/EBPδ leads to the expression of C/EBPα and peroxisome proliferator-activated receptor-γ (PPARγ) [9]. Gain and loss of function studies have identified PPARγ, a nuclear hormone receptor and a transcription factor, to be necessary and sufficient for adipogenesis. Studies involving mouse embryonic fibroblasts (MEFs) lacking C/EBPα have shown that, ectopic expression of PPARγ in these cells induced adipogenesis, however, C/EBPα could not rescue adipogenesis in the absence of PPARγ [10], thus establishing PPARγ as the master regulator of adipogenesis [11]. Although, transgenic expression of PPARγ was sufficient to induce adipogenesis and lipid accumulation, C/EBPα is necessary for acquiring insulin sensitivity [10]. Crucial signaling pathways in adipogenesis have been demonstrated to converge on the regulation of PPARγ expression or activity [11]. The complex transcriptional cascade of that regulates adipogenesis is summarized in Figure 1.2.

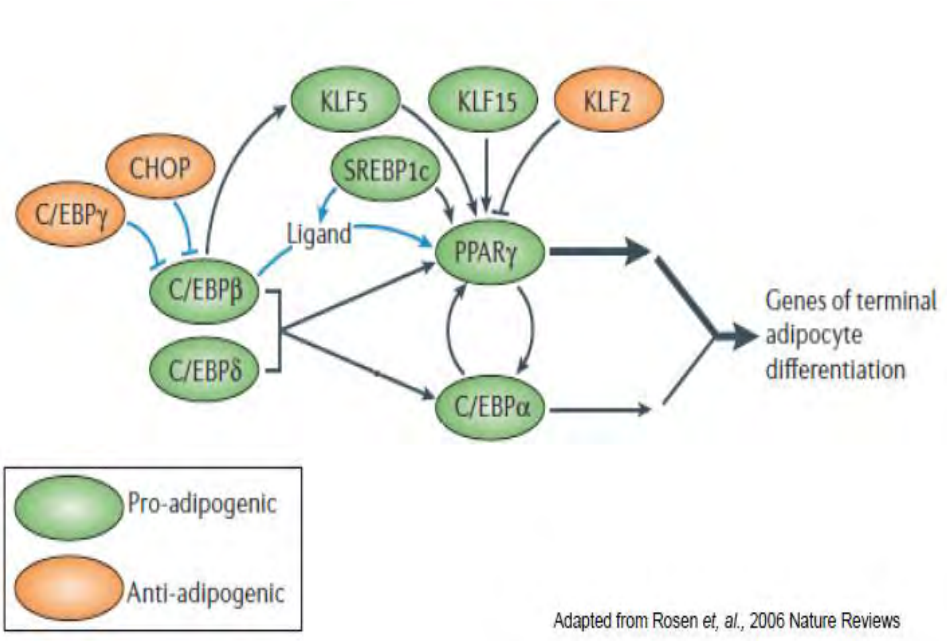


FIGURE 1.2: A complex transcriptional cascade regulates adipogenesis. PPARγ is at the core of the transcriptional cascade that regulates adipogenesis. The expression of PPARγ is regulated by several pro-adipogenic and anti-adipogenic factors. PPARγ itself is activated by an unknown ligand. C/EBPα is regulated through a series of inhibitory protein-protein interactions. Several members of KLF family of ttransciption factors participate in adipogenesis. Black lines – effects on gene expression. Blue lines – effects on protein activity.

Cell cycle proteins also function as cofactors of adipogenesis. CDK6 binds to and activates PPARγ, through phosphorylation [12]. CDK4 is also involved in activating PPARγ without affecting phosphorylation [5]. To the contrary, CDK1 has been shown to inhibit PPARγ function [13]. Several members of the family of transcription factors Krüppel-like factors (KLFs) also regulate adipogenesis. While KLF 5 & 15 promote adipogenesis [14], KLF 2 & 7 inhibit adipose differentiation [6, 15-17]. KLF15 also induces the expression of the insulin sensitive glucose transporter GLUT4 [17]. The differential recruitment of co-repressors and co-activators to PPARγ promoter region maintains adipogenesis. TRAP220 (also called as PPAR-binding protein; PBP) is a known binding partner of PPARγ [18]. PRIP (PPAR interacting protein) has been identified as a co-activator of PPARγ [19]. NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor) function as co-repressors of PPARγ [20]. Co-repressors recruit HDACs (histone deacetylases) to target promoters thereby blocking transcription. PPARγ is required for the release of histone deacetylase from C/EBPα promoter, for C/EBPβ to induce the expression of C/EBPα [21].

There are three major subfamilies of MAPKs: the extracellular signal-regulated kinases (ERK1/2), the Jun amino terminal kinases (JNK1-3) and the p38 kinases (p38 α, β, γ). The initial studies performed to analyze the role of ERK MAP kinase in adipogenesis was contradictory, however, further studies demonstrated that ERK1 activity is required for the proliferative phase of adipogenesis, and this signaling cascade has to be turned off during the second phase of terminal differentiation [22]. The

important role played by ERK1 in adipocyte differentiation is further demonstrated by the impaired adipogenesis exhibited by the mouse embryonic fibroblasts (MEFs) derived from ERK1-/- animals [23]. Furthermore, the residual adipogenesis seen in ERK1-/- cells is not inhibited by pharmacological ERK inhibitor. These studies demonstrated that ERK2 possibly has no function in adipocyte differentiation. A positive role for p38 MAPK in adipogenesis was described by Engelman and colleagues where, chemical inhibition of p38 decreased 3T3-L1 differentiation [24, 25]. However, expression of an upstream activator of p38 for long duration resulted in adipocyte cell death. To the contrary, p38 mediated CHOP phosphorylation that subsequently inhibits C/EBP results in inhibition of adipocyte differentiation [26]. JNK has not been implicated in regulation of adipocyte differentiation. Together, these studies have put forward a complex cascade of events that involves a delicate balance of interactions between several different families of transcription factors, cell cycle checkpoint proteins, co-repressors and co-activators that participate in the process of adipocyte differentiation. Along with many other hormones and proteins, insulin plays an important and major role in regulating adipogenesis.

Insulin Signaling

Insulin action is initiated once insulin interacts with its cell surface receptor. Preadipocytes express more of IGF1 (insulin like growth factor-1) receptors than insulin receptors. This changes as the adipocytes differentiate. Insulin functions through IGF-1 signaling in the early stages of adipogenesis. The insulin receptor (IR) is composed of

two extracellular α subunits and two transmembrane β subunits that are linked together by disulphide bonds. Upon insulin binding to the α subunit, a conformational change is induced that results in the autophosphorylation of a number of tyrosine residues present in the β subunit [27]. The first adaptor protein identified was the insulin receptor substrate-1 (IRS-1); it contains phosphotyrosine-binding (PTB) domains that recognize these residues [28, 29], and is recruited through it‘s pleckstrin homology (PH) domain. Insulin receptor interacts with other adaptor proteins, including IRS-2, -3 and -4 along with SHC, GAB2, DOCK1/2, APS [30-32]. However, the majority of insulin‘s metabolic effects appear to be mediated by IRS-1 and IRS-2. Receptor activation leads to the phosphorylation of key tyrosine residues on IRS proteins, some of which are recognized by the Src homology 2 (SH2) domain containing proteins [30]. There are at least 23 known tyrosine residues phosphorylated on IRS protein [33]. The regulatory subunit of PI3K (p85 – a lipid kinase) and the growth factor receptor bound protein-2 (GRB2) form the two main branches of insulin signaling, leading to the activation of Akt (also known as protein kinase-B (PKB)) and mitogen activated protein kinases (MAPKs) respectively. While Akt regulates utilization, production and uptake of glucose, MAPKs regulate cell growth, gene expression and differentiation [34]. A brief summary of insulin signaling is depicted in Figure 1.3.

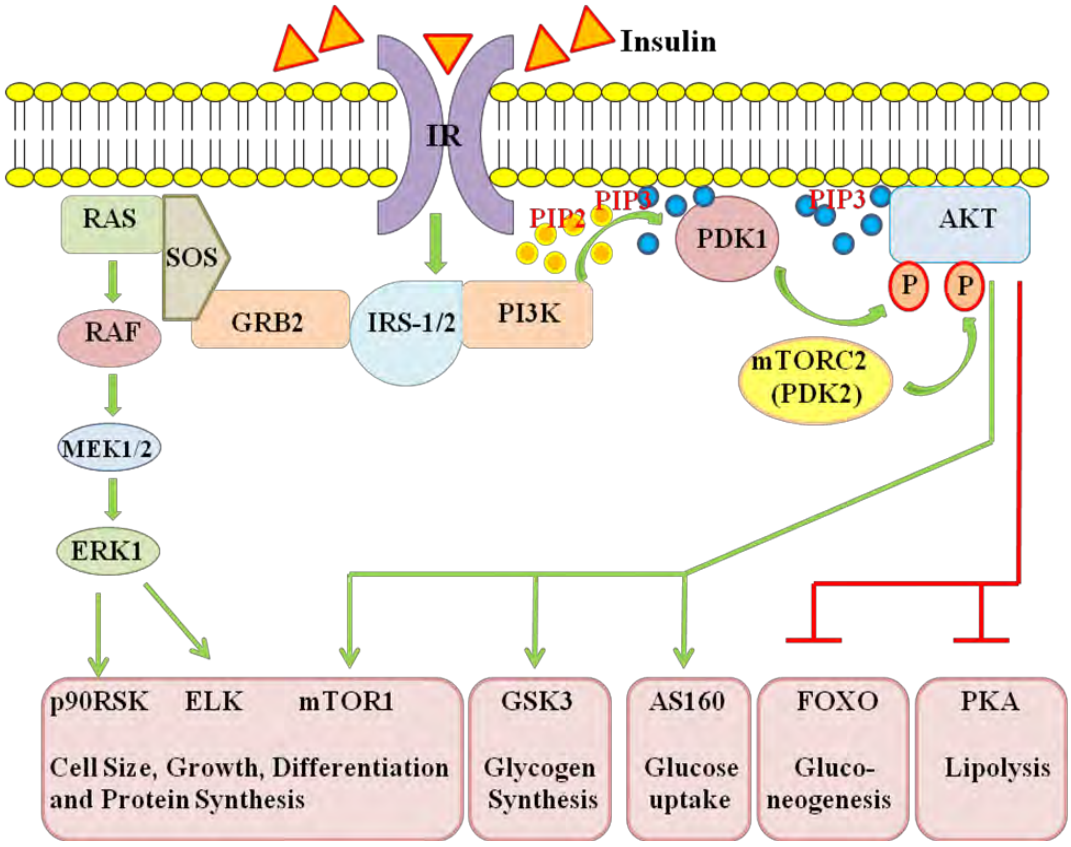


FIGURE 1.3: Insulin Signaling Mediated Regulation of Metabolic Pathways. Insulin binding to the insulin receptor (IR) stimulates its kinase activity that leads to the activation of insulin receptor substrate 1 (an adaptor protein) (IRS1). Tyrosine phosphorylated IRS1 functions as a docking site for the growth factor receptor bound protein 2 (GRB2) and phosphatidylinositol 3-kinase (PI3K). By a cascade of activating events extracellular-signal regulated kinase [35] pathway is activated by GRB2. Protein kinase B (Akt) pathway is activated by the PI3K mediated signaling events. Downstream targets of ERK and Akt regulate the indicated processes. Glycogen synthesis is stimulated in the muscle and liver; glucose uptake is regulated in the muscle and adipose tissue, gluconeogenesis is inhibited in the liver; lipolysis is inhibited in the adipose tissue.

Insulin Activation of PI3K/Akt Pathway

There are multiple isoforms of PI3Ks that are classified into three classes. Akt is activated by Class IA PI3Ks that consists of p110β catalytic subunit and p85α adaptor/regulatory subunit in insulin sensitive cells. The catalytic and regulatory subunits are derived from alternate splicing of three genes, resulting in 3 catalytic and 8 regulatory subunits [34]. The observations that, chemical inhibitors of PI3K like LY294007 and Wortmannin, as well as ectopic expression of dominant negative PI3K, could completely abrogate insulin stimulated Akt activation, underscores the requirement of PI3K in insulin signaling [36]. Stimuli that induce IRS tyrosine kinase activity lead to the activation of PI3Ks, to phosphorylate the 3‘- OH of inositol ring in inositol phospholipids, generating 3‘-phosphoinositides (PI); thus, resulting in three lipid products PtdIns3-monophosphate, PtdIns(3,4)-diphosphate (PIP2) and PtdIns(3,4,5)-triphosphate (PIP3) [37].

PDK1 was identified by its ability to phosphorylate Akt, and this function required the presence of PIP2 or PIP3 in vitro, thus giving the name 3‘-phosphoinositide-dependent kinase-1 (PDK1) [38, 39]. Ubiquitously expressed, this 63kDa serine/threonine kinase is a constitutively active cytosolic protein. PIP3 once generated by activated PI3K, directs the translocation of PDK1 to the membrane where, it activates PKB (Akt) by phosphorylating Thr308 on the activation loop. Complete activation of Akt requires the phosphorylation of a second site Ser473 present on the hydrophobic motif. The identity of the kinase, considered as PDK2, that phosphorylates this second site on

Akt has been obscure. Recent studies have identified mTOR2 complex (consisting mTOR, Rictor, mLST8) as the elusive PDK2 [40]. Insulin stimulated Akt-Ser473 phosphorylation was prevented in the mouse models with genetic ablation of Rictor in fat cells [41], as well as studies with muscle specific Rictor knockout [42]. Interestingly, mTOR2 complex activates Akt, while mTOR1 complex (made up of mTOR, Raptor and mLST8) is one of the downstream effectors of Akt [43]. Akt is a 57 kDa serine/threonine protein kinase, and is homologous to the protein kinases A and C (PKA & PKC). Insulin stimulated metabolic functions of Akt are elicited by phosphorylation of substrates, that include (i) AS160 – functions to regulate glucose uptake through GLUT4 membrane translocation in fat and muscle [16] GSK3 – mediates glycogen synthesis regulation in liver and muscle [16] inhibition of FOXO nuclear translocation, thus preventing gluconeogenesis [15] TSc1 & TSc2 complex that in turn modulate mTOR1 complex to regulate cell size, growth & differentiation and protein synthesis (v) PKA – to inhibit lipolysis. Several of these pathways involved in the regulation of insulin‘s metabolic functions also participate either directly or indirectly in the process of adipogenesis.

Insulin Activation of MAPK Pathway

The second branch of insulin signaling through IRS is to the GRB2 adaptor protein and its effectors [44]. Shc (*src* homology 2/α-collagen-related) is a ubiquitously expressed cytosolic protein that is stimulated by several growth factors and cytokines, competes with IRS as substrate of insulin receptor. Insulin induced Shc tyrosine phosphorylation stimulates Shc/GRB2 association [45] [45]. Son of sevenless [46], a

guanine nucleotide-exchange factor is constitutively associated with GRB2. SOS catalyzes the GDP to GTP exchange, thus activating RAS, a membrane bound GTPase. Activated RAS then recruits and activates RAF1 kinase (v-raf-leukemia viral oncogene 1), that in turn activates MEK1/2 (mitogen activated protein kinase kinase 1 and 2. Extracellular-signal regulated kinases 1 and 2 (ERK1/2) are phosphorylated and activated by MEK1/2 [47, 48]. ERK1/2 then phosphorylate many substrates that include ELK1, an ETS (the ternary complex) family member [48]. ELK1 forms complexes with serum response factors and promotes the transcription of serum response element containing genes that then regulate cell growth and proliferation [49]. These functions are also mediated by p90 ribosomal S6K (p90S6K), another substrate of ERK1/2, that activates ribosomal S6 protein to stimulate protein synthesis [50]. Interestingly, JNK activation is independent of the PI3K catalytic subunit binding to the p85 subunit; instead it involves the small GTPase cell division control protein 42 (CDC42) [51]. However, insulin stimulated JNK activation results in insulin resistance that would be discussed later. The mechanism of insulin signaling mediated p38 activation is not known. Similar to the Akt branch of insulin signaling, the MAP kinases also play an important role not only in the metabolic actions of insulin but also regulate adipogenesis [52].

Role of mTOR in Metabolic Regulation

Adipose tissue is an information treasure trove for the nutritional status of the whole organism. Possibly, by employing a nutritional sensor, adipocytes recognize, assimilate and decipher their own metabolic status that is later relayed to other metabolic

tissues. It would not be erroneous to speculate that adipocytes might actually utilize mammalian target of rapamycin (mTOR) to this end. Being a master regulator of cell growth, TOR plays a central role in development & aging and is associated with disorders like cancer, cardiovascular disease, obesity and diabetes. Many cellular processes including protein synthesis, ribosome biogenesis, nutrient transport and autophagy are controlled by mTOR. Studies from yeast, worms, flies as well as mammalian cell lines and animal models have provided the basic understanding of mTOR biology. First identified as a target of the anti-fungal & anti-proliferative drug rapamycin in yeast, and later also observed in many mammalian cells, mTOR is an atypical serine/threonine protein kinase, that belongs to the phosphatidylinositol kinase related kinase (PIKK) family [53-57]. Structurally, mTOR is made up of 20 tandem HEAT (a protein-protein interaction structure), domains on the N-terminus, followed by FAT (FRAP, ATM and TRRAP) domain, FRB (FKBP12/rapamycin binding) domain, Kinase domain and FATC (FAT c-terminus) on the C-terminus [58]. mTOR knockout mice are embryonic lethal, demonstrating the importance of mTOR in cell growth and proliferation [59-61]. Upstream regulators and downstream targets of mTOR are summarized in Figure 1.4.

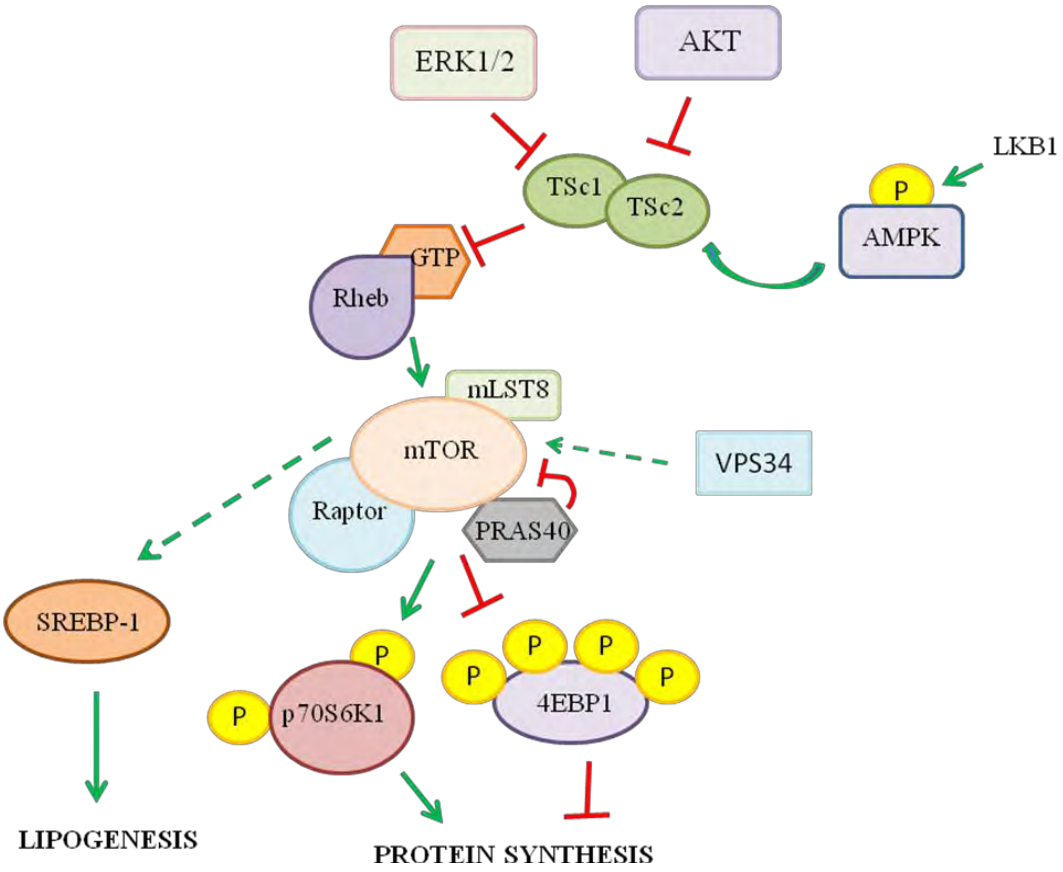


FIGURE 1.4: Upstream and Downstream of mTORC1 Signaling in Mammalian Cells. The mammalian target of rapamycin complex 1 (mTORC1) consists of mTOR, Raptor, mLST8 and PRAS40. Through tuberous sclerosis complex 1 &2 (TSc1/TSc2) – Ras homologue enriched in brain (Rheb) axis mTORC1 integrates growth factor signaling mediated by protein kinase B (Akt), extracellular-regulated kinase 1 & 2 (ERK1/2), cellular energy levels through AMP Kinase (AMPK), and amino acid signaling through VPS34, to regulate protein translation by phosphorylating S6K1 and 4EBP1 and to stimulate lipogenesis by activating sterol regulatory element-binding protein 1 (SREBP1).

Studies by McKnight and his coworkers which identified FKBP51, as an immunophilin that bound to rapamycin and inhibited clonal expansion in 3T3-L1 preadipocytes was the first to implicate mTOR in adipogenesis [62]. Moreover, studies by Sorisky and colleagues have shown that rapamycin exhibited potent negative effects on triacylglycerol accumulation as well as C/EBPα and PPARγ protein expression in the later stages of adipogenesis after clonal expansion, suggesting the continued requirement of mTOR for normal adipocyte function [63]. Furthermore, recent studies have demonstrated that the amino acid stimulated mTOR and insulin stimulated PI3K/Akt pathways operated in parallel to regulate PPARγ activity, illuminating a molecular mechanism linking nutrient status and growth factor signaling to adipogenesis [64]. mTOR, in a nutrient sensitive mechanism, also regulates insulin stimulated IRS activity positively. Although, IRS1 is phosphorylated on several tyrosine residues, in response to insulin, and numerous serine residues on IRS1 are identified to be inhibitory phosphorylation sites (that would be discussed later), however, phosphorylation of IRS1 on Ser302 is the only stimulatory serine residue published in the literature so far. White and coworkers have shown that through a rapamycin and LY294007 sensitive mechanism, insulin/IGF-1 stimulates IRS1 Ser302 phosphorylation, and this plays an important role in insulin-stimulated IRS1 tyrosine phosphorylation and signal transduction [65].

mTOR Binding Partners

Initial work using yeast, by M. Hall and his coworkers have identified several mTOR interacting proteins. The two functionally and structurally distinct multiprotein mTOR complexes are named as mTORC1 (complex 1) and mTORC2 (complex 2) [66, 67]. mTORC1 comprises of the mTOR, Raptor, mLST8 and the recently identified PRAS40 forming the insulin responsive, rapamycin sensitive complex [68-71]. The rapamycin insensitive complex mTORC2 is made up of mTOR, Rictor, mLST8 and the recently identified protein mSIN1 [72-74].

mLST8 (also known as GβL) is the common component of both complexes mTORC1 & 2. It is the mammalian ortholog of *S. cerevisiae* Lst8p and consists of seven WD40 repeats with high sequence similarity to β subunits of heterotrimeric G proteins. GβL interacts constitutively with mTOR kinase domain, and is necessary for the formation of nutrient-sensitive interaction with Raptor [69]. Regulatory associated protein of TOR (Raptor) is a ubiquitously expressed, evolutionarily conserved 150 kDa protein, that forms a tight complex with mTOR under nutrient and growth factor depleted condition, thus regulating mTOR kinase activity [70]. Although there are conflicting studies, there is a consensus that Raptor functions as a scaffold protein that recruits mTOR to its downstream substrates through their mTOR signaling (TOS) motifs [68].

Recent studies have identified six different ser/threonine phosphorylation sites on Raptor that are responsible for regulation of its function. The phosphorylation events follow a hierarchy, with Ser863 being the priming site [75]. Oncogenic Ras, phorbol

esters, growth factors as well as mitogenic signals through the MAPK pathway to activate p90 ribosomal s6 kinase (p90RSK), that phosphorylates Raptor to regulate mTORC1 activity [76]. However, in a very recent study Roux and colleagues have shown that ERK1/2 can directly phosphorylate Raptor, and mutation of these sites impaired mTORC1 kinase activity, thus demonstrating another novel means of mTOR regulation [76]. AMPK (5‘ AMP activated protein kinase) has also been shown to directly phosphorylate Raptor, which results in binding of 14-3-3 protein and possibly leads to either sequestration to a different cellular compartment or degradation of Raptor [77].

Similar to mTOR knockout, Raptor knockout mice are embryonic lethal. Tissue specific Raptor knockout mouse models have provided immense knowledge, as to the role of mTORC1 in regulating metabolic functions of the whole organism. Adipose-specific Raptor knockout resulted in lean mice with substantially less adipose tissue, and the animals were protected from DIO and insulin resistance [78]. The leanness was attributed to enhanced energy expenditure due to mitochondrial uncoupling, thus providing evidence for a possible role of mTOR in energy homeostasis. However, muscle specific Raptor knockout resulted in dystrophy and down regulation of genes involved in mitochondrial biogenesis [79]. These studies have shown that mTOR activity is essential for cell growth and proliferation while highlighting the tissue specific functions of mTOR with wide metabolic implications.

PRAS40 was first identified as a subunit of mTORC1. Further studies have established that PRAS40 functions as a negative regulator of mTORC1 activity, by

directly binding to the TOR kinase domain [71, 80]. However, there is also evidence showing that PRAS40 associates with mTOR through a Raptor mediated interaction. PRAS40 has also been proposed to consist of TOS motif and functions as mTOR substrate, and may compete with the known mTOR substrates [81]. Supporting this hypothesis, mTOR was found to phosphorylate PRAS40 on the serine 183 residue. Upon activation, Akt is known to directly phosphorylate PRAS40 on threonine 246, and hence the name proline rich substrate of Akt1-40 (PRAS40). This phosphorylation possibly leads to binding of 14-3-3 protein and thus is believed to release the inhibition of PRAS40 on mTOR [80, 82].

TSC1/TSC2 Mediated mTOR Regulation

Insulin stimulation leads to the activation of Akt that in turn results in the regulation of numerous effectors through phosphorylation. TSC2 (also known as tuberin) is one of the well documented substrates of Akt, through which, signals from the insulin receptor are transmitted to mTOR. TSC1 (also known as hamartin) and TSC2 physically associate and function as a complex, and TSC1 is necessary to stabilize TSC2 and to prevent from ubiquitin mediated degradation [35]. Their function as tumor suppressor was identified from observations made in autosomal dominant tumor syndrome [83]. The 140 kDa TSC1 and the 200 kDa TSC2 proteins share homology neither with each other nor with other proteins. However, TSC2 contains a GAP domain and has been demonstrated to exhibit GTPase activity. The TSC1-TSC2 complex regulates a small G-protein called ras homologue enriched in brain (Rheb), to modulate mTOR activity [84].

Rheb is a member of the Ras superfamily, is expressed ubiquitously in mammals. Studies from fission yeast have demonstrated that the interaction between mTOR and Rheb requires, Rheb to be GTP bound. The GAP domain in TSC2 complex stimulates the intrinsic GTPase activity in Rheb, thereby converting Rheb-GTP to Rheb-GDP [85-87]. Upon growth factor stimulation, Akt activation leads to the phosphorylation and cytosolic sequestration of TSC2 by 14-3-3 proteins, thus relieving the repression of Rheb-mTOR signaling. Recent studies using TSC2 null mouse embryo fibroblasts have shown that, despite insulin resistance and lack of Akt activity, there was increase in adipogenic capacity. This observed enhancement in adipogenesis was dependent on the mTOR activity [88]. Furthermore, TSC2 mutants lacking all five known Akt phosphorylation sites blocked insulin-stimulated mTORC1 signaling in reconstituted TSC2 null cells, and this mutant also inhibited adipogenesis.

PI3K/AKT Independent mTOR Regulation

Through numerous phosphorylation events, TSC1-TSC2 complex senses and integrates signals from several different pathways, to regulate its downstream effector, mTOR. Mitogen stimulation activates RSK and ERK, which then directly phosphorylate TSC2 at many serine and threonine residues, to inhibit its function [89, 90]. TSC1 is inhibited upon phosphorylation by cyclin dependent kinase 1 (CDK1) and inhibitory κ-B kinase β (IKKβ) [91, 92]. Decrease in energy status is reflected in the increase of cellular AMP levels that bind to AMP kinase (AMPK), which is further activated by the kinase LKB1, a tumor suppressor [93]. One of the mechanisms by which, mTOR senses the

intracellular energy status is through the direct phosphorylation of TSC2 by AMPK. However, the effect of this phosphorylation on GAP activity toward Rheb has not been demonstrated. Moreover, TSC2 phosphorylation by AMPK primes the complex for GSK3β mediated activation by phosphorylation that is downstream to Wnt signaling pathway but not that of Akt [94].

Recent studies have shown that the glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH), under low glucose conditions binds to and sequesters Rheb, thus inhibiting mTOR signaling. But high glycolytic flux was found to suppress GAPDH-Rheb interaction, thus restoring Rheb mediated mTOR activation. This GAPDH mediated control of mTOR signaling was observed independent of TSC1/TSC2 as well as AMPK, thus opening a novel means of regulating mTOR activity [95]. Initial observations demonstrating mTOR as an amino acid sensor, came from studies in *Saccharomyces cerevisiae*, the budding yeast, which lack TSC1 and TSC2 homologues. The observations provided the first proof for nutritional signaling to mTOR independent of these proteins. Furthermore, the class II PI3K, vacuolar protein sorting 34 (VPS34) and MAP4K3 (a sterile 20 related kinase), have been recently recognized to mediate amino acid cues to mTOR [96, 97]. In an elegant study Sancak and coworkers have demonstrated that Rag GTPases bind to Raptor and mediate amino acid signaling to mTOR. Interestingly, Rag proteins instead of directly activating mTOR, localized it to a Rheb containing intracellular compartment [98]. These studies have opened new paradigms of nutrient signaling, to regulate metabolic functions independent of insulin

stimulation, although, earlier studies have recognized insulin-Akt-TSC2 signaling independent activation of mTOR.

mTOR Substrates

Upstream activating signals through Akt-TSC1/TSC2-Rheb dependent mechanism results in mTOR phosphorylation on Ser2448 that in turn leads to Ser2481 autophosphorylation, considered the major indicator of mTOR intrinsic kinase activity [43, 99, 100]. Activated mTOR phosphorylates the protein translation regulators p70 ribosomal S6 Kinase-1 (S6K1) and EIF4E- binding protein-1 (4E-BP1) the well characterized mTOR substrates [101-104]. S6K is an AGC family protein kinase, synthesized as two isoforms, produced from two alternate translation initiation start sites. The larger isoform is localized to the nucleus, while the smaller isoform referred to as S6K1 is localized to the cytosol [105]. S6K1 is activated by two phosphorylation events one, by mTORC1 at Thr389 on the c-terminal hydrophobic motif; second, by PDK1 at Thr229 on the T loop of the kinase domain [106-109]. Activated S6K1 subsequently phosphorylates ribosomal S6 protein and eIF4B, to regulate protein synthesis. S6K1 is not only implicated in cell growth, protein synthesis and ribosome biogenesis, but has also been show to regulate life span [110]. Unlike mTOR and Raptor knockout mouse models, S6K1 knockout mice are viable. S6K1 whole body knockout mice are 20% smaller in size, owing to the role played by S6K1 in cell growth and protein synthesis [111]. Although, earlier studies suggested S6K1 to regulate translation of 5‘ TOP mRNA, that encode protein translation machinery [112]; however, their translation was not

effected in the S6K1 knockout mice, suggesting either a compensatory mechanism by other proteins, or that the earlier observations were an artifact of cell culture model systems. Additionally, the S6K1 knockout mice were protected from diet and age induced obesity owing to enhanced fatty acid β-oxidation. Furthermore, the animals were hypersensitive to insulin but were not hypoglycemic when fasted, as they exhibited smaller β cell mass, reiterating the role of S6K1 in cell growth. Tissue specific knockout mouse models would be needed to completely analyze the role of S6K1 in regulating energy homeostasis.

4E-BP1, the other well known substrate of mTOR regulates protein synthesis, by sequestering eIF4E, the cap-binding protein, rendering it unavailable to the mRNA, for protein translation. Once bound to the Raptor-mTORC1 complex, 4E-BP1 is regulated by a hierarchical phosphorylation, leading to the dissociation of 4E-BP1 from eIF4E. Priming phosphorylation on Thr37/46 that depends on the RAIP (the single letter code for its amino acid sequence) motif leads to the subsequent phosphorylation of Ser64; followed by Thr70, which are regulated through the mTOR-signaling (TOS) motif [113-116]. CDK1 has also been shown to phosphorylate Thr70 during cell cycle regulation. Phosphorylation at Thr37/46 is maintained by amino acids and is modestly regulated by insulin stimulation, and is also insensitive to rapamycin. While Ser64 is highly responsive to insulin, Thr70 is variably receptive [117-119]. Although, the role of 4E-BP1 in protein translation is well established, the consequences on metabolic functions are not well known. Since 4E-BP1 is a negative regulator of protein synthesis, ablation of this protein in mice should render the animals anabolic. To the contrary, 4E-BP1 knockout mice were

hypoglycemic, exhibited reduced WAT, and displayed increased metabolic rate, increased mRNA and protein expression of uncoupled protein 1 (UCP1) and PPARγ coactivator-1 α (PGC1α) respectively. This study has placed 4E-BP1 in the forefront of adipogenesis and regulation of adipose function [120]. Surprisingly, 4E-BP1 and 4E-BP2 double knockout mice exhibited elevated sensitivity to diet-induced obesity that was due to increased adiposity as a result of accelerated adipogenesis driven by increased expression of C/EBPα,δ and PPARγ, accompanied by reduced energy expenditure, reduced lipolysis and greater fatty acid reesterification. These animals also displayed insulin resistance, which was due to elevated S6K1 activity [121].

The central role played by mTOR in cell growth and cell size regulation has been revealed by studies using rapamycin treatment that resulted in a reduced cell size phenotype. The rapamycin induced cell size decrease was rescued completely or partially by the expression of a rapamycin-resistant mutant of mTOR or a partial-rapamycin-resistant S6K1, respectively [122]. Additionally, in the absence of rapamycin, ectopic expression of S6K1 or eIF4E increased cell size; co-expression had additive effect. Moreover, expression of a phosphorylation site-defective mutant of 4E-BP1 reduced cell size [123]. These studies in mammalian cells, together with many other observations in yeast, drosophila and worms have established two independent but synergistic modes of cell size regulation by mTOR, through S6K1 and 4E-BP1.

mTOR Regulation of Protein Synthesis

mTOR regulates cell growth by integrating the nutrient signaling and the growth factor signaling pathways. The major role of mTOR in stimulating cell growth is through regulation of protein synthesis. Protein synthesis involves two main steps initiation and elongation, which are complex processes engaging several factors. Very briefly, eukaryotic initiation factor-4E (eIF4E) binds to the 5‘ cap region, allowing the formation of translation initiation factor complexes at the 5‘ end of the mRNA. Eukaryotic initiation factor-4G (eIF4G), the scaffold protein binds to eIF4E and interacts with the helicase eukaryotic initiation factor-4A (eIF4A) and poly(A)-binding protein PABP, as well as several other proteins, that leads to the circularization of the mRNA leading to the initiation of protein synthesis.

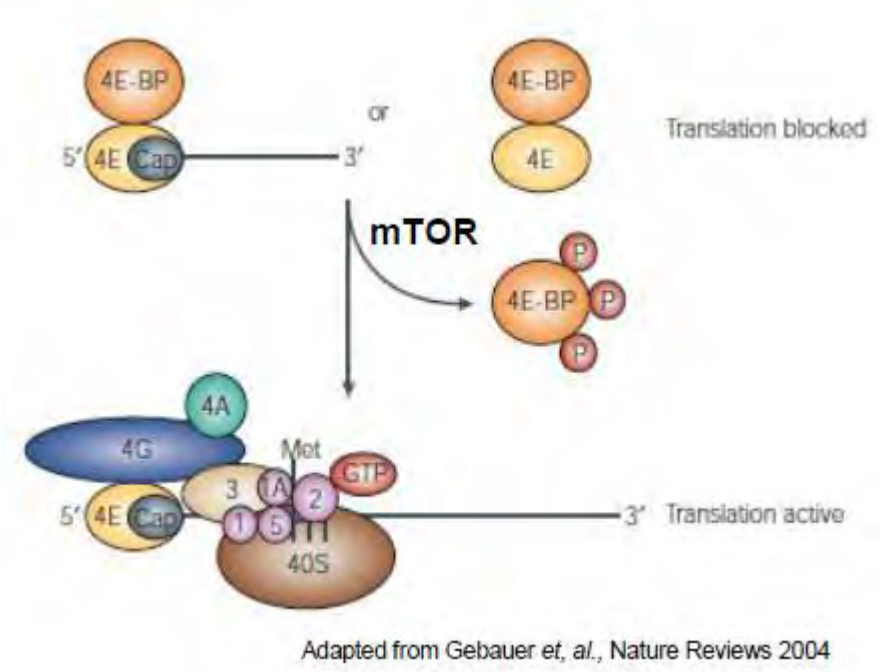


FIGURE 1.5: mTOR mediated control of protein synthesis. 4D-BPs bind to eIF4E and prevent its interaction with eIF4G and thus inhibits translation. Phosphorylation of 4E-BP by mTOR releases the protein from eIF4E that allows its interaction with eIF4G and thereby allows translation to proceed.

mTOR regulation of 4E-BP1 through phosphorylation renders it inactive thus relieving the inhibition on eIF4E to commence the formation of initiation complex, as summarized in Figure 1.5. During the elongation process, eukaryotic elongation factor-1 and factor-2 (eEF1 & eEF2) promote the translocation, where ribosome moves by one codon on the mRNA to facilitate peptidyl-tRNA migration from the ribosomal A site to P site after the formation of the new peptide bond. Phosphorylation of eEF2 by eEF2 kinase (eEF2K) renders it inactive. mTOR phosphorylates eEF2K on Ser366, leading to its inactivation, in a p70S6K1 dependent mechanism and thus promotes protein synthesis. This site is also a target for RSKs, the downstream effectors of ERK1/2 signaling pathway. mTOR also regulates eEF2K through Ser 359 and Ser78 phosphorylation. AMPK mediated direct phosphorylation of eEF2K decreases the rate of elongation. These processes are involved in the acute regulation of protein synthesis [124] (and the references there in). Interestingly, the long term cellular capacity for protein synthesis is regulated by the increase in the number of ribosomes and other components of translational machinery. The mRNAs for these factors are believed to contain pyrimidine tracts at the 5‘ end of mRNA (5‘-TOP) that suppresses translation under basal conditions. However, the role of mTOR in regulation of 5‘TOP containing mRNA is controversial. Earlier studies showed that these 5‘TOP sequences are regulated by p70S6K1, although later studies showed that, translation of 5‘TOP mRNA is not compromised in conditions were both isoforms of S6K1 are absent [125, 126].

Rapamycin is known to inhibit mTOR function. Studies have demonstrated that rapamycin treatment substantially inhibited protein synthesis in short-term treatment of 1-

2 hours. Much of what we today know about mTOR signaling pathway has been studied in HEK293T (human embryonic kidney) cells. In these cells, rapamycin pretreatment decreased both the insulin as well as phorbol ester stimulated protein synthesis by about 50% [127]. However, in primary rat cardiomyocytes, rapamycin pretreatment lead to >50% decrease in insulin as well as phenylephrine (hypertrophic α-agonist PE) induced protein synthesis [128]. These results demonstrate the cell type specific regulation of protein synthesis. Moreover, the ~40% rapamycin insensitive component of protein synthesis in these observations, was found to be significant and thus provided evidence of mTOR independent protein synthesis regulation or points to a rapamycin insensitive output of mTOR.

Glucose Metabolism - Glucose Uptake

Glucose homeostasis is preserved by the precise regulation of glucose uptake by the metabolic tissues as well as production by the liver. While liver and muscle each take up a third of an oral glucose load; of the remaining third, a bulk is taken up by the nervous system including the brain, and the rest is shared between erythrocytes and other tissues and organs of the body. Glucose is taken up by different tissue by facilitated diffusion that involves transport proteins called glucose transporters (GLUTs). Class I proteins comprise GLUT1-GLUT4, the well characterized glucose carriers. GLUT1 is highly expressed in erythrocytes and endothelial cells, and is responsible for basal glucose uptake. Expression of this transporter is inversely related to the glucose levels. GLUT2 is expressed by renal tubular cells, liver and pancreatic β cells, as well as the

epithelial cells of the small intestine [129]. Even though GLUT2 is not an insulin regulated glucose transporter, insulin stimulated glucose uptake is enhanced in the liver; this could be due to the feed forward regulation of the glycogenesis and glycolysis pathways that increase the glucose flux into hepatocytes [130]. GLUT3 comprises the main glucose transporter in the neurons and the brain. GLUT4 found in the adipose tissue and skeletal & cardiac muscle is the well studied insulin regulated glucose transporter; although, fatty acids are the fuel of choice for heart muscle.

Insulin stimulated clearance of glucose is partly depended on the ability of adipose and muscle to take up glucose in response to insulin. The role of GLUT4 in achieving this is demonstrated by numerous genetic mouse models. Adipose and muscle tissue specific over expression or deletion of GLUT4 protected from or promoted glucose intolerance & diet induced diabetes respectively [131-133]. Decreased insulin sensitivity and tendency toward diabetes was observed in GLUT4 heterozygous +/- mice; and this was overcome through muscle specific over expression of GLUT4 [134, 135]. Studies using cellular models revealed that GLUT4 translocation to the plasma membrane that results in glucose uptake is regulated by insulin through PI3K/AKT signaling mediated mechanism [136, 137]. Recent efforts have identified RAB GAP, AS160, as an AKT substrate, that regulates GLUT4 translocation [138-140]. Upon insulin stimulation, activated AKT phosphorylates AS160, thus releasing GLUT4 from the retained intracellular compartment [141]. Although RNAi mediated AS160 depletion released only part of GLUT4 intracellular pool, raising the possibility for the existence of other AKT substrates that could regulate GLUT4 retention [142]. However, knockin mutation

of AS160 on the insulin mediated phosphorylation site led to glucose intolerant and altered GLUT4 trafficking in mice [143]. Recent study in cultured adipocytes identified pleckstrin homology domain containing protein (PHLDB1) as a novel enhancer of insulin stimulated Akt activation as well as GLUT4 translocation [144]. Interestingly, chronic activation of ERK or p38 MAPK pathway induced GLUT1 expression while GLUT4 was suppressed that resulted in increased basal glucose transport but diminished insulin stimulated transport [145].

Regulation of Glycogen Synthesis

The glucose taken up by the metabolic tissues is destined to diverse end products, depending on the target organ. Whereas in the muscle it leads to glycogen synthesis, it results in triglyceride synthesis in the adipose; however, in the liver both glycogenesis as well as lipogenesis occur. Upon uptake, glucose is converted to glucose-6-phosphate (G-6-P) by hexokinase in the liver, and glucokinase in the muscle. G-6-P is either completely oxidized to generate ATP through several steps involving committed as well as rate limiting reactions, or stored as glycogen by the insulin sensitive enzyme glycogen synthase (GS). Insulin stimulated activation of Akt results in phosphorylation and subsequent inactivation of its substrate glucose synthase kinase-3 (GSK3), a key modulator of GS activity. Interestingly, increased GSK-3 activity was observed in diabetic humans [146], and animals [147] that could lead to deregulation of GS activity. Studies in mice using genetic ablation of GSK3 have shown that decrease in GSK3 activity towards GS is responsible for the increase in glycogen synthesis in the muscle

and liver [148]. Furthermore, GSK3 chemical inhibitors were shown to reduce blood glucose levels, by increasing glycogen synthesis in rodents.

In the adipose, dietary carbohydrates, glucose in particular, contributes to triglyceride synthesis by two ways. One, glucose is converted to glycerol 3-phosphate, to form the glycerol backbone of triacylglycerols. Two, glucose is catabolized to pyruvate, which in a pyruvate dehydrogenase mediated reaction, is converted to Acetyl CoA that forms the substrate for *de novo* lipogenesis (DNL). Upon feeding, insulin stimulates lipogenesis in the liver through this second mechanism.

Gluconeogenesis

Decrease in blood glucose as well as insulin levels after about an hour of food intake trigger the increase of glucagon concentrations. Glucagon is a peptide hormone synthesized and secreted by the α-cells of islets of Langerhans, stimulates glycogen breakdown, termed as glycogenolysis. G-6-P generated by this process can be utilized for ATP synthesis through the glycolytic pathway, in muscle and also in liver. However, liver as well as kidney are the two unique organs that can remove the phosphate on G-6-P using the enzyme G-6-Phosphatase, to generate glucose, to be utilized by other cells.

In conditions of fasting, blood glucose levels are maintained by liver and to a lesser extent kidney, through gluconeogenesis; a process by which glucose is generated from non-carbohydrate sources such as lactate, amino acids and glycerol. Lactate produced from glucose utilization in the muscle and RBC, glucogenic amino acids

(except leucine and lysine) generated by protein degradation in the muscle, glycerol and fatty acids from the adipose are taken up by the liver and utilized as substrates for gluconeogenesis. Most of these substrates are converted to pyruvate so as to generate glucose; however, since glycerol enters late into the gluconeogenic pathway, it is converted to glucose directly. Whether fatty acids can be converted to glucose in animals is a subject of debate [149]. However, odd-chain fatty acids can be oxidized to yield propionly CoA, a precursor for succinyl CoA that can then be converted to pyruvate to enter gluconeogenesis. Although glyoxylate cycle utilizing fatty acids generates substrates for glucose synthesis [150], the existence of this pathway is not established in humans.

Regulation of Gluconeogenesis

Gluconeogenesis is inhibited by insulin, by suppressing the expression of two important enzymes involved in this process, phosphoenolpyruvate carboxykinase (PEPCK) [151] and glucose-6-phosphatase (G-6-Pase) [152, 153]. While PEPCK catalyzes one of the rate limiting steps, G-6-Pase catalyzes the final step of gluconeogenesis. Glucagon, glucocorticoids and catecholamines activate the expression of these enzymes during fasting, stress and exercise respectively [154, 155] [156] [157].

Activated PI3K plays a central role in insulin-mediated suppression of gluconeogenesis. This is supported by the observations made using the pharmacological inhibitors wortmannin and LY-294002, where both basal and cAMP or dexamethasone induced inhibition of PEPCK and G-6-Pase gene expression by insulin was abolished

[152, 158-160]. Furthermore, adenoviral mediated over expression of a dominant negative mutant of PI3K increased PEPCK & G-6-Pase gene expression as well as in vivo hepatic glucose production[161]. Several studies investigated the signaling mechanism downstream to PI3K and observed a decrease in the gene transcription of PEPCK and G-6-Pase in hepatoma cells as well as in primary hepatocyte cultures, upon AKT overexpresssion [158, 159, 162]. Additionally, several proteins including Foxo1 and cAMP response element-binding protein (CREBP) have been identified to mediate this Akt effect.

DAF-16 the *C. elegans* homolog of the mammalian proteins Foxo1, 3 & 4 was identified by genetic studies as the insulin sensitive AGE1 (PI3K homolog) downstream effector [163]. Foxo proteins are transcription factors that have been found to be phosphorylated by Akt at three conserved serine/threonine residues, rendering the proteins inactive and leading to their nuclear exclusion [164-170]. Direct binding of these factors to the promoter region of PEPCK & G-6-Pase has been demonstrated using electromobility shift assay as well as reporter gene analysis. Furthermore, over expression of wild type or a dominant negative mutant of Foxo1 led to increase and suppression of G-6-Pase expression respectively [159, 164, 171]. In an insulin resistant mouse model, haploinsufficiency of Foxo1 correlated with blood glucose and insulin normalization. Additionally, expression of a constitutively active Foxo1 increased PEPCK and G-6-Pase expression resulting in elevated fasting blood glucose levels [172]. Additionally, studies have shown that insulin stimulated signaling events result in dissociation of glucocorticoid receptor (GR) and Foxo1 from PDK4 promoter [173].

Together, these observations have established the insulin-PI3K-AKT signaling to regulate Foxo1 activity, as the mechanism for regulation of hepatic gluconeogenesis.

Treatment of hepatoma cells with AICAR, a pharmacological activator of AMPK the cellular energy sensor, mimicked insulin effect on G-6-Pase and PEPCK gene expression [174]. Moreover, the widely used drug Metformin has been show to exhibit some of its anti-diabetic effects through AMPK activation that is required for the increase in the expression of SHP (small heterodimer partner), which inhibits C/EBPα function towards PEPCK promoter, and thus downregulates gluconeogenesis [175-177]. These studies show the convergence of multiple signaling pathways and several modes of regulating PEPCK and G-6-Pase gene expression that in turn regulate hepatic glucose production.

Lipid Metabolism - Lipogenesis

The process of lipid synthesis from either glucose (de novo lipogenesis) or reesterification of dietary fatty acids to triglycerides is termed as lipogenesis. Both these processes of lipid synthesis are employed by hepatocytes and adipocytes. The pathway of reactions involved and the enzymes that mediate triglyceride synthesis from glucose are well documented. Glucose through the glycolytic pathway is converted to pyruvate that through a series of reactions in the mitochondria leads to the synthesis of acetyl-CoA. Two carbon unit additions to this intermediate in the cytoplasm, mediated by enzymes that form a multienzyme complex called fatty acid synthase (FAS), leads to the synthesis of fatty acyl-CoA. Thus synthesized fatty acids are esterified with glycerol to form

triglycerides. Dietary lipids as well as the triglycerides synthesized in the liver, complex with lipoproteins, phospholipids and cholesterol to form very low density lipoproteins (VLDLs) that are released into the blood stream in the fasting state as well as postprandially. Dietary lipids are transported by the intestinal mucosa as chylomicrons, that contain triacylglycerols and apolipoproteins, including apoB-48, apoA-1, II and IV. In the bloodstream, chylomicrons acquire apoC-II and apoE from plasma HDLs (high density lipoproteins). In the capillaries of adipose tissue and muscle, by the action of lipoprotein lipase (LPL), present on the surface of capillary endothelial cells, chylomicrons similar to VLDLs from the liver, release their fatty acids; the glycerol backbone of the triacyglycerols is returned to the liver and kidneys by the blood. The fatty acids taken up by the adipocytes are reesterified to synthesize triacylglycerol. The glycerol backbone is provided by dihydroxyacetone phosphate (DHAP), a glycolytic intermediate.

Regulation of Lipogenesis

Insulin potently stimulates lipogenesis by increasing the uptake of glucose, as well as by activating lipogenic enzymes. The flux through lipogenesis is increased by insulin signaling that leads to activation of pyruvate dehydrogenase (PDH) by dephosphorylation; that catalyzes the conversion of pyruvate to acetyl-CoA. Acetyl-CoA carboxylase is similarly activated through dephosphorylation by insulin signaling, to increase malonyl-CoA synthesis from acetyl-CoA. Using adipocytes in culture and transgenic mice expressing reporter gene driven by FAS, Kim and his colleagues

demonstrated insulin stimulated, PI3K/Akt signaling dependent regulation of FAS expression [178]. Serum response element binding proteins (SREBPs 2, 1a & 1c) are a family of transcription factors that regulate the expression of genes involved in cholesterol and fatty acid synthesis. Studies involving mice with SREBP-1a or 1c over expression in the liver showed elevated expression of lipogenic genes, leading to the conclusion that SREBP-1 activates lipogenesis in liver [179]. To the contrary, adipose specific SREBP-1c transgenic mice exhibited no change in the expression of genes involved in fatty acid and triglyceride synthesis; although expression of genes implicated in cholesterol metabolism were elevated. Strikingly, adipose tissue mass was reduced in these mice, that was accompanied by decreased expression of PPARγ and C/EBPα [180]. These observations raise the possibility of differential regulation of SREBP-1 functions in liver and adipose tissue. Recent studies have implicated protein kinase C beta (PKCβ) in the insulin-stimulated regulation of hepatic SREB-1c and its target lipogenic genes. RNAi or pharmacological inhibitor mediated silencing of PKCβ inhibited SREBP-1c activation [181].

Expression of lipogenic genes is downregulated by polyunsaturated fatty acids by inhibiting the mRNA expression as well as the proteolytic processing of SREBP-1 [182-185]. Studies using adenovirus mediated constitutively nuclear FOXO1 expression in the liver, demonstrated stimulation of hepatic lipogenesis, through inhibition of PPARα [186]. This is further supported by the increased lipid content observed in the liver of PPARα knockout mice [187]. Furthermore, studies using luciferase assays demonstrated that PPARα activation could suppress LXR-SREBP-1c pathway, to inhibit lipogenic gene

expression in the liver [188]. Together, these observations show that SREBP1 regulation forms the important mechanism for hepatic lipid synthesis. Although insulin is the major stimulus for lipogenesis, an insulin independent mechanism of lipid synthesis in the liver has been proposed. Under insulin resistance conditions, in the liver, IR/PI3K/Akt signaling to inhibit gluconeogenesis, and to stimulate glycogen synthesis is imparied; however, SREBP1 mediated lipogenesis remains active. Recent studies have identified mTOR to be responsible for this paradox. Even in the absence of insulin signaling to Akt in the liver, nutrient signaling is believed to stimulate mTOR activity towards SREBP1 that mediates increase in lipogenesis [189]. It remains to be seen if adipose lipid synthesis is regulated in a similar manner.

Lipolysis & Fatty Acid Oxidation

During states of energy deprivation, fat stores from adipose tissue are mobilized to meet the energy requirement of the organism. To this end, triacylglycerols are hydrolyzed to fatty acids and glycerol that are released to be used as energy substrates. Although at steady state, the adipose lipid pool is in a constant state of flux leading to a futile cycle of lipolysis and reesterification [190]. Triacylglycerol (TAG) is sequentially hydrolyzed to diacylglycerol (DAG), then to monoacylglycerol (MAG), releasing fatty acid at each step and finally glycerol. In the fasting state, the fatty acids generated by lipolysis in the adipose tissue forms the major sources of VLDL synthesis in the liver.

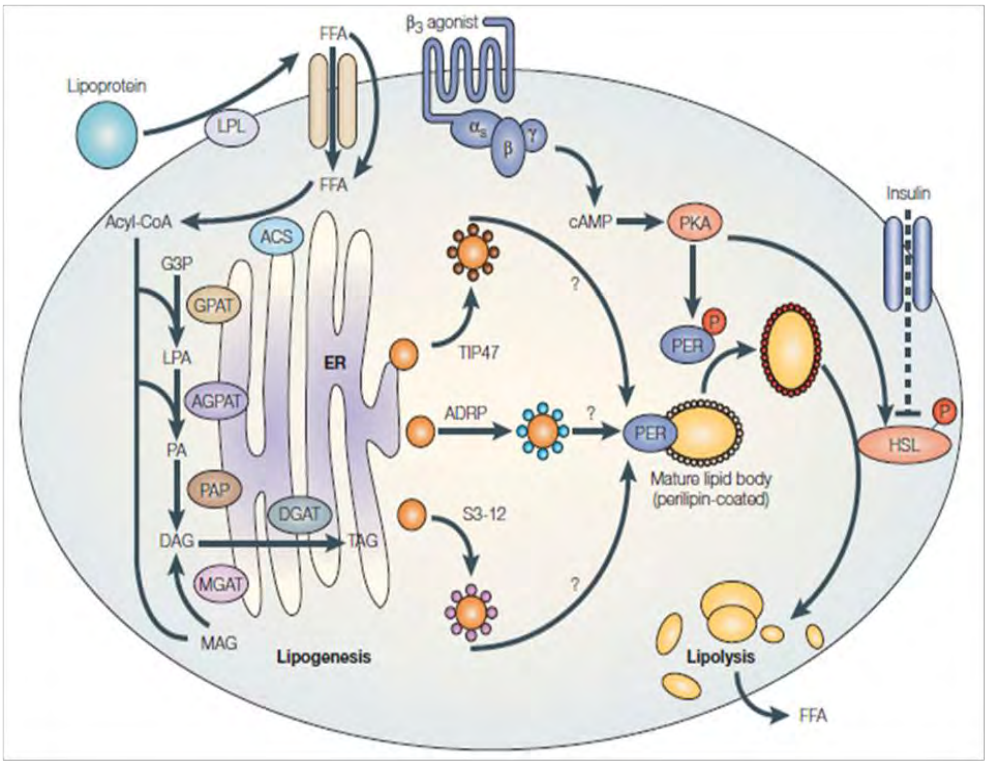
This process of lipolysis is acutely regulated by hormones, neurotransmitters and other effector molecules. The catecholamine norepinephrine is an important stimulator of

fasting induced lipolysis. It binds to the β-adrenergic receptors that are coupled with G-protein coupled receptor-s (Gs-proteins) on the adipocyte plasma membrane and thus transmits activating signal to adenylyl cyclase to generate cyclic AMP (cAMP). Upon binding to protein kinase A (PKA), cAMP activates the kinase to phosphorylate its substrates. Glucagon also activates lipolysis through adenylyl cyclase mediated mechanism [191]. Activated PKA phosphorylates and stimulates hormone sensitive lipase (HSL) translocation from the cytosol to the lipid droplet [192] . HSL was considered the major adipose lipase to initiate lipolysis until recently [193]. The accumulation of DAG observed in HSL null mice demonstrated the existence of HSL independent TAG lipase activity [194, 195]. This enzyme was identified as adipose triglyceride lipase (ATGL). However, serum TAG and non-esterified fatty acid (NEFA) levels were decreased in the HSL-null mice. This indicates that HSL-independent lipolysis is not adequate for the maintenance of fatty acid output for energy substrates and VLDL synthesis [196, 197]. The accumulation of MAGs in an in-vitro reaction using isolated HSL, lead to the identification of MAG-lipase (MAGL) [198].

ATGL is a TAG specific lipase, whose expression is induced upon fasting and inhibited upon refeeding, in mice. Glucocorticodis have been shown to increase the expression of ATGL in a concentration dependent manner in 3T3-L1 adipocytes [199]. Furthermore, the observed decrease in the expression of ATGL in the genetically obese ob/ob and db/db mice, suggests a possible role for this lipase in the development of obesity [199]. Interestingly, ATGL over expression in adipose tissue increases lipolysis, and expression of genes involved in FA oxidation and thermogenesis is elevated as well,

resulting in resistance to diet-induced obesity in mice [200]. Perilipin is one of the earliest identified lipid associated proteins. It coats the lipid droplet and functions to prevent unrestrained basal lipolysis [192]. This is supported by the constitutively elevated basal lipolysis observed in perilipin null mice [201]. As expected, over expression of perilipin in 3T3-L1 adipocytes resulted in increased TAG stores and also lead to reduced lipolyis [202]. Similar to HSL, perilipin is also phosphorylated by PKA upon adrenergic stimulus [192]. Mutations in these phosphorylation sites of perilipin blunted stimulated lipolysis [203]. Presence of perilipin on the lipid droplet appears to be necessary for PKA-mediated stimulation of HSL translocation [204]. However, recent evidence shows that perilipin phosphorylation is not necessary for HSL translocation [203].

Lipolysis mediated synthesis of fatty acids by the adipocytes is not required in the fed condition. Insulin levels are elevated upon feeding and this hormone is believed to be a major inhibitor of lipolysis. The ability of insulin to lower cellular cAMP levels and therefore decrease PKA activity accounts for its ability to inhibit lipolysis. This is achieved by AKT dependent phosphorylation and activation of phosphodiesterase 3B (PDE3B) [208]. PDE3B hydrolyzes cAMP thus reducing its cellular levels leading to the inactivation of PKA and thus HSL and possibly perilipin activity. This signaling mechanism appears to be disrupted in type 2 diabetic patients contributing to elevated serum free fatty acid levels [209]. The general process of fat storage and lipolyis is summarized in the Figure 1.6.



Adapted from (Shi & Burn Nature Reviews 2004)

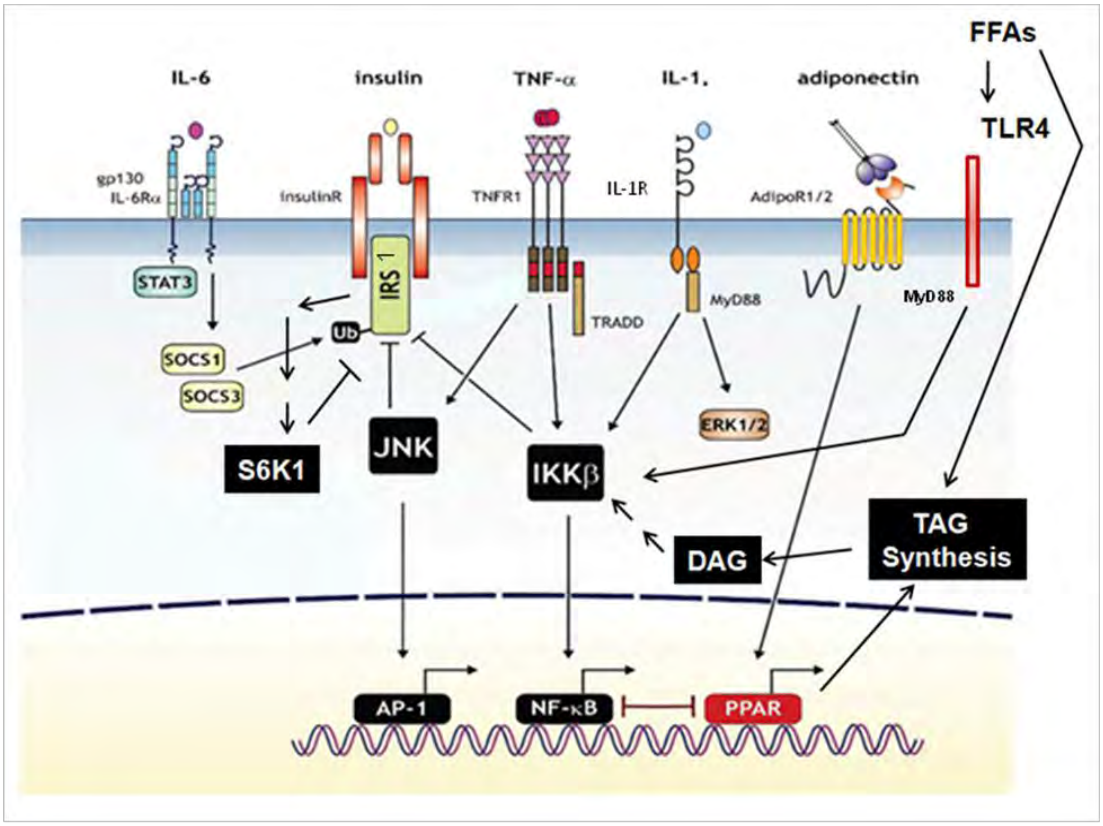
FIGURE 1.6: Lipid Storage and Lipolyis in Adipocytes. Free Fatty Acids (FFAs) released from lipoporteins by lipoprotein lipase mediated catalysis enter adipocytes through passive diffusion and active transport. Inside the cell, FFAs are first converted to acyl-CoA by acyl-CoA synthase (ACS). Glycerol-3-phosphate (G3P) generated by glucose metabolism is acylated by glycerol-3-phosphate acyltransferase (GPAT) and acylglycero-3-phosphate acyltransferase (AGPAT) sequentially and is converted to diacylglycerol (DAG) by phosphatidic-acid phosphohydrolase (PAP). Monoacylglycerol (MAG) is acylated by MAG acyltransferase (MGAT). DAG synthesized by these two mechanisms is then acylated by diacylglycerol acyltransferase (DGAT) to generate triacylglycerol (TAG). Lipid droplets generated from the ER are coated by proteins such as perilipin (PER), adipose differentiation-related protein (ADRP) and tail interacting protein of 47 kDA (TIP47). β-adrenergic receptor activation mediated cyclic-AMP (cAMP) dependent protein kinase A (PKA) activation leads to PER and hormose-sensitive lipase (HSL) phosphorylation, resulting in hydrolysis of TAG to FFAS that are released from the adipocytes. Insulin signaling inhibits lipolysis. LPA, lysophosphatidic acid. PA, phosphatidic acid.

Insulin Resistance

The inability of insulin to elicit cellular responses is termed as insulin resistance. In the muscle and adipose tissue, insulin resistance leads to reduced glucose uptake and storage as glycogen and triglycerides respectively. In the liver it results in decreased glycogen synthesis and storage as well as failure to suppress glucose production. Deregulation of all these metabolic functions combine to increase serum concentrations of glucose and FFAs, characteristic feature of insulin resistance. Unrestrained insulin resistance can progress to type 2 diabetes.

In addition to genetic factors in both humans and model systems, insulin resistance has been observed to be caused by several acquired factors and conditions, including and not limited to obesity, increased levels of FFAs, chronic inflammation, sepsis and aging. Drugs such as glucocorticoids are also implicated in insulin resistance. Even though, most of the factors that cause insulin resistance are observed in conditions of obesity, and studies using mouse model systems have revealed several deregulated metabolic pathways, the exact cause of obesity associated insulin resistance still remains unknown. Although insulin resistance observed in conditions of obesity mainly refers to resistance to insulin-stimulated glucose disposal [210], interestingly, all obese individuals do not exhibit insulin resistance, a paradox that needs rigorous investigation. Elegant studies in mouse model system, using knockout, knockin and a combination of both, have contributed immensely to the vast knowledge that we have today about the pathways, mechanisms and possible contributing factors in insulin resistance and associated

metabolic disorders however, much remains to be known. The signaling mechanisms that are dysfunctional, the role of various metabolites & tissue specific secreted products, and the cross talk between different metabolic tissues, which are involved in mediating insulin resistance, will be discussed. The mechanisms involved in the regulation of these processes are briefly summarized in the Figure 1.7.



Modified from (Tilg & Moschen Molecular Medicine 2008)

FIGURE 1.7. Regulation of Insulin Resistance – Mediators and Pathways. Insulin signaling to p70S6K1 results in the activation of negative feedback loop and IRS1 serine phosphorylation. Diacylglycerol (DAG) generated from TAG hydrolysis activates several proteins which subsequently activate inhibitor of κappa B protein kinase-β (IKKβ) to inhibit IRS1. TNFα signaling pathway stimulation activated JNK and IKKβ that leads to the inhibitory phosphorylation of insulin receptor substrate 1 (IRS1). IKKβ is also activated by interleukin-1 receptor (IL-1R) and toll like receptor 4(TLR4) stimulation. IKKβ activation results in nuclear factor-κB (NF-κB) nuclear translocation and inflammatory gene expression. IL-6 mediated suppressor of cytokine signaling 1 (SOCS1) and SOCS3 activation results in ubiquitin-mediated IRS1 degradation.

Role of Insulin

Binding of insulin to the insulin receptor, leading to tyrosine phosporylation of IRS forms the initial events in the insulin signaling cascade. However, prolonged insulin stimulation in cells leads to IRS phosphorylation on serine residues, leading to the inhibition of insulin signaling, suggesting a feed-back mechanism possibly involving downstream proteins in the signaling pathway. Although several serine residues on IRS are implicated in regulation of insulin sensitivity, the inhibitory phosphorylation of Ser307 of IRS functions as the key player in insulin resistance. This serine residue is located in the phospho-tyrosine binding domain that is responsible for IRS interaction with the insulin receptor and its phosphorylation inhibits the interaction. Several protein kinases phosphorylate IRS on Ser307, including the mTORC1 substrate S6K1, cJUN n-terminal Kinase (JNK), inhibitory κ B Kinase β (IKKβ), protein kinase θ (PKCθ), protein kinase ε (PKCε), GSK3 [211]. Phosphorylation of IRS Ser307 possibly leads to its degradation and thus termination of insulin signaling; since IRS1 with a point mutation at Ser307 is resistant to degradation following long term insulin stimulation [212]. Furthermore, recent work from Shulman‘s group demonstrated that mice carrying alanine substitution in IRS1 serines 302, 307 and 361 were protected from high fat diet induced insulin resistance in the muscle [213]. To the contrary, White and coworkers showed that IRS1 Serine 307 promoted insulin sensitivity in mice, as mice with mutation of this serine to alanine developed severe insulin resistance and exhibited impaired muscle insulin signaling [214]. However, insulin resistance can also be induced by direct

inhibition of insulin receptor tyrosine phosphorylation or inhibition of a downstream effector such as Akt.

Role of S6K1 in Inducing Insulin Resistance

Wild-type mice fed on high fat diet, as well as the genetic obese mouse model ob/ob have been shown to exhibit elevated S6K1 activity as well as increased IRS1 inhibitory phosphorylation. Furthermore, S6K1 has been identified to directly phosphorylate IRS1 on Ser307 as well as Ser636/639 and Ser1104, leading to its inactivation and thus resulting in insulin resistance [215, 216]. This is supported by the observations of increased insulin sensitivity due to decreased IRS1 inhibitory phosphorylation in an S6K1 deficient mouse model. Additionally, these animals maintained normal fasting glucose levels and were protected against obesity due to enhanced β-oxidation. Moreover, S6K1 gene ablation resulted in reduction of the adipose tissue weight [111]. Loss of 4e-BP1 and 4e-BP2 in mice, the other well known downstream effector of mTOR, increased their sensitivity to DIO, exhibited enhanced adiposity and insulin resistance that was associated with increased S6K1 activity in muscle, liver and adipose tissue; that was accompanied by increase in inhibitory IRS1 phosphorylation [121]. These studies highlight the direct role played by S6K1 in insulin resistance.

Role of Adipokines in Mediating Insulin Sensitivity and Resistance

Adipose tissue is now considered not just as an energy storage depot but is recognized as an active endocrine organ. The protein hormones synthesized and secreted by adipocytes are termed as adipocytokines (adipokines), and include adiponectin, leptin, retinol binding protein 4 (RBP4), tumor necrosis factor-α (TNFα), IL-6, and MCP-1. TNFα and IL-6 are secreted by other cell types as well and are considered as cytokines.

Adiponectin

Adiponectin also named Acrp30 is expressed exclusively by the adipocytes and is the most abundant protein secreted by the adipocytes beginning at an intermediate stage of adipogenesis [217]. It is a 30kDa protein, and forms aggregates of trimer, hexamer, and 12-18 mer as well. Proteolytic cleavage at amino acids 110 generates a c-terminal globular protein, although the full length form predominates [218, 219]. Plasma levels of adiponectin are observed to be decreased with obesity, insulin resistance, atherosclerosis, and type-2 diabetes in both mice and humans [220-222]. Administration of full length as well as the globular forms of adiponectin in obese mouse models resulted in insulin sensitization. Studies using adiponectin transgenic mice exhibited partial amelioration of insulin resistance and diabetes and endogenous glucose production was suppressed [223]. As expected, mice deficient for adiponectin were glucose intolerant and insulin resistant [224]. Furthermore, adiponectin is also implicated in stimulating insulin secretion by the pancreas and thus could alter glucose metabolism, along with its insulin sensitizing effects.

Interestingly, adiponectin plays an important role in protection from endothelial damage and mediates anti-inflammatory action, by potently suppressing TNFα or hyperglycemia induced IKKβ activation [225]. The role of these inflammatory mediators in insulin sensitivity will be discussed later. Adiponectin levels are also inversely correlated with the risk for developing several types of cancers. Work from various groups implicated adiponectin in tumor derived angiogenesis regulation. Although there are studies against, as well as in support of this hypothesis, observations made by Lodish and Sun suggest a role for adiponectin in macrophage infiltration and regulation of tumor growth [226].

The metabolic effects of adiponectin are mediated by AMPK and ACC, where, upon activation by phosphorylation, these effectors cause increased glucose transport and lipid oxidation in the muscle and inhibit gluconeogenesis in the liver. Adiponectin also leads to increase in expression of PPARα and of its target genes, resulting in decreased muscle and liver triglyceride content. Thiazolidinediones (TZDs) stimulate adiponectin secretion by adipocytes. The improved insulin sensitivity exhibited upon TZD administration is possibly in part due to the increased adiponectin secretion observed in mice and humans.

Leptin

Leptin is produced predominantly in white adipocytes, as a product of the obese (ob) gene. Six leptin receptor isoforms are generated by alternate splicing of the db gene of which, Ob-R is the long isoform, expressed predominantly in the hypothalamus [228].

Leptin signaling is considered most important for the regulation of food intake and energy homeostasis [229-231]. The main targets of leptin are the appetite centers in the brain where, leptin receptor activation of the anorexigenic (catabolic) and inhibition of the orexigenic (anabolic) signaling regulates energy intake and expenditure. Leptin effects are also observed on insulin action in peripheral tissues, as well as on pancreatic β-cells and blood vessels [228, 232, 233].

Activated leptin receptor stimulates several downstream signaling pathways such as, Janus kinase (JAK) and STAT (signal transducers and activators of transcription) signaling, RAS-Raf mediated MAPK pathway as well as IRS-PI3K pathway, that modulate gene transcription and ion channel functions in the brain. The inability of leptin to induce a response in the target organs, even in the presence of high concentrations of leptin, as observed in obese individuals is termed as leptin resistance. The ability of leptin to activate STAT is inhibited in the high fat diet fed condition as well. Impaired leptin transport across the blood-brain-barrier, as well as induction of suppressors of cytokine signaling-3 (SOCS-3) and protein tyrosine phosphatase 1B (PTP-1B) expression, can block leptin signaling. Increased sensitivity to leptin and resistance to obesity is observed in SOCS-3 and PTP-1B deficient animals [234-237].

Mice expressing mutant leptin receptors deficient in downstream signaling to JAK-STAT pathway exhibit severe hyperphagia and obesity. However, these animals have only a mild effect on glucose homeostasis, unlike the leptin receptor deficient db/db mice [238]. These observations suggested two independent modes of leptin mediated

effects; while JAK-STAT signaling regulates body weight and food intake, leptin-stimulated PI3K signaling regulates glucose metabolism. Leptin also modulates TAG accumulation in adipose, as well as liver, muscle and pancreas through a direct effect on AMPK, and an indirect effect through central neural pathway, and thus improves insulin sensitivity [239-241].

Role of Fatty Acids and Lipid Mediators in Promoting Insulin Resistance

High post-prandial circulating concentration of FFAs is an indicator of insulin resistance. Insulin stimulated IRS1 tyrosine phosphorylation, PI3K activity as well as GLUT4 translocation is inhibited in the muscle in conditions of increased FFA accumulation, either due to increased uptake or decreased FA oxidation. Increased TAG synthesis due to excess fatty acid availability also raises the levels of the intermediates DAG and acyl-CoA. Studies have shown that elevated levels of DAG and acyl-CoA, in the muscle result in activation of PKCθ, leading to IRS1 serine phosphorylation. Confirming this, studies in PKCθ knockout mice showed prevention of FFA induced decrease in IRS tyrosine phosphorylation as well as PI3K activity [242]. These results are further supported by the 3-4 hours of lipid infusion induced whole body insulin resistance observed in healthy humans [243]. However, different mechanisms are involved in the FFA mediated lipotoxic effects in liver and muscle that regulate whole body insulin sensitivity. Short-term high fat feeding (3 days) in rats resulted in hepatic lipid accumulation and insulin resistance, while muscle remained insulin sensitive. In these insulin resistant livers, tyrosine kinase activity of IR was negatively regulated by PKCε,

thus leading to inhibition of IRS2 activation [244]. Additionally, silencing of PKCε using anti sense oligonucleotides, prevented high fat diet induced hepatic insulin resistance [245].

Inflammation

The body‘s first response to tissue damage caused either by pathogens, toxic chemical stimuli or tissue injury is termed as inflammation. Acute inflammation involves a short-term response resulting in healing that is mediated by leukocyte infiltration, removal of the stimulus and tissue repair. To the contrary, chronic inflammation that is associated with obesity, arthritis, type 1 & type 2 diabetes and cardiovascular disease, is prolonged, dysregulated and involves active inflammation and an attempt to repair damaged tissue. In obese conditions, adipose tissue is considered to be an important initiator of inflammation. Inflamed adipose tissue synthesizes and secretes cytokines and chemokines that function in an endocrine or a paracrine fashion. Obese mice and human adipose tissue is infiltrated with macrophages that can comprise to greater than 40% of the total cells [246]. Bone marrow derived macrophages are recruited to the adipose tissue in obesity, and the macrophage content correlates to the degree of obesity. The resident as well as recruited adipose tissue macrophages (ATMs), upon activation secrete chemokines to recruit more macrophages, in a feed-forward mechanism to increase ATM content and chronic inflammation. The inflammatory cytokines and chemokines, secreted by macrophages further add to the development of insulin resistance in the adipose as well as in other metabolic tissues.

Immune Cell Recruitment

Immune cells are recruited to the adipose tissue in response to chemotactic molecules, small proteins of 8-10kDa termed chemokines. Other molecules such as small peptides (defensins), lipids (prostaglandins, lysophospholipids and leukotrienes) and complement factors (C5a and C3a) also can act as chemo-attractants. These molecules mediate cellular effects through their interaction with cell surface receptors on target cells, majority of which are G-protein coupled receptors. Enhanced expression of macrophage chemoattractant protein-1 (MCP-1) expression, both protein and mRNA, has been observed in all the adipose depots of diet induced obese mice [247, 248]. Correlating to this, expression of F4/80 and CD68, macrophage cell surface receptor proteins is also elevated in these tissues. Furthermore, enhanced macrophage migration was observed in response to mesenteric adipose tissue conditioned media, in an in vitro assay, which was blocked by an antibody against MCP-1 [249]. Although these studies establish that an adipose derived factor could be responsible for the recruitment of immune cells to the adipose tissue, contradicting results are observed by investigators in MCP-1 gene knockout mouse models and studies using the MCP-1 receptor, CCR2 (chemokine chemo-attractant receptor-2) ablated mice [250].

In mouse models and humans, adipocyte size is correlated to ATM content [246]. Interestingly, adipose cell death has been proposed as a stimulus for ATM accumulation and this was observed in lipoatrophy mouse model. Adipose hypertrophy leads to hypoxia resulting in cell death that could trigger ATM recruitment. Genetic inactivation

of a pro-apoptotic molecule Bid, prevented adipocyte apoptosis and subsequent macrophage infiltration, and protected from systemic insulin resistance and hepatic steatosis. Moreover, ATMs are observed to form crown like structures (CLS) associated with apoptotic adipocytes and these CLS are observed to be multinucleated cells with large lipid droplets. However, adipocyte cell death is not found to be elevated in obese humans [251]. Basal lipolyis is considered to be enhanced in obesity and this is more true in visceral than compared to subcutaneous adipose tissue and also correlates with the ATM accrual [252, 253]. Contrary to the studies where adipose apoptosis was considered the cause for ATM enrollment, Kosteli and coworkers have recently demonstrated that, increase in circulating concentrations of FFAs due to enhanced lipolysis recruits macrophages to the adipose tissue, as a means to sequester the released lipids. Furthermore, these ATMs were responsible for the attenuation of lipolysis induced FFA release, as pharmaceutical agent mediated macrophage depletion lead to an increase in the expression of genes involved in lipolysis [254].

Two main phenotypes of ATMs have been identified: the classically activated macrophages that secrete pro-inflammatory cytokines such as TNFα, IL-1 IL-6 and the alternatively activated anti-inflammatory macrophages. These two kinds of macrophages are also categorized as M1 or M2 type based on their cell surface receptors, although there is no clear consensus. The alternatively activated macrophages are considered to be the adipose tissue resident macrophages that are involved in the repair and remodeling. High fat diet induced obese conditions in mouse models increase proportion of classically activated macrophages [255]. Although the precise mechanism of the increase and the

manifestation of changed activation state is not know. However, studies using macrophage specific PPARγ ablation in mice have demonstrated impaired maturation of alternatively activated macrophages that lead to DIO, insulin resistance and glucose intolerance [256].

Cytokines

In disease states, adipocytes as well as the recruited immune cells, specifically macrophages secrete pro-inflammatory cytokines. Several cytokines produced by the macrophages, are known to stimulate lipolysis in adipocytes. The FFAs thus generated further activate the macrophages to secrete pro-inflammatory cytokines, triggering a vicious cycle. TNFα (Tumor Necrosis Factor-α) is one of the major pro-inflammatory cytokines secreted by activated macrophages. Although, adipocytes are capable of expressing and secreting TNFα, studies have shown that, ATMs are the major source of TNFα in conditions of obesity-associated inflammation.

Role of Tumor Necrosis Factor α (TNF α) in Mediating Insulin Resistance

Cachectin, a mediator of tumor-induced cachexia observed in some cancer patients who survived bacterial infections, was later identified as a factor produced by macrophages, which inhibited lipoprotein lipase (LPL) activity that is required for fat storage. Amino acid sequencing identified cachectin as tumor necrosis factor-α (TNFα), an inflammatory cytokine with anti-tumor and immune functions. TNFα is produced as a

transmembrane protein of 26kDa that has autocrine and paracrine functions. Upon cleavage by metalloprotease, a secreted homotrimer is released [257]. Prevention of this maturation of TNFα, by metalloprotease inhibitors protected mice against endotoxin mediated lethality [258] . Two distinct receptors TNFα receptor-1 (TNFR1) and TNFα receptor-2 (TNFR2) mediate the functions of the cytokine. Binding of TNFα trimer leads to the trimerization of the receptor monomers, leading to its activation and subsequent intra-cellular signaling. Both receptor TNFR1 and 2 are ubiquitously expressed although in different ratios, depending on the cell type. These receptors differ in their amino acid composition as well as in their intracellular binding partners.

NFκB Regulation

TNFα binding to its receptor, leads to the activation of two major transcription factors, activator protein-1 (AP-1) and NFκB, to regulate the expression of inflammatory response genes. NFκB is a family of transcription factors comprising of 5 members, p50, p52, p65(RelA), RelB and c-Rel. The general term NFκB refers to a heterodimer complex usually made up of p50-RelA. The gene regulatory function of NFκB is mediated by RelA. In the unstimulated state, NFκB is retained in the cytosol by forming a complex with inhibitor κ-B protein α (IκB-α). In the classical NFκB signaling pathway upon stimulation by TNFα (or other cytokines), activation of NFκB upstream kinase IKKβ (inhibitor κ-B kinase-β), results in the subsequent phosphorylation, ubiquitination and degradation of IκBα. Now, NFκB is free to translocate to the nucleus and transcribe genes for cytokines, growth factors, cell adhesion molecules as well as pro and anti-

apoptotic factors. IKKβ exists in a heterotrimeric complex containing IKKα, β and γ. The alternate NFκB pathway is active in immune cell types like B cells, where IKKα mediates the phosphorylation of IκB-α and thus its degradation.

Studies in 3T3-L1 adipocytes have demonstrated that TNFα promotes tyrosine phosphorylation of IRS1 and its subsequent binding to PI3K. In these studies, the authors observed a synergetic effect of insulin and TNFα on IRS1 tyrosine phosphorylation [259]. Furthermore, TNFα stimulated NFκB activation has been shown to require Akt mediated IKKβ phosphorylation [260]. This is further supported by the study where, PTEN blocked the TNFα mediated NFκB activation, through suppression of PI3K/Akt signaling [261]. However, the PI3K/Akt/mTOR signaling cascade is implicated in the TNFα mediated insulin resistance, through IRS1 serine 636/639 phosphorylation. Rapamycin treatment or ectopic expression of PTEN or an Akt kinase-dead mutant inhibited TNFα stimulated IRS1 serine phosphorylation [262]. Furthermore, TNFα mediated apoptosis and caspase-8 activation was inhibited in mouse hepatocytes with S6K1 deletion [263]. These observations show that TNFα utilizes the insulin receptor downstream signaling cascade to promote pro-inflammatory gene expression as well as insulin resistance. Study by Kim and coworkers identified GβL (mLST8), mTOR binding partner, as a negative regulator of TNFα stimulated NFκB signaling. GβL was shown to directly interact with IKK and inhibit its phosphorylation [264]. Recent study by these authors further demonstrated that, GβL mediated IKKβ dephosphorylation is through its interaction with protein phosphatases. Protein phosphatase 4 (PP4), PP2A and PP6 were identified as GβL binding partners [265].

IKKβ has been shown to play an important role in promoting insulin resistance. Salicylate treatment inhibited IKKβ activation and improved insulin sensitivity in rodent models of obesity and insulin resistance. In addition, HFD fed and ob/ob mice were protected from insulin resistance in IKKβ heterozygous (IKKβ+/-) deleted condition. Moreover, IKKβ +/- mice were protected from FFA induced insulin resistance [266]. Supporting these observations, studies have shown that, the lipid intermediate DAG stimulates PKCθ and PKCδ that subsequently phosphorylate IKKβ [267]. Furthermore, PKCθ knockout mice were protected from high fat diet induced insulin resistance [242]. Mice with liver specific deletion of IKKβ developed muscle and adipose insulin resistance in response to HFD, aging and obesity, although they retained hepatic insulin sensitivity. The same authors also analyzed the NFκB pathway in the myeloid cells; absence of IKKβ in myeloid cells protected mice from whole body insulin resistance [268]. These studies show that IKKβ plays an important role in mediating local as well as systemic insulin resistance.

TNFα mRNA expression was found to be enhanced in the adipose tissue of obese human subjects when compared to lean individuals and this correlated with the plasma insulin levels [269]. Tsigos and coworkers observed 2 fold increase in the circulating concentration of TNFα in insulin resistant humans compared to controls [270]. However, no correlation was observed between TNFα mRNA levels and BMI in men and women in other studies [271, 272]. Neutralization of TNFα improved insulin sensitivity and lowered plasma lipid and glucose levels in obese rats [273]. In addition, deletion of TNFα receptors or TNFα protected mice against DIO and insulin resistance [274, 275].

Neutralizing TNFα, using anti TNFα antibody, failed to improve insulin sensitivity in humans [276, 277]. Moreover, patients who received TNFα neutralizing antibody to treat rheumatoid arthritis exhibited improved glycemia, but after the completion of treatment there was a relapse [278, 279]. Although circulating levels of TNFα are found to be very low in insulin resistant humans and mouse models, however, liver and muscle local concentration of TNFα were observed to be elevated in insulin resistance condition [280, 281]. Saghizadeh and colleagues observed six fold elevated TNFα mRNA levels in muscle biopsies from type 2 diabetic subjects [282]. These studies show a possible role of locally produced TNFα in mediating muscle insulin resistance, which is supported by the observation that muscle insulin resistance was reversed using anti-TNF treatment in insulin resistant rats accompanied by decrease in TNFα protein in the muscle but not in fat [281].

Activator Protein-1 (AP-1) Regulation

Regulation of AP-1 transcription factor expression as well as activation is the other downstream effect of TNFα signaling cascade. AP-1 transcription factor is a heterodimeric protein complex comprising members of JUN, FOS and ATF protein families. These transcription factors control a number of cellular processes including differentiation, proliferation, and apoptosis. The JUN family consists of c-Jun, JunB and JunD; while the FOS family is made up of c-Fos, FosB, Fra-1 and Fra-2. These proteins are involved in regulating cell proliferation and differentiation [283]. Furthermore, studies have shown that immediately after adipocyte differentiation induction, expression

of c-Jun, c-Fos, Jun-B, Fos-B and Fra-1 is induced [284]. Additionally, insulin stimulated Fra-1 expression in 3T3-L1 adipocytes requires both mTOR and the MAPK pathways [285]. Recent studies have implicated ΔFosB (a naturally occurring truncated form of FosB) as a negative regulator of adipogenesis both *in vivo* and *in vitro* [286]. Transgenic expression of ΔFosB resulted in increased energy expenditure and promoted insulin sensitivity [287].

In response to TNFα stimulus, MAPKs, specifically JNK and p38 are activated which in turn induce the expression of AP-1 transcription factors. These MAPKs also phosphorylate the AP-1 proteins in response to stimulus. AP-1 gene expression is also stimulated by ERK1 and ERK2 MAPKs in response to phorbol esters. Three distinct JNKs exist; JNK1 and JNK2 are ubiquitously expressed while JNK3 is restricted to the brain, heart and testis. JNKs are activated by threonine and tyrosine phosphorylation by MKK4 and MKK7. Through a cascade of upstream kinase mediated phosphorylation events, MKK7 and MKK4 are activated, primarily in response to cytokines and environmental stress respectively. JNKs are also activated in response to other cellular stress such as osmotic stress, ultra-violet light, reactive oxygen species, as well as endoplasmic reticulum stress (ER stress). Activated JNK regulates its substrates such as cJUN, ATF-2 and other cytosolic and nuclear proteins through phosphorylation. JNK can be stimulated through toll like receptor-4 (TLR4) mediated activation. TLR4 is the receptor for lipopolysaccharide (LPS) and plays an important role in innate immunity. Stimulation of this pathway induces cytokine expression and activates proinflammatory pathways. FFAs mediate insulin resistance through activation of JNK cascade through

TLR4 pathway. TLR4 deficient mice are protected from lipid-induced suppression of muscle insulin signaling and systemic glucose metabolism. Furthermore, TLR4 deficient female mice exhibit increased obesity but are partially protected from HFD induced insulin resistance [288].

In diet induced obese mice, as well as leptin deficient ob/ob mice, expression of TNFα is increased and this is associated with enhanced JNK activity. JNK binds to, as well as phosphorylates IRS1 and IRS2 on the Ser307 residue, resulting in IRS1 inactivation. This is further supported by a study in which JNK1 knockout mice are protected from high fat diet induced insulin resistance as well as IRS1 Ser307 phosphorylation. Moreover, these animals were protected from HFD induced obesity and adipose tissue expansion. Although JNK1 in hematopeietic cells mediates pro-inflammatory cytokine synthesis in response to HFD and induces insulin resistance, however, mice deficient for JNK1 in myeloid cells and hematopeietic cells did not differ from the control mice, in their glucose and insulin response. Interestingly, adipose specific JNK1 knockout mice were protected from HFD induced insulin resistance in the liver that was due to decreased IL-6 synthesis by the adipose tissue.

p38 MAPK exists as four isoforms α, β, γ and δ. Similar to JNK, p38 is also activated by phosphorylation in response to cellular stress. Through the MAPK cascade, activated MKK6 phosphorylates all the p38 isoforms, while MKK3 activates p38α, γ and δ, and MKK4 activates only p38α. Studies in cultured endothelial cells have shown that TNFα mediated insulin resistance by inhibition of PI3K/Akt/eNOS (endogenous nitric

oxide synthase) pathway, is a p38 MAPK dependent function [289]. Furthermore, recent studies in hepatocytes demonstrated the important role played by JNK and p38 MAPK pathway in palmitate induced hepatic insulin resistance [290].

Role of Interleukins in Promoting Insulin Resistance - Interleukin-1

The interleukin -1 family of cytokines consists of interleukin-1α (IL-1α) and interleukin-1β (IL-1β), two low molecular weight proinflammatory cytokines. IL-1β is produced as a ~31kDa precursor that is proteolytically processed by caspase-1, to its active 17.5 kDa form; however, both the precursor and the mature forms of IL-1α are biologically active. Calpain, a calcium-activated cysteine protease mediates the processing of IL-1α. The two cytokines also differ in the cell types where they are produced. While IL-1α is constitutively produced by epithelial cells and keratinocytes, variety of other cells such as macrophages, monocytes, fibroblasts, endothelia cells, can synthesize and secrete this cytokine, but only upon stimulation. In vitro studies have implicated IL-1α in a wide variety of functions including, induction of pro-collagen type I & III synthesis, prolifereation of fibroblasts, collagenase secretion and IL-6, induction of TNFα release by endothelial cells and many more. In vivo, it induces synthesis of proteases and subsequent proteolysis of muscle, stimulates acute phase protein synthesis, fibroblast and lymphocyte proliferation and induces fever. In contrast, IL-1β is produced by activated macrophages and is an important mediator of inflammatory response and other cellular functions, including cell proliferation & differentiation and apoptosis. Inspite of these differences, the two cytokines share the same receptor interleukin-1

receptor I and II (IL1R1 & IL1RII). Only IL1R1 has a biological functional, while IL1RII lacks a cytosolic domain and is non functional. IL-1 receptor antagonist, another member of IL1 family also binds to the IL1R1, but does not induce a cellular response and thus antagonizes the effects of IL-1α and β.

IL-1β exerts its biological functions through IL1R1/IKK/NFκB pathway, and has been demonstrated to utilize ERK, JNK and p38 MAPKs. Recently IL-1β has been shown to be involved in mediating insulin resistance. IL-1β concentrations are found to be elevated in nondiabetic offspring of diabetic individuals and this correlates with metabolic syndrome [291]. Furthermore, studies have shown that, individuals with elevated circulating levels of both IL-1β and IL-6 are at more risk of developing type 2 diabetes, than of IL-6 levels alone. Additionally, in the visceral adipose tissue of obese subjects, and epididymal adipose tissue of diet induced obese and genetically obese (ob/ob) mouse models, increased expression of IL-1β and IL1R1 is observed. Studies in 3T3-L1 as well as human adipocytes demonstrated IL-1β to stimulate ERK, JNK and p38 MAPK phosphorylation, as well as IκBα degradation. However, expression of C/EBPα, AP2 and PPARγ was found to be unaffected in acute treatment, but inhibited in chronic stimulation. Long-term treatment with IL-1β inhibited adiponectin mRNA as well as protein expression. Additionally, insulin stimulated glucose uptake and GLUT4 expression were inhibited. This was possibly due to the observed specific decrease in IRS1 mRNA expression and diminished tyrosine phosphorylation, however, IRS2 tyrosine phosphorylation was not affected. Furthermore, pharmacological inhibition of

ERK1/2 prevented the IL-1β mediated suppression of insulin stimulated Akt, as well as AS160 and GLUT4 translocation to the plasma membrane [292, 293].

The role of IL-1α in insulin resistance is not known. Although, in a recent study in 3T3-L1 adipocytes, IL-1α treatment, within 15 minutes induced activation of IKK, JNK, ERK as well as p70S6K1 and also caused IRS1 serine phosphorylation on several residues. Pharmacological inhibitor induced inhibition of these kinases prevented serine phosphorylation of IRS1. However, IL-1α alone did not inhibit insulin stimulated glucose uptake or Akt phosphorylation, but exhibited a synergistic inhibition in the presence of IL-6 [294]. Owing to the effects observed on insulin signaling in IL-1α and IL-1β treatment condition, ablation of IL1RI should render animals insulin sensitive. To the contrary, Garcia and coworkers observed age induced obesity in IL1RI knockout mice. Additionally, the animals were glucose intolerant, insulin resistant and partially leptin resistant [295]. Furthermore, studies in mice with IL-1R antagonist gene ablation made the mice lean and exhibited increased energy expenditure [296]. In a second study, IL-1R antagonist knockout animals exhibited decreased levels of serum insulin levels and were resistant to diet-induced obesity [297]. Together, these studies show that IL-1 mediated regulation of insulin signaling and whole body metabolism is a complex phenomenon and more detailed analysis is needed to make definitive conclusions.

Interleukin-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine with pleotropic effects, and has tissue specific functions that differ in humans and rodents. Although, IL-6 promotes

chronic inflammation, it displays anti-inflammatory properties as well, during acute inflammatory stimuli. Due to the contradicting effects exhibited by IL-6, understanding its role in insulin resistance is quite complicated. During states of obesity-associated inflammation, increased secretion of IL-6 by the adipocytes is observed. Additionally, elevated levels of circulating IL-6 correlates with adiposity in humans, however, it does not reflect insulin sensitivity. Although, experiment from cell culture model systems and studies using rodents demonstrate that IL-6 promotes insulin resistance. Furthermore, IL-6 neutralizing antibody treatment improved hepatic insulin signaling in ob/ob mice. Surprisingly, IL-6 deficient mice became obese and insulin resistant, which could be due to increased TNFα and leptin levels. To the contrary, IL-6 over expression caused obesity and insulin resistance in mice. Despite these contradicting observations, it is interesting to note that IL-6 induces lipolysis in the adipose tissue and increases FFA for muscle oxidation during exercise. IL-6 administration was found to increase glucose uptake in human muscle. In the liver, IL-6 signaling through the JAK/STAT pathway leads to the induction of suppressor of cytokine signaling 3 protein (SOCS3) and thus suppresses insulin signaling. Studies in cultured hepatocytes have demonstrated a role for IL-6 to induce gluconeogenesis and in vivo studies have further corroborates this where increased glucose output was observed. A recent study in mice has shown that IL-6 expression in the adipose is regulated by JNK1 and adipose specific knockout of this MAPK decreased high fat diet induced increase in circulating IL-6 levels and protected the animals from hepatic insulin resistance that was due to decreased SOCS3 function.

Role of PPARs in Insulin Sensitivity and Resistance

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors, were first identified in Xenops as receptors that induce the proliferation of peroxisomes. Three types of PPARs have been identified PPARα, PPARδ (β) and PPARγ. PPARα is expressed in liver, kidney, heart, muscle, adipose as well as in other cell types. PPARα is activated by long chain fatty acids and plays an important role in the transcriptional regulation of genes involved in peroxisomal and mitochondrial fatty acid oxidation pathways. Mice deficient for PPARα had impaired fatty acid uptake and oxidation in the liver, exhibited hypoglycemia and increased inflammation. While the decrease in glycemia is due to shift from fat oxidation to glucose utilization, increase in NFκB activity and expression of inflammatory genes and pro-inflammatory lipid mediators caused elevated inflammatory state. Treatment with fibrates, synthetic PPARα agonists improved serum lipid levels, due to enhanced hepatic fatty acid uptake and oxidation.

PPARδ is expressed as two splice variants and is expressed in wide range of tissues. The major function of PPARδ is regulation of fatty acid oxidation and thermogenesis in muscle and also mediates muscle fiber type programming. PPARδ knockout mice die at early age due to placental and mylenations defects. PPARδ is not required for fat storage in adipose tissue, as fat mass was not reduced in adipose specific PPARδ deletion. Additionally, PPARδ has been shown to suppress macrophage derived inflammation as well as mediate alternate activation, thus regulating whole body insulin

sensitivity. Furthermore, studies have demonstrated its role in decreasing hepatic glucose output, and increasing fatty acid transport and oxidation in the heart.

By differential promoter usage PPARγ gene gives rise to three different transcripts. PPARγ1, expressed in all tissues is encoded by exon A1, A2 and exon 1-6. PPARγ2 is 30 amino acids longer and is expressed mainly in the adipose tissue and is encoded by exon B and exon 1-6. While PPARγ3 is identical to PPARγ1, its expression is restricted to human white adipocytes and few other cell types and is encoded by exon A2 and exon 1-6. However, exon A1 and A2 are not translated. Ligand mediated activation of PPARγ and its dimer formation with retinoic X receptor (RXR) results in transcription of genes containing PPREs (PPARγ response element).

The role of PPARγ as an essential component of glucose homeostasis, lipid metabolism, regulation of inflammation and atherosclerosis is supported by vast data [298]. As discussed earlier, PPARγ plays a central role and is required for adipogenesis. PPARγ deficiency in mature adipocytes of mice lead to a significant reduction in both brown and white adipose tissue mass and was accompanied by hyperlipidemia elevated serum TG, FFAs and decreased adiponectin and leptin levels. Furthermore, these animals exhibited hepatic insulin resistance and lipid accumulation. However, muscle insulin sensitivity was maintained on chow diet but the mice developed insulin resistance on HFD [299].

Along with its primary role in adipose tissue, PPARγ plays a central role in other insulin sensitive tissues such as muscle and liver, to maintain whole body insulin

sensitivity, even though it is expressed at low levels in these tissues. Loss of muscle PPARγ resulted in adipose tissue and liver insulin resistance [300]. While decreased hepatic steatosis, increased adiposity, hyperlipidemia and insulin resistance was observed in the liver PPARγ deficient animals. Furthermore, PPARγ was found to be required for macrophage alternate activation; as, myeloid specific depletion of this nuclear receptor resulted in DIO, insulin resistance, and glucose intolerance [256]. These studies have demonstrated the important role played by PPARγ in regulating the communication between different metabolic tissues.

Regulation of PPARγ

Interestingly, in adipocytes, TNFα suppressed adipogenic gene expression, but stimulated preadipocyte as well as cytokine gene expression, which were mediated by NFκB activity [301]. TNFα mediated decrease in expression of lipogenic genes as well as its function leading to dedifferentiation of mature adipocytes is believed to be through repression of PPARγ gene expression. It is well known that C/EBP/delta binds to and activates PPARγ promoter. TNFα exhibits its inhibitory effects on PPARγ in a mechanism involving decrease in C/EBPδ expression [302]. Additionally, TNFα stimulated NFκB association to PPARγ inhibits the ligand dependent transcriptional activity of PPARγ. Furthermore, TNFα mediated IκBα degradation resulted in translocation of cytoplasmic histone deacetylase-3 (HDAC3) to the nucleus, which then bound to PPARγ and prevented its transcriptional activity. Studies from several groups have demonstrated inhibition of adipogenesis through ERK1/2 and JNK mediated PPARγ

Ser112 (γ2) and Ser82 (γ1) phosporylation [303-306]. Moreover, DIO was prevented in downstream tyrosine kinase-1 (Dok1) knockout mice, by ERK mediated PPARγ Serine112 phosphorylation [307]. Recently, TNFα has been shown to mediate PPARγ protein cleavage through caspase-3, -6 and -8 activation, which resulted in PPARγ nuclear exclusion in cultured cells [308]. Additionally, AMPK mediated PPARγ phosphorylation represses both the ligand-dependent and independent transactivating functions [309]. Taken together, these studies suggest that TNFα potently downregulates PPARγ function in fat cells and NFκB as well as MAPKs seem to play an important role in this process.

MAP4K4

Mitogen activated protein kinase kinase kinase kinase-4 (MAP4K4), a serine/threonine that belongs to a large family of protein kinases related to yeast Ste20p (sterile 20 protein) kinase. The mammalian STE20 family consists of about 28 kinases related in their catalytic domains. Based on their domain structure, these proteins are classified into two classes, the p21-activated protein kinases (PAKs) and the germinal center kinases (GCKs). The GCK class or proteins are categorized under eight groups GCK-1 to GCK-VIII. MA4PK4 mouse ortholog HGK (hepatocyte progenitor kinase-like/GCK-like kinase), is part of the GCK-IV group of kinases along with three other members TNIK, MINK and NRK. This group of kinases is implicated in various intracellular regulatory functions, including cell cycle progression, differentiation, transcription, apoptosis, cytoskeleton rearrangement and cell motility [310].

MAP4K4 was first identified as a kinase interacting with the SH3 domains of Nck, an adaptor protein composed of one SH2 and three SH3 domains and was named NIK (Nck interacting kinase) [311, 312]. MAP4K4 shares a high homology in its kinase domain and C-terminal domains, with TNIK and MINK while the intermediate domain is variable. The C-terminal domain is a citron homology domain (CNH), is important for protein-protein interaction. NIK was shown to interact with MEKK1 as well as cytoplasmic domain of ß1-integrin receptors through this domain. *misshapen* (*msn)* in *Drosophila melanogaster* and *mig-15* in *Caenorabditis elegans*, are MAP4K4 orthologs, which share about 80% amino acid identity in both kinase and CNH domains.

Initial studies using transient over expression of NIK identified MAP4K4 as a stress-activated protein kinase (SAPK/JNK) activating kinase, functioning upstream to MEKK1. Furthermore, using transient transfection of HGK in 293T cells, it has been demonstrated that TNF-alpha stimulated MAP4K4 activity specifically towards JNK but not ERK1/2 or p38 MAPK [312]. Recent studies in human skeletal muscles further supported this, where silencing MAP4K4 prevented TNF-alpha stimulated JNK activation. However in this study, the authors demonstrated that TNF-alpha regulated ERK1/2 activation in a MAP4K4 dependent manner [313]. MAP4K4 was found to interact with Rap2 (Ras-related protein family member) through its CNH domain. Rap2 in the GTP bound form enhanced MAP4K4 induced JNK activation [314]. Although, numerous studies have demonstrated JNK activation by exogenously expressed MAP4K4, there are no studies in the literature till date showing endogenous MAP4K4 mediated JNK activity. Studies from our laboratory identified TNFα stimulated TNFR1

signaling to c-Jun and ATF2 in a JNK1/2 and p38 SAP kinase dependent mechanism, to regulate Map4k4 gene expression in 3T3-L1 adipocytes, thus establishing MAP4K4 as a JNK1/2 transcriptional target [315].

Stimulation of ephrin receptor family, which are cell surface ligands involved in cell migration and tissue remodeling has been shown to activate NIK, implicating MAP4K4 in cell morphogenesis [316]. Consistent with this, MAP4K4 knockout mice (NIK-/-) exhibit embryonic lethality during early embryogenesis, with mesodermal differentiation and cell migration defects [317]. Although JNK has been identified as a downstream effector of MAP4k4, owing to the differences in the time line, when the embryos die post-gastrulation, (Nik-/- die between day 9.5-10.5 while JNK1&2-/- die at 11.5), and the morphological differences observed in the two kinds of embryos, the embryonic lethality observed in the NIK-/- mice might not be due to JNK effects [318]. Moreover, MAP4K4 has been shown to be associated with several types of cancers and also has been identified as a promigratory kinase [319]. Together, these functions of MAP4K4 are possibly through activation of p38 MAPK that subsequently inhibits E-Cadherin protein, thus preventing its inhibitory function on epithelial to mesenchymal transition (EMT) and mesoderm migration [320].

MAP4K4 expression has been observed to be elevated in the preadipocytes of obese individuals, thus preventing adipocyte differentiation and maturation leading to insulin resistance [368]. Recent studies have implicated MAP4K4 in TNF-alpha induced insulin resistance. Silencing MAP4K4 in myotubes from insulin-resistant type 2 diabetic

patients restored insulin stimulated glucose uptake to the level observed in healthy subjects. Furthermore, the authors demonstrated complete prevention of TNF-alpha induced insulin resistance on Akt, AS160 as well as IRS1, which was through MAP4K4 dependent JNK1/2 and ERK1/2 activation [321]. Moreover, MAP4K4 has been shown to play a role in beta cell function. Human and rat primary pancreatic beta cells were protected from TNF-alpha induced inhibition of glucose stimulated insulin secretion, as well as decrease in IRS-2 expression in the absence of MAP4K4. Additionally, MAP4K4 was required for the TNF-alpha activation of Akt, AS160, ERK, JNK and p70S6K1. However, NFκB and p38 MAPK were unaffected [322]. Interestingly, recent study from our laboratory has identified MAP4K4 as a mediator of cytokine expression and thus assigning a new role for this MAPK in inflammation. Silencing MAP4K4 using an innovative siRNA delivery system in vivo, the authors demonstrated protection from LPS induced lethality that was due to inhibition of TNFα and IL-1β production [323]. Interestingly, Tang and colleagues from our laboratory, in an siRNA mediated screen in 3T3-L1 adipocytes, identified MAP4K4 as a novel protein kinase involved in regulation of insulin stimulated glucose uptake. Silencing MAP4K4 lead to an increase in GLUT4 expression accompanied by significantly enhanced glucose uptake. Furthermore, C/EBPα and PPARγ protein expression, as well as triglyceride content were observed to be elevated in the absence of MAP4K4 [324]. This study was the first to recognize the important role of MAP4K4 as a negative regulator of insulin stimulated hexose transport, PPARγ synthesis and hence modulates adipose function. Map4k4 functions in different cell types are summarized in Figure 1.8.

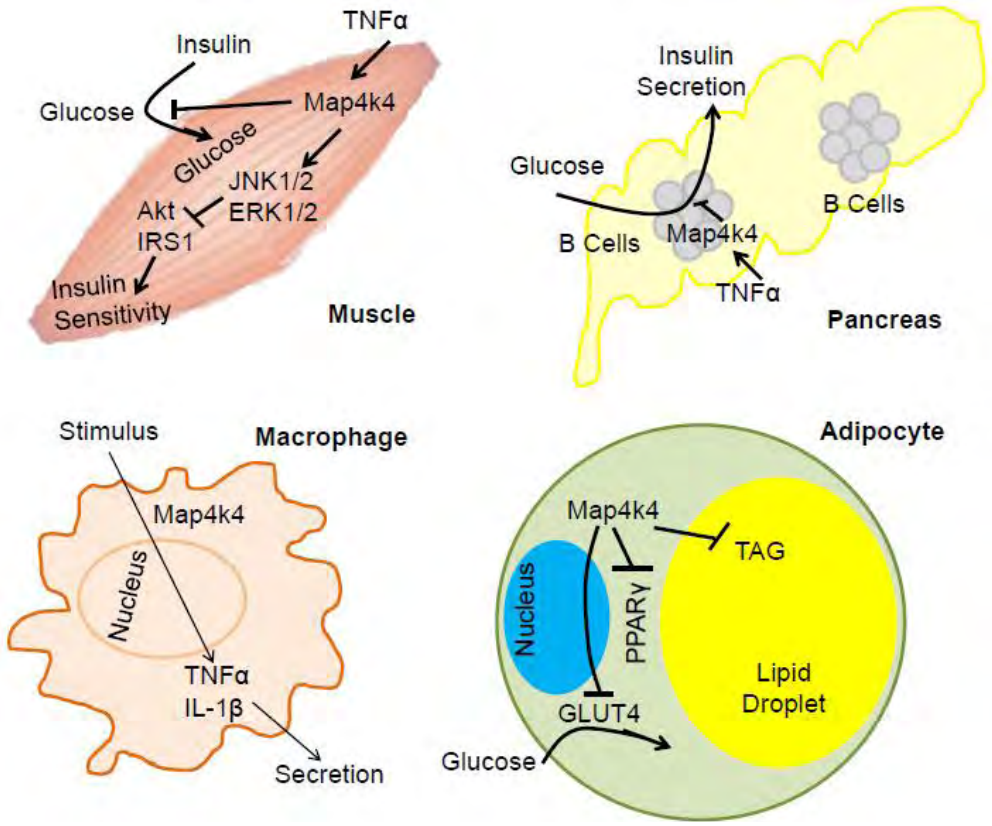


FIGURE 1.8. Role of Map4k4 in Inducing Insulin Resistance. In the muscle Map4k4 inhibits insulin stimulated glucose uptake, and mediates TNFα induced insulin resistance. TNFα induced inhibition of glucose-stimulated insulin secretion is mediated by Map4k4 in the pancreatic beta cells. Map4k4 regulates cytokine expression in macrophages and thus plays a role in mediating inflammation. In the adipocyte, GLUT4 expression, insulin stimulated glucose uptake, PPARγ expression and TAG synthesis are inhibited by Map4k4.

Specific Aims

Studies from our laboratory have identified Map4k4 as a negative regulator of insulin stimulated glucose uptake and adipogenesis. Furthermore, Map4k4 was also found to regulate PPARγ expression. PPARγ is considered to play a central role in modulating adipogenesis and is also the target of the diabetic medication TZDs [325]. Understanding the mechanism by which Map4k4 regulates PPARγ expression could lead to better manage obesity induced insulin resistance and associated complications.

Map4k4 is also implicated in TNFα synthesis, as well as TNFα signaling to induce insulin resistance in muscle. Additionally, Map4k4 is also responsible for the TNFα induced inhibition of glucose stimulated insulin secretion by beta cells. Given the involvement of Map4k4 in inflammatory pathways, insulin signaling & secretion, insulin resistances, as well as preliminary studies implicating Map4k4 in regulation of adipose function, I embarked on determining the role of Map4k4 in regulating adipocyte function in-vitro as well as in-vivo. To address this RNAi strategy was employed for the in vitro study and a recently published novel strategy was utilized in the in vivo investigation. The specific aims of these studies were:

To identify the signaling pathway by which Map4k4 regulates PPARγ protein expression in vitro, utilizing 3T3-L1 adipocytes as model system.

To determine the role of Map4k4 in regulating adipose function in vivo, using a mouse model system with adipose-specific Map4k4 gene silencing

CHAPTER II: *Map4k4* Negatively Regulates Peroxisome Proliferator-activated Receptor (PPARγ) Protein Translation by Suppressing the Mammalian Target of Rapamycin (mTOR) Signaling Pathway in Cultured Adipocytes

Disclaimer: All experiments were performed by the author except for Figure 1 which was done in colaboration with Adilson Guilherme and me; Figure 2 and Figure 3A were done by Adilson Guilherme; and Figure 6 was done in colaboration with Liting Xue and me. Anil Chawla prepared the construct to generate adenovirus for Map4k4 overexpression. Xiaoqing Tang made the original observation that Map4k4 gene silencing enhances PPARγ protein level. I did all the statistical analysis in this chapter.

This Chapter is in the format published: **Kalyani V. P. Guntur,** Adilson Guilherme, Liting Xue, Anil Chawla, and Michael P. Czech1. *Map4k4* Negatively Regulates Peroxisome Proliferator-activated Receptor (PPAR)γ Protein Translation by Suppressing the Mammalian Target of Rapamycin (mTOR) Signaling Pathway in Cultured Adipocytes. J Biol Chem 285: 6595-6603.

Abstract

The receptor peroxisome proliferator-activated receptor γ (PPARγ) is considered a master regulator of adipocyte differentiation and promotes glucose and lipid metabolism in mature adipocytes. We recently identified the yeast Sterile 20 (Ste20) protein kinase ortholog, *Map4k4*, in anRNAinterference-based screen as an inhibitor of PPARγ expression in cultured adipocytes. Here, we show that RNA interference-mediated silencing of *Map4k4* elevates the levels of both PPARγ1 and PPARγ2 proteins in 3T3-L1 adipocytes without affecting PPARγ mRNAlevels, suggesting that *Map4k4* regulates PPARγ at a post-transcriptional step. PPARγ degradation rates are remarkably rapid as measured in the presence of cycloheximide ($t1/2$=2 h), but silencing *Map4k4* had no effect on PPARγ degradation. However, depletion of *Map4k4* significantly enhances [35S]methionine/cysteine incorporation into proteins, suggesting that *Map4k4* signaling decreases protein translation. We show a function of *Map4k4* is to inhibit rapamycin-sensitive mammalian target of rapamycin (mTOR) activity, decreasing 4E-BP1 phosphorylation. In addition, our results show mTOR and 4E-BP1 are required for the increased PPARγ protein expression upon *Map4k4* knockdown. Consistent with this concept, adenovirus-mediated expression of *Map4k4* decreased PPARγ protein levels and mTOR phosphorylation. These data show that *Map4k4* negatively regulates PPARγ post-transcriptionally, by attenuating mTOR signaling and a 4E-BP1-dependent mechanism.

Introduction

Adipose tissue has long been recognized as the major storage depot for excess food-derived energy and has more recently been identified as an endocrine organ critical in the hormonal regulation of energy homeostasis [326]. Dysfunctions in these roles, which occur as a result of adipose tissue expansion in obesity, appear to be key elements in the development of insulin resistance and ultimately type 2 diabetes [327]. Maintenance of adipose tissue functions, including lipid sequestration as triglyceride stores, depends in part on appropriate levels of expression of genes required for triglyceride synthesis and storage. Many such genes are regulated by the nuclear hormone receptor PPARγ a nuclear hormone transcription factor. It is considered a master regulator of adipocyte differentiation [328] and essential for the increase in adipose mass associated with obesity [329]. Additionally, PPARγ is activated by lipid ligands [330] and plays an important role in glucose homeostasis and regulation of lipid metabolism in mature adipocytes. Understanding the regulation of PPARγ, a major metabolic modulator, would provide a better comprehension of its role in whole body homeostasis. PPARγ expression could be regulated at different levels, transcription, protein turnover and degradation, and possibly translation. Tumor necrosis factor-α mediated IκB kinase stimulation activates NF-κB signaling thus potently decreasing PPARγ transcription [301, 331, 332]. Studies have shown that phosphorylation of PPARγ promotes its degradation through a ubiquitin-proteasome-dependent pathway [333, 334]. Stephens and co-workers have shown that PPARγ protein has a rapid turnover, and interferon γ treatment further enhanced the protein turnover [334].

In a screen for regulators of adipocyte functions, including glucose uptake, we recently identified *Map4k4* as an inhibitor of PPARγ expression [324]. *Map4k4* is a serine/threonine protein kinase that belongs to a large group of protein kinases related to *Saccharomyces cerevisiae* Ste20. The potential involvement of *Map4k4* as an element in obesity-associated adipocyte dysfunction was suggested by the observations that it is acutely activated [335] and increased in expression by tumor necrosis factor-α [315], a cytokine overproduced in adipose tissue associated with obesity in animal models as well as human patients [269]. Silencing *Map4k4* has been shown to improve glucose uptake and insulin sensitivity as well as triglyceride synthesis in cultured adipocytes [324]. In our previous studies, enhanced expression of PPARγ upon *Map4k4* gene silencing in 3T3-L1 adipocytes was observed [324]. However, our preliminary data suggested that this enhancement is not the result of increased PPARγ mRNA levels. We have thus focused on the synthesis of PPARγ protein as a potential control point for *Map4k4* effects. Of the three phases of protein translation, initiation is a complex process that is mediated by many different proteins [336, 337]. Global control of protein synthesis is achieved by changes in the phosphorylation state of initiation factors or their interacting regulators. One of the first steps of initiation of protein synthesis and a rate-limiting step is the formation of a ~~–t~~ernary complex" by Met-tRNA and GTP-bound eIF2-α. Another means of translation regulation involves modulation of eIF4e (cap-binding protein) availability that is achieved by eIF4e-binding proteins called 4e-BPs. Hypophosphorylated 4e-BPs bind to eIF4e and prevent interaction between the 43 S preinitiation complex and mRNA that leads to inhibition of translation initiation.

Association of eIF4e and the scaffold protein eIF4G is through a small domain also present in 4e-BPs. Nonphosphorylated 4e-BP binds tightly to eIF4E preventing its association with eIF4G. Phosphorylated 4e-BP is inactive and dissociates allowing eIF4e to engage eIF4G to form the complex that facilitates translation initiation [338, 339]. Mammalian target of rapamycin (mTOR) is a conserved Ser/Thr kinase and a major regulator of cell growth and proliferation via modulating protein synthesis in response to environmental cues. 4E-BP1 and S6K1 are well known downstream targets ofmTORand are phosphorylated upon insulin stimulation [340]. Insulin is a major upstream effector of mTOR that enhances protein synthesis as part of regulating anabolic processes in response to glucose. Thus, impaired mTOR signaling might play a role in the development of glucose and insulin resistance and type 2 diabetes. In this study we have examined the role of *Map4k4* in regulating PPARγ protein and embarked on identifying the players that are regulated by *Map4k4* that potentially would regulate PPARγ protein expression.

EXPERIMENTAL PROCEDURES

Materials—Antibodies against eIF2-α (total (catalog no. 9722) and phospho-Ser-51 (catalog no. 9721)), 4E-BP1 (total (catalog no. 9452), phospho-Thr-37/46 (catalog no. 9459), Ser-65 (catalog no. 9451), and Thr-70 (catalog no. 9455)), 4e-BP2 (catalog no. 2845), and mTOR (total (catalog no. 2972) and phospho-Ser-2448 (catalog no. 2971)) were purchased from Cell Signaling Technology, Inc. Antibody against PPARγ (catalog no. sc-7273) was purchased from Santa Cruz Biotechnology. Antibody against actin was

purchased from Sigma (catalog no. A2228). Rapamycin was purchased from Calbiochem (catalog no. 553210). All siRNA was purchased from Dharmacon (Lafayette, CO). [35S]Met protein labeling mix was purchased from Perkin Elmer Life Sciences.

Cell Culture and siRNA Transfection—3T3-L1 preadipocytes were cultured and differentiated into adipocytes as described previously [341]. For siRNA transfection, cells 4 days post-induction of differentiation were used as described previously (11). Briefly, $5.625*10^5$ cells were electroporated using 7.5 nmol of siRNA for scrambled, *Map4k4*, or 1.87 nmol of siRNA for 4E-BP1 and 4e-BP2 and then plated in 4 wells of a 24-well plate. Cells were recovered in complete Dulbecco‘s modified Eagle‘s medium (10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin) and were cultured for 48 or 72 h after the transfection before beginning the experiments.

[35S]Met/Cys Labeling—3T3-L1 adipocytes 4 days post-differentiation induction were transfected with 7.5 nmol of scrambled or *Map4k4* siRNA. 56 h later, cells were starved for methionine and cysteine using methionine- and cysteine-free Dulbecco‘s modified Eagle‘s medium. 16 h later, 45 μCi/ml [35S]methionine/cysteine protein labeling mix was added to the cells for 1, 3, or 6 h. Cell lysates were prepared, and total DNA and RNA were harvested. An equal volume of protein was analyzed to measure 35S incorporation on a scintillation counter. Cell lysate was resolved on SDS-PAGE and transferred onto a nitrocellulose membrane, and 35S incorporation was detected by autoradiography. The incorporation of radioactivity was normalized to total DNA content of that sample.

Rapamycin Treatment—For 3T3-L1 adipocytes, 72-h post siRNA transfections were treated with the indicated concentration of rapamycin for 0.5, 1, or 2 h. In all experiments, cells were washed with ice-cold phosphate-buffered saline and harvested on ice as described previously [342]. Protein samples were separated on a 7% (for mTOR) or a 15% (for 4e-BP) SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were then analyzed by Western blot analysis. Changes in phosphorylation were determined through densitometry using Adobe Photoshop and normalized for loading against the nonphosphorylated protein or actin as appropriate.

Cell Size Determination—3T3-L1 adipocytes after transfection with the appropriate siRNA were seeded on coverslips in a 12-well plate. After 72 h, cells were washed in ice-cold phosphate- buffered saline. The cells were fixed in 4% formaldehyde at room temperature for 0.2 h, quickly rinsed three times in ice-cold phosphate-buffered saline, briefly dried, and mounted on Vectashield mounting medium (H1000). The coverslip was sealed using nail polish. Cell images were collected under a microscope (Zeiss inverted microscope) using bright field at X20 magnification. Saved images were analyzed for cell size by measuring the cell circumference of 150 randomly picked cells using the ImageJ program (National Institutes of Health). Average cell circumference for each siRNA transfection was calculated and graphically represented using Microsoft EXCEL.

Isolation of RNA and Quantitative Real Time PCR—RNA isolation was performed according to the TRIzol reagent protocol (Invitrogen). Briefly, media were

aspirated, and the cells were washed once with ice-cold phosphate-buffered saline. 1 ml of TRIzol reagent was added to each well. The concentration and the purity of the RNA were determined by measuring the absorbance at 260/280 nm. To further determine the quality of the RNA, 1µg of total RNA was run on a 1% agarose gel, and the quality of the 28 S and 18 S ribosomal bands was inspected visually. cDNA was synthesized using 1 µg of RNA and the iScript cDNA synthesis kit (Bio-Rad). The cDNA was synthesized according to the protocol provided by the manufacturer in a 20 µl reaction volume. For real time PCR, 1 µl of the synthesized cDNA was loaded into 1 well of a 96-well plate for detection of a specific target gene. Primers were designed with primer bank [343] and listed in Table 2.1. 36B4 was used as an internal control because its expression did not change with the silencing of the genes used in this study. 10 pmol of forward and reverse primer along with 12.5 µl of the iQ SYBR Green Supermix (Bio-Rad) was added to each well along with DNase/RNase-free water for a final volume of 25 µl. Samples were analyzed on the MyIQ real time PCR system (Bio- Rad). Relative gene expression was determined using the (Δ)*Ct* method [344].

Adenovirus Expression—Four days after differentiation, 3T3-L1 adipocytes were infected with the indicated amount of viral particles. Briefly, 10,000 cells were infected using 40 µl (1.43 $*10^{12}$ particles/ml) of HA control virus or 3HA-*Map4k4*-HA virus. 72 h post-infection, cells were washed with ice-cold phosphate- buffered saline and harvested on ice as described previously (21). Protein samples were separated on a 7% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were then analyzed by Western blot analysis. Changes in total protein level or phosphorylation were

determined through densitometry using Adobe Photoshop and normalized for loading against actin or the nonphosphorylated protein as appropriate. The virus was generated as described previously [345].

Statistics—The characteristics of the outcomes were evaluated by visual inspection of histograms generated using Microsoft EXCEL, and the observed effects were evaluated by either one-way or multifactorial analysis of variance [79]. Computations were performed using the ezANOVA program. Regression analysis was performed using Microsoft EXCEL. The data are presented as the means ± S.E.

RESULTS

Map4k4 Gene Silencing Enhances PPARγ Protein Expression at a Translational Step—We have previously shown that depletion of *Map4k4*/NIK enhances PPARγ expression in cultured adipocytes [324]. To confirm the increase in PPARγ upon *Map4k4* gene silencing, we examined the protein level of PPARγ in 3T3-L1 adipocytes upon siRNA-mediated depletion of *Map4k4* mRNA (Fig. 2.1A). At 72 h post-transfection, cell lysates were prepared from either scrambled or *Map4k4* siRNA-transfected 3T3-L1 adipocytes, and PPARγ protein levels were examined by SDS-PAGE and immunoblotting with PPARγ antibody. *Map4k4* gene silencing resulted in a 2-fold increase in PPARγ1 as well as PPARγ2 protein levels (Fig. 2.1B), consistent with our previously published results [324]. To determine whether the increase in the protein level is due to increased PPARγ transcription, RNA was isolated from similarly treated cells, and PPARγ as well as *Map4k4* mRNA levels were compared using quantitative real time

PCR. *Map4k4* gene expression was decreased by about 80% (Fig. 2.1 D and 2.E); however, the expression of PPARγ mRNA did not change upon *Map4k4* gene silencing (Fig. 2.1C). These results suggest that *Map4k4* regulation of PPARγ levels is mediated through increased translation and/or decreased degradation of PPARγ protein.

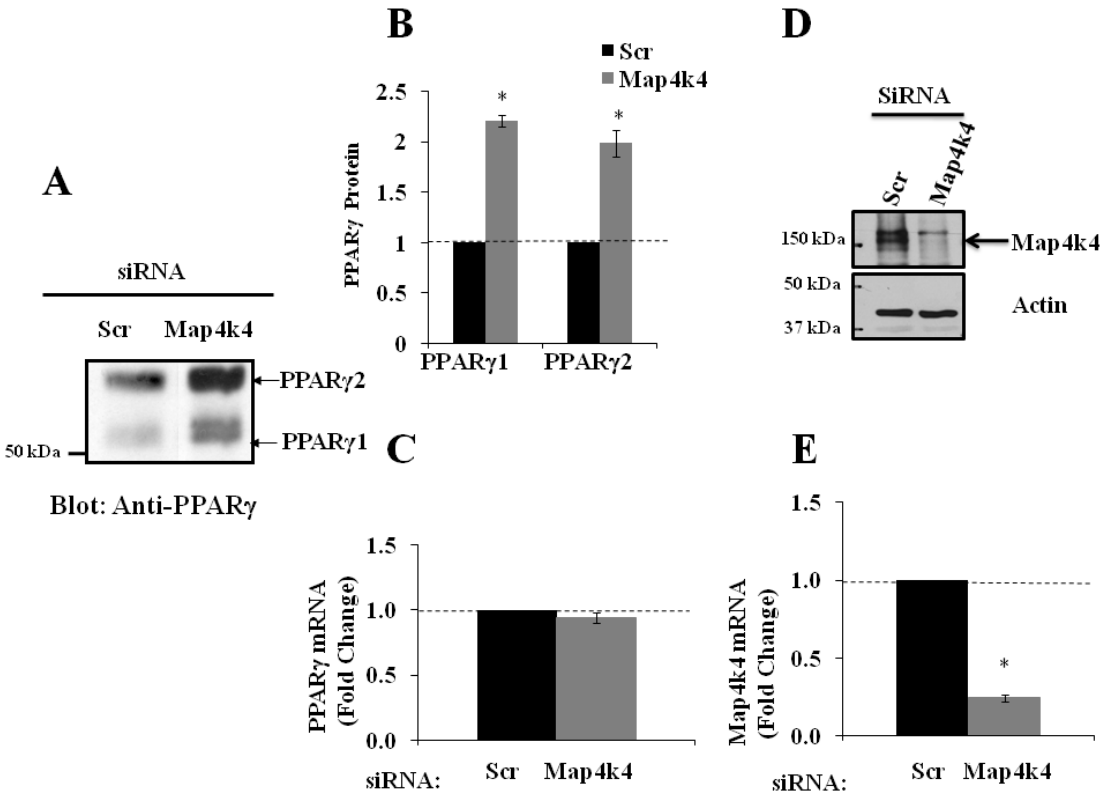


FIGURE 2.1. Gene silencing of *Map4k4* enhances PPARγ protein but not PPARγ mRNA in 3T3-L1 adipocytes. 3T3-L1 adipocytes 4 days post-differentiation induction, were transfected with 7.5 nmol of either scrambled (*Scr*) or *Map4k4* siRNA. 72 h later, cell lysates were examined by Western blot (*A*) and densitometry analysis for PPARγ1 and PPARγ2 (*B*). Total RNA was harvested and analyzed by quantitative real time PCR for PPARγ mRNA expression (*C*) and *Map4k4* mRNA expression (*E*). *D, Map4k4* protein expression. *, $p<0.01$ when compared with scrambled siRNA-transfected samples by ANOVA ($n = 4$).

To test whether *Map4k4* silencing decreased degradation of PPARγ protein, 3T3-L1 adipocytes were transfected with scrambled or *Map4k4* siRNA and were then treated with 5 μg/ml of the protein synthesis inhibitor cycloheximide for the indicated times. Cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-PPARγ antibody. As expected, initial PPARγ protein levels were enhanced with *Map4k4* gene knockdown. In the absence of protein synthesis, we observed a rapid decrease in PPARγ protein levels with time (half-life 1.5–2 h) [334, 346] in scrambled as well as *Map4k4* siRNA-treated conditions (Fig. 2.2A), indicating protein degradation. Densitometry (Fig. 2.2B) and regression analysis (Figure. 2.3) revealed that despite the higher initial level, *Map4k4* gene silencing does not prolong the half-life of PPARγ proteins. These results show that the increase in PPARγ protein level is not due to decreased degradation upon *Map4k4* gene knockdown. Rather, increased protein synthesis is the most likely mechanism for enhanced PPARγ protein levels.

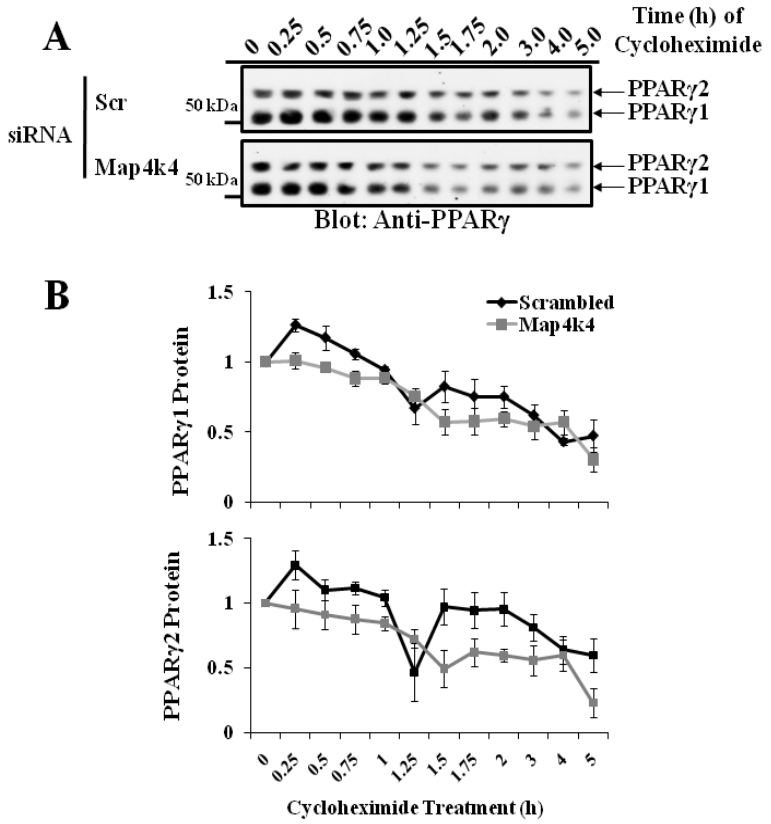


FIGURE 2.2. Depletion of *Map4k4* does not prolong the half-life of PPARγ protein in 3T3-L1 adipocytes. 3T3-L1 adipocytes 4 days post-differentiation induction were transfected with 7.5 nmol of either scrambled (*Scr*) or *Map4k4* siRNA. 72 h later, the cells were treated with 5 μg/ml cycloheximide for the indicated times. Cell lysates were examined by Western blot (A) and densitometry analysis (B) for PPARγ1 and PPARγ2. Densitometry is representative of three independent experiments.

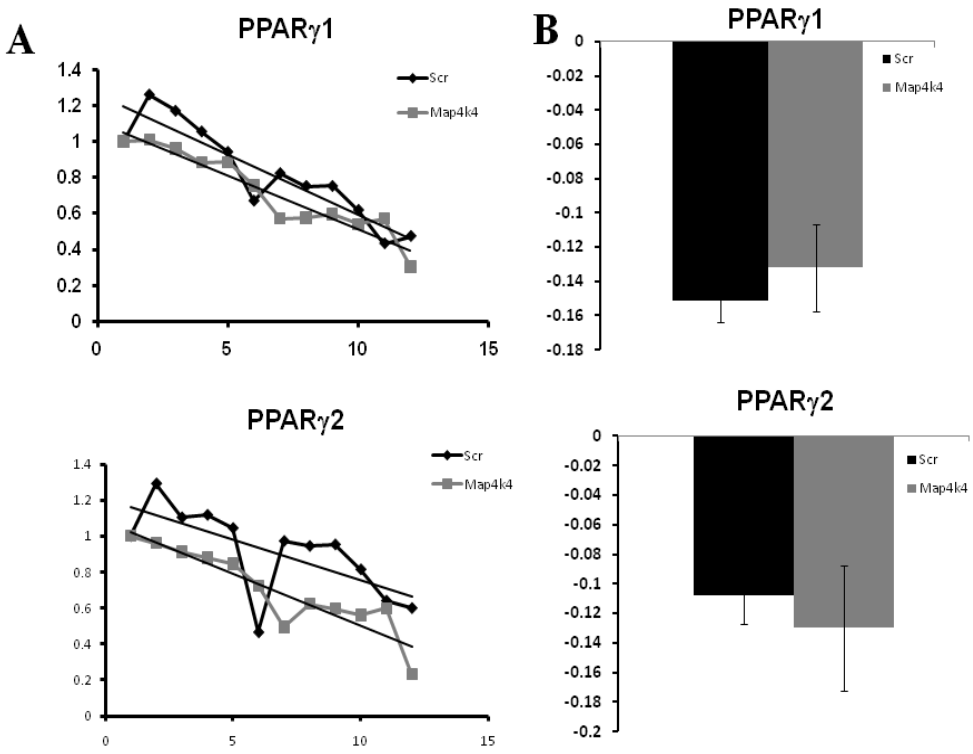


Figure 2.3. Regression Analysis. Graphical representation of regression analysis (A). No significant differences in the slope for PPARγ1 or PPARγ2, between scrambled or Map4k4 siRNA transfected conditions (B) (n=3).

Protein Synthesis and mTOR Activation Are Up-regulated upon Map4k4 Gene Silencing—To determine whether enhanced PPARγ protein levels with inhibition of *Map4k4* expression is due to an increase in general protein synthesis, we exposed 3T3-L1 adipocytes for 1, 3, or 6 h to [35S]methionine after 72 h of scrambled or *Map4k4* siRNA transfection. Total protein and RNA were harvested. Equal volumes of protein lysates were analyzed for 35S incorporation by scintillation counting, and counts were normalized to total DNA content analyzed in parallel. *Map4k4* gene silencing resulted in about 30% higher protein synthesis as indicated by increased 35S incorporation at all time points tested (Fig. 2.4A). These results suggest that the increase in PPARγ protein levels after *Map4k4* gene silencing is the result of increased protein synthesis. Thus, *Map4k4* appears to suppress protein translation in cultured adipocytes. We then sought to determine the pathways though which *Map4k4* might modulate protein synthesis. mTOR is an evolutionarily conserved protein kinase that is a central component of the signaling mechanism regulating cell growth and proliferation by modulating protein synthesis [340]. This is achieved by regulating phosphorylation of effectors of protein synthesis, including the mRNA translation repressor 4E-BP1 and the ribosomal S6 kinase S6K1 by inactivation and activation, respectively. Studies in rat skeletal muscle have suggested that modulation of Ser-2448 phosphorylation by upstream regulators of mTOR have an important role in the control of mTOR kinase activity and protein synthesis [347]. We thus measured mTOR (Ser-2448) phosphorylation to test whether *Map4k4* negatively regulates protein synthesis through modulation of the mTOR activation. 72 h after

scrambled or *Map4k4* siRNA transfection, cell lysates were analyzed by immunoblotting using phospho-mTOR (Ser-2448) antibody. *Map4k4* gene silencing significantly enhanced basal mTOR phosphorylation without changing total mTOR levels (Fig. 2.4 B and C). These results suggest that *Map4k4* could function as a negative regulator of mTOR activity via modulation of mTOR (Ser-2448) phosphorylation (Fig. 2.4D).

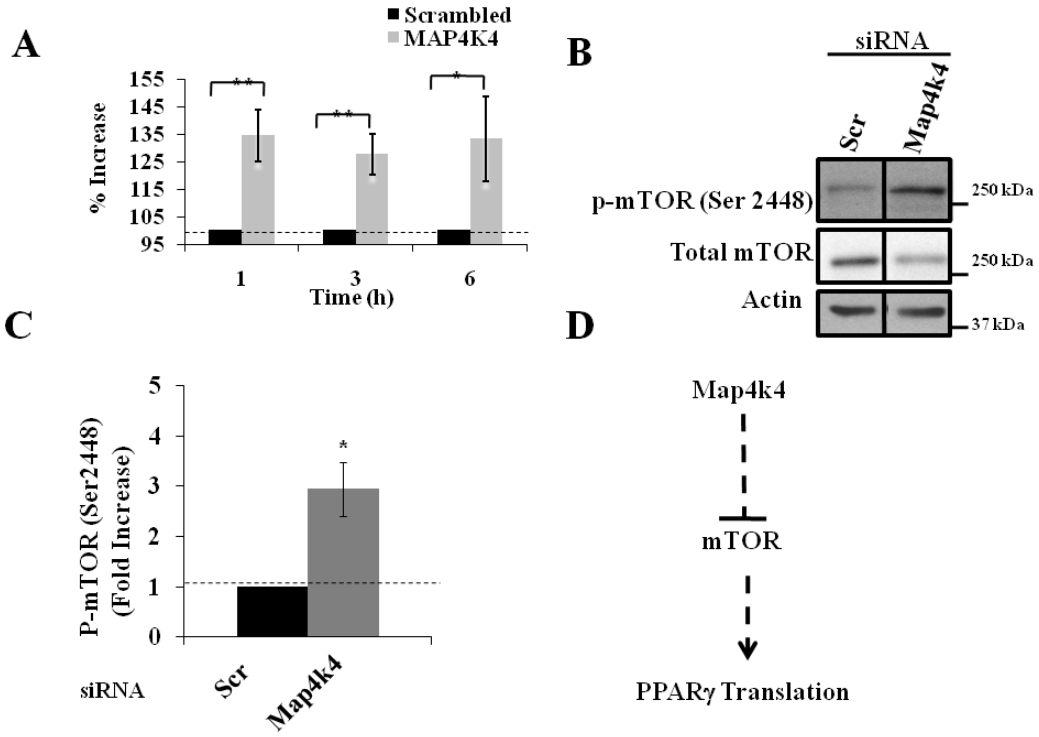


FIGURE 2.4. *Map4k4* gene silencing enhances protein synthesis and mTOR phosphorylation. 3T3-L1 adipocytes were transfected with 7.5 nmol of either scrambled (*Scr*) or *Map4k4* siRNA 4 days post-differentiation induction. 72 h later, [^{35}S]Met/Cys protein labeling mix was added to cells for 1, 3, and 6 h. A, Graphical representation of ^{35}S incorporation as detected by a scintillation counter (n =3). The counts/min from each knockdown condition was normalized to total DNA content of that sample. Cell lysates were examined for phospho-mTOR (Ser-2448) by Western blot (B) and densitometry analysis (C). D, Model depicting possible mechanism of PPARγ translational regulation through mTOR. Densitometry and reverse transcription-PCR are representative of three independent experiments. **, $p < 0.01$, *, $p < 0.05$ when compared with scrambled siRNA-transfected samples by ANOVA.

Map4k4 Depletion Enhances 4E-BP1 Phosphorylation—Because siRNA-mediated suppression of *Map4k4* expression promotes activation of mTOR, we sought to examine whether downstream targets of mTOR involved in protein synthesis, such as 4E-BP1 and p70S6K1, were also affected by *Map4k4* gene depletion. 4E-BP1 is a translational repressor protein that inhibits cap-dependent translation by binding to the eIF4e translation initiation factor [348]. mTOR-mediated hyperphosphorylation of 4E-BP1 disrupts this interaction and results in activation of cap-dependent translation [339, 349]. To determine whether *Map4k4* gene silencing results in increased protein translation via enhanced 4E-BP1 phosphorylation, 3T3-L1 adipocytes were transfected with scrambled or *Map4k4* siRNA, and then cell lysates were harvested and analyzed for changes in the phosphorylation state of 4E-BP1 protein by immunoblotting using phospho-4E-BP1 (Thr-36/45/69 and Ser-64) antibodies. As depicted in Fig. 2.5 A, *Map4k4* gene silencing enhanced 4E-BP1 phosphorylation at all sites tested. Depending on the extent of phosphorylation, 4E-BP1 migrates as three distinct bands, α (hypo-phosphorylated form), β, and γ (hyper-phosphorylated forms), on SDS-PAGE. Densitometry analyses revealed that there is a significant increase in the phosphorylation of sites that are responsible for the formation of slower migrating β as well as γ forms of 4E-BP1 upon *Map4k4* gene silencing (Fig. 2.5 B). We also examined other downstream targets of mTOR involved in the regulation of translation. S6K1 is another downstream effector of mTOR and is known to be the enzyme that phosphorylates ribosomal protein S6 (rpS6), a component of the small (40 S) ribosomal subunit [338]. S6K1 Thr-389 phosphorylation by mTOR allows phosphorylation within the catalytic domain resulting

in kinase activation [60]. To test if *Map4k4* gene silencing mediates changes in S6K1 phosphorylation, cell lysates from the above-mentioned experiment (as that in Fig. 2.5) were subjected to SDS-PAGE and immunoblot analysis. Surprisingly, *Map4k4* gene silencing does not enhance S6K1 phosphorylation at Thr-389 (Fig. 2.6) despite the activation of mTOR. To test if *Map4k4*-mediated increases in PPARγ protein synthesis are due to regulation of other translation initiation factors, we also examined the changes in the phosphorylation of eIF2-α by SDS-PAGE and immunoblotting using phospho-eIF2-α antibody (Ser-51). There was no significant change in the phosphorylation state of eIF2-α (Ser-52 in mouse) upon *Map4k4* gene silencing (Fig. 2.7). These results suggest that the mechanism whereby *Map4k4* suppresses protein synthesis is primarily through down-regulation of mTOR and activation of 4E-BP1. Taken together, these results put forward a new mechanism of *Map4k4*-mediated regulation of PPARγ protein synthesis in an mTOR-dependent manner, through 4E-BP1.

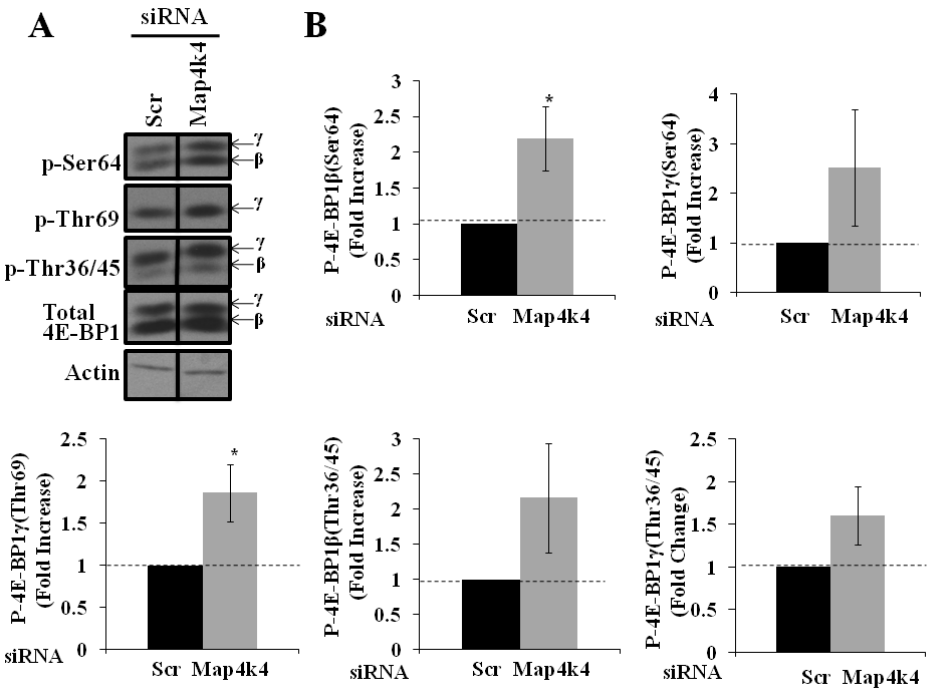


FIGURE 2.4. *Map4k4* gene silencing in 3T3-L1 adipocytes enhances 4E-BP1 phosphorylation. 4 days postdifferentiation induction, 3T3-L1 adipocytes were transfected with 7.5 nmol of either scrambled (*Scr*) or *Map4k4* siRNA. 72 h later, cell lysates were examined for phospho-4E-BP1 (Thr-36/Thr-45) by Western blot (A) and densitometry analysis (B). *, $p < 0.05$ when compared with scrambled siRNA-transfected samples by ANOVA. Densitometry is representative of three independent experiments.

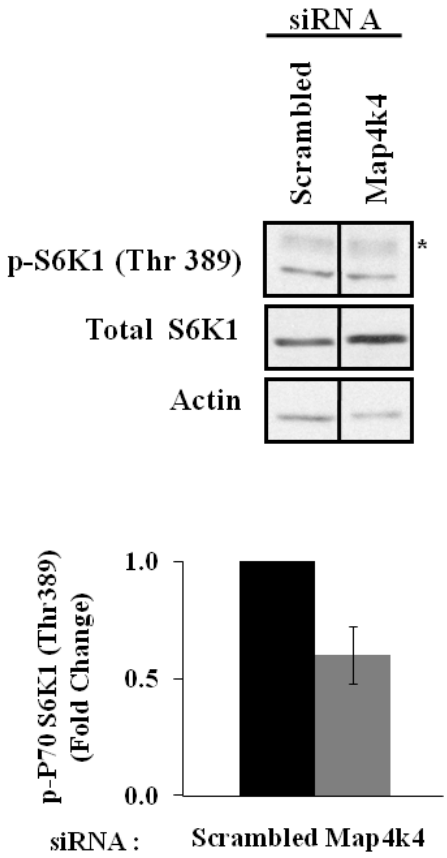


Figure 2.6. Map4k4 gene silencing does not enhance p70S6K phosphorylation. 3T3-L1 adipocytes were transfected with 7.5nmol of either scrambled, or Map4k4 siRNA 4 days post differentiation induction. 72 h later cell lysates were examined for phospho p70S6K (Thr389) by Western blot and densitometry analysis. Densitometry is representative of three independent experiments.

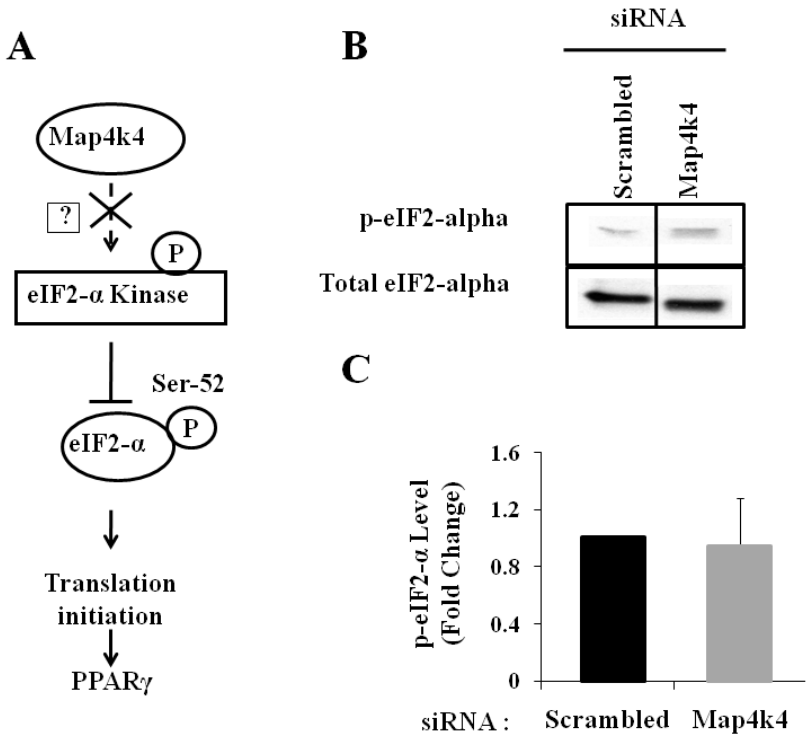


Figure 2.7. Map4k4 gene silencing does not change eIF2-α phosphorylation. (A) Hypothetical model for eIF2-α regulation by Map4k4. 3T3-L1 adipocytes were transfected with 7.5nmol of either scrambled, or Map4k4siRNA 4 days post differentiation induction. 72 h later cell lysates were examined for phospho eIF2-α (Ser52) by Western blot (B) and densitometry (C) analysis. Densitometry is representative of three independent experiments.

4E-BP1 and 4E-BP2 Are Required for Map4k4 Regulation of PPARγ Protein—4E-BP1 gene silencing should render eIF4e constitutively active, and hence protein synthesis should be enhanced. The eIF4e-binding protein exists in three isoforms 4E-BP1, 4E-BP2, and 4E-BP3. Isoforms 1 and 2 are well studied, and they share similar phosphorylation sites [350]. To test if general protein synthesis is affected in the absence of 4E-BP1, we examined 35S incorporation in 3T3-L1 adipocytes after silencing 4E-BP1 and 4e-BP2. Scrambled or *Map4k4* siRNA individual transfections were used as negative and positive controls, respectively. Total protein and RNA were harvested 72 h later. Equal volumes of protein were analyzed for 35S incorporation, using a scintillation counter. Global protein synthesis was found to be enhanced as reflected by significant increases in [35S]methionine incorporation, upon 4E-BP1 gene depletion (data not shown), similar to *Map4k4* gene silencing. To test if PPARγ protein synthesis is enhanced upon 4E-BP1 and 4E-BP2 gene silencing, cell lysates were analyzed for changes in PPARγ protein levels as well as efficiency of gene silencing of 4E-BP isoforms using SDS-PAGE and immunoblotting analysis. *Map4k4* gene depletion resulted in significantly enhanced PPARγ1 as well as PPARγ2 protein levels as expected (Fig. 2.8A). Because 4E-BP1 is known to be a negative regulator of protein translation [351], phosphorylation of 4E-BP1 renders it inactive; silencing 4E-BP1 should mimic the hyperphosphorylated inactive state of 4E-BP1. Surprisingly, 4E-BP1 and 4E-BP2 knockdown did not result in an enhancement of either PPARγ1or PPARγ2 protein levels as revealed by Western blot (Fig. 2.8A) and densitometry analysis (Fig. 2.8B).

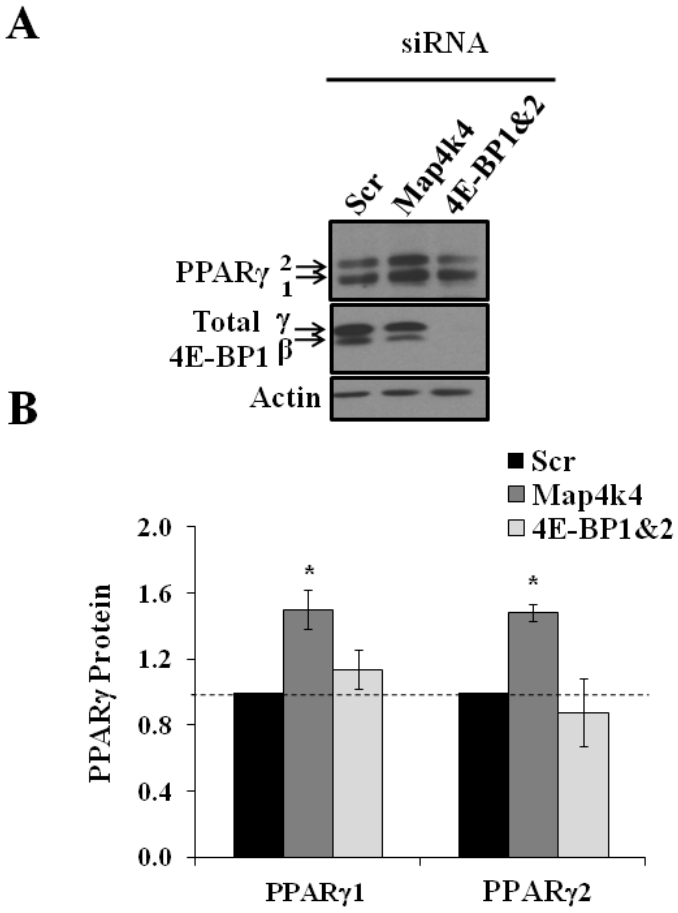


FIGURE 2.8. 4E-BP1 and 4e-BP2 silencing does not enhance PPARγ protein level. 3T3-L1 adipocytes were transfected with 7.5 nmol of scrambled (*Scr*) or *Map4k4* and 1.87 nmol of (each) 4E-BP1 and 4e-BP2 siRNA. 72 h later, cell lysates were examined for PPARγ protein levels as well as for efficiency of 4E-BP1 and 4e-BP2 gene silencing by SDS-PAGE followed by Western blot (A) and densitometry analysis (B). *, $p < 0.05$ when compared with scrambled siRNA transfected samples by ANOVA. Densitometry is representative of three independent experiments.

To determine whether expression of 4E-BP1 is necessary for the increase in PPARγ protein level mediated by *Map4k4* silencing, a triple knockdown strategy using siRNA against *Map4k4* together with 4E-BP1 and -2 was employed. 72 h after siRNA transfection, cell lysates were examined by Western blot (Fig. 2.9A), and densitometry analysis (Fig. 2.9B) for changes in PPARγ protein level. *Map4k4* siRNA treatment caused an enhancement in PPARγ protein level as seen in Fig. 2.1A. Furthermore, the triple knockdown resulted in no enhancement of PPARγ protein level; additionally, enhancement in PPARγ protein level in response to *Map4k4* silencing is attenuated in the absence of 4E-BP1 and 4E-BP2. These results suggest an important role for 4E-BP1 and 4E-BP2 in *Map4k4* signaling in this pathway.

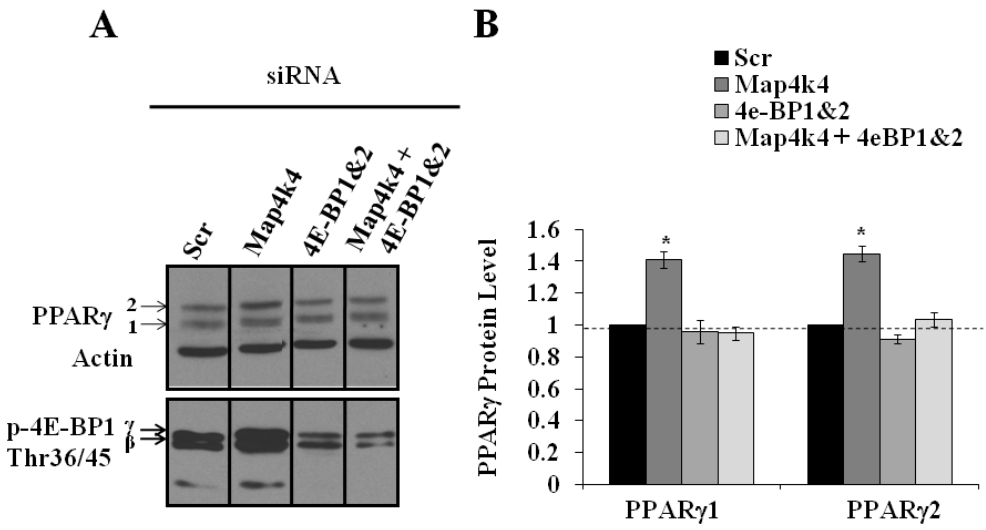
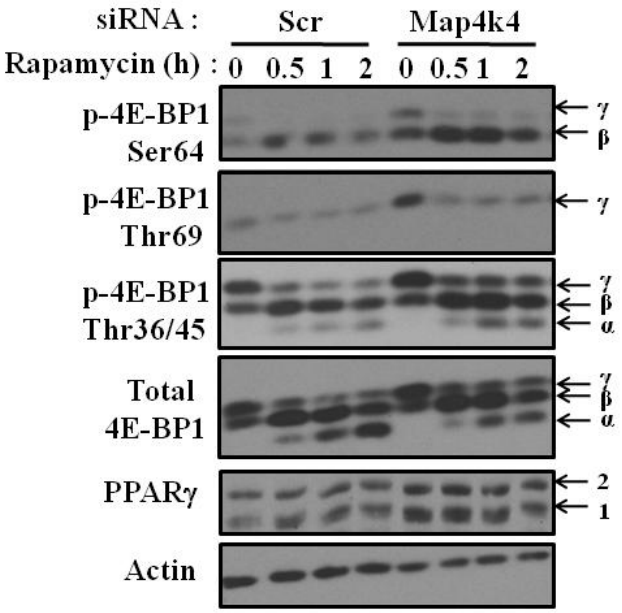


FIGURE 2.9. 4E-BP1 and 4e-BP2 are required for increased PPARγ protein levels upon *Map4k4* gene silencing. 3T3-L1 adipocytes were transfected with 7.5 nmol of either scrambled (*Scr*) or *Map4k4* and 1.87 nmol of 4E-BP1 and 4e-BP2 siRNA. 72 h later cell lysates were examined for PPAR_ protein levels by Western blot (A) and densitometry analysis (B). *, $p < 0.01$ when compared with scrambled siRNA transfected samples by ANOVA. Densitometry is representative of three independent experiments.

Up-regulation of PPARγ Level upon Map4k4 Depletion Is Suppressed by mTOR Inhibition—As phosphorylation ofmTORas well as 4E-BP1 is enhanced upon *Map4k4* knockdown, we tested to see if this increase in 4E-BP1 phosphorylation is mediated by mTOR. Rapamycin is known to be a potent inhibitor of mTOR function [352] and should result in dephosphorylation and activation of 4E-BP1. We thus treated 3T3-L1 adipocytes with 20 nM rapamycin for the indicated times, beginning 72 h after transfection with scrambled or *Map4k4* siRNA. Cell lysates were analyzed by SDS-PAGE and immunoblotting using the respective antibodies (Fig. 2.10A). Phosphorylation of mTOR (Ser-2448) as well as 4E-BP1 (at Thr-69, Ser-64, and Thr-36/45) was enhanced upon *Map4k4* gene silencing as expected, and both PPARγ1 and PPARγ2 protein levels were found to be increased. In the scrambled siRNA-transfected cells (control), treatment with rapamycin resulted in dephosphorylation of 4E-BP1 at all the phosphorylation sites tested in a time-dependent manner. Similarly, rapamycin-induced 4EBP1 dephosphorylation (in all the sites tested) was not prevented in *Map4k4* silenced cells (Fig. 2.10A & B). At the same time, PPARγ1 and PPARγ2 protein levels showed a significant decrease in expression upon rapamycin treatment in a time-dependent manner (Fig. 2.7A & B). These results are consistent with the hypothesis that enhanced 4E-BP1 phosphorylation that is observed upon *Map4k4* gene silencing is mediated through an mTOR-dependent mechanism that regulates PPARγ protein synthesis.

A


siRNA :
Scr
Map4k4
Rapamycin (h) : 0 0.5 1 2 0 0.5 1 2
p-4E-BP1 Ser64
p-4E-BP1 Thr69
p-4E-BP1 Thr36/45
Total 4E-BP1
PPARγ
Actin
γ
β
α
2
1

B

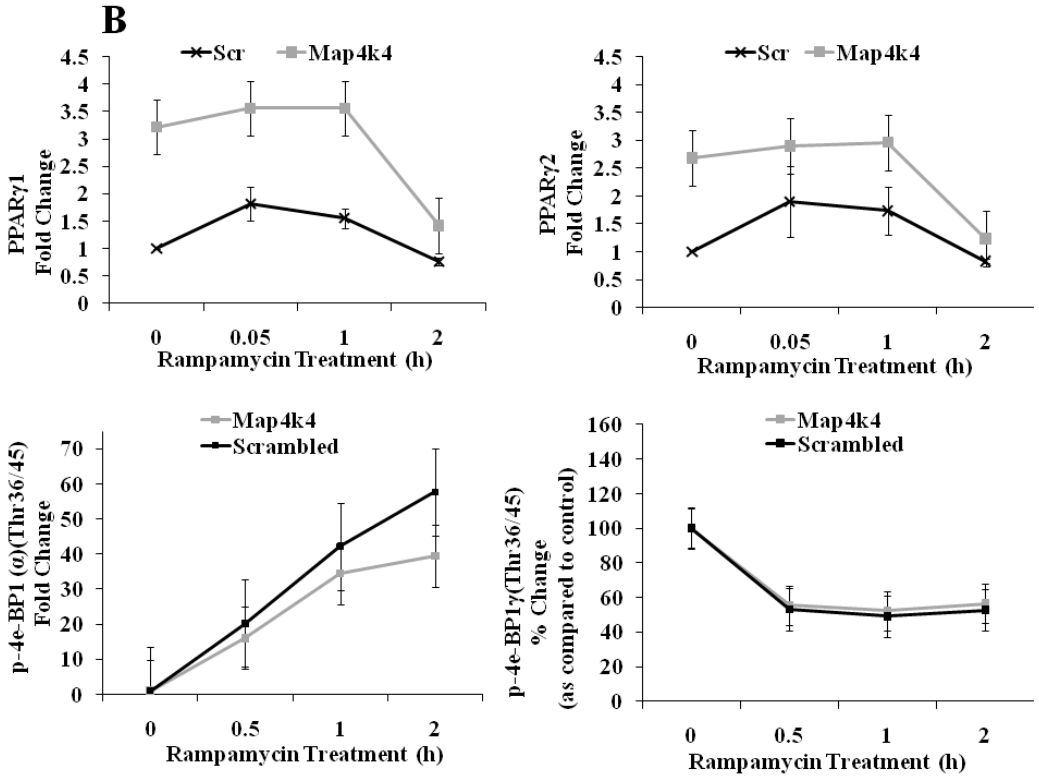

Scr
Map4k4
PPARγ1 Fold Change
PPARγ2 Fold Change
Rampamycin Treatment (h)
Scrambled
p-4e-BP1 (α)(Thr36/45) Fold Change
p-4e-BP1γ(Thr36/45) % Change (as compared to control)

FIGURE 2.10. Rapamycin inhibits *Map4k4* regulation of 4E-BP1 phosphorylation and PPARγ expression. 3T3-L1 adipocytes 4 days post-differentiation induction were transfected with 7.5 nmol of either scrambled (*Scr*) or *Map4k4* siRNA. 72 h later, cells were treated with 20 nM of rapamycin for 0.5, 1, or 2 h. Cell lysates were examined for the appearance of the hypophosphorylated form (4E-BP1α), and changes in the phosphorylation levels of 4E-BP1 at Ser-64, Thr-69, and Thr-36/45 were analyzed using appropriate antibodies by Western blot (A) (representative blots) and densitometry (B) analysis ($n = 5$). *, $p < 0.05$ when compared with scrambled siRNA transfected samples by ANOVA.

Enhanced Cell Size upon Map4k4 Gene Silencing in 3T3-L1 Adipocytes—mTOR is known to be a central regulator of cell growth [57]. To determine whether up-regulation of mTOR activity upon *Map4k4* gene silencing affected cell growth, we measured the changes in cell size upon siRNA-mediated knockdown of these genes in 3T3-L1 adipocytes. After transfection of adipocytes with scrambled or specific siRNAs, bright field cell images were collected, and cell size was analyzed by measuring the circumference of 150 randomly chosen cells. Cell size was found to be significantly enhanced upon *Map4k4* gene silencing (Fig. 2.11), consistent with increased growth as a result of increased mTOR activity.

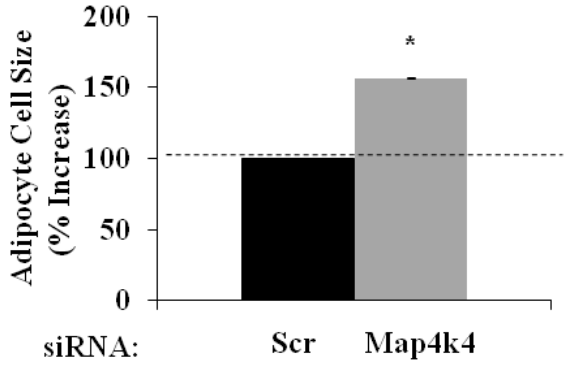


Figure 2.11. Depletion of Map4k4 enhances cell size in 3T3-L1 adipocytes. 4 days post differentiation induction, scrambled or Map4k4 siRNA transfected cells were seeded on cover slips. 72 h later the cells were fixed in 4% formaldehyde and cell images were captured under a microscope and cell size was measured using NIH ImageJ program. Histogram represents cell size of average of three independent experiments. *=$p<0.05$ when compared with scrambled siRNA transfected samples by ANOVA.

Adenovirus-mediated Map4k4 Overexpression Decreases mTOR Phosphorylation Levels as well as PPARγ Protein— Because siRNA-mediated suppression of *Map4k4* expression enhanced PPARγ expression, as well as promoted activation of mTOR in cultured adipocytes, we hypothesized that transgenic expression of *Map4k4* would have the opposite effect. To test this, 72 h post-infection, cell lysates were prepared from either control adenovirus (Ad-control) or *Map4k4* adenovirus (Ad-*Map4k4*) (3HA-*Map4k4*-HA)-infected 3T3-L1 adipocytes. We first assessed the level of *Map4k4* evoked by adenovirus-mediated gene transfer. Analysis of cell lysates harvested from cells infected with the *Map4k4* adenoviral vector (Ad-*Map4k4*) revealed efficient production of the 150-kDa protein. The protein level of *Map4k4* was examined using both anti-*Map4k4* and HA antibody by SDS-PAGE and immunoblotting (Fig. 2.12A). Increases in *Map4k4* expression with higher multiplicities of infection (data not shown) were observed. PPARγ protein levels and mTOR phosphorylation at Ser-2448 were examined by Western blot analysis with PPARγ and phospho-mTOR antibody, respectively. Adenovirusmediated expression of *Map4k4* resulted in a modest but significant decrease in PPARγ1 as well as PPARγ2 protein levels (Fig. 2.12, A and B) without any change in the mRNA (data not shown). At the same time, there was also a diminished basal mTOR phosphorylation at Ser-2248, although total mTOR levels were unchanged (Fig. 2.12A). These results support the hypothesis that *Map4k4* could function as a negative regulator of mTOR activity toward PPARγ protein synthesis via modulation of mTOR (Ser-2448) phosphorylation.

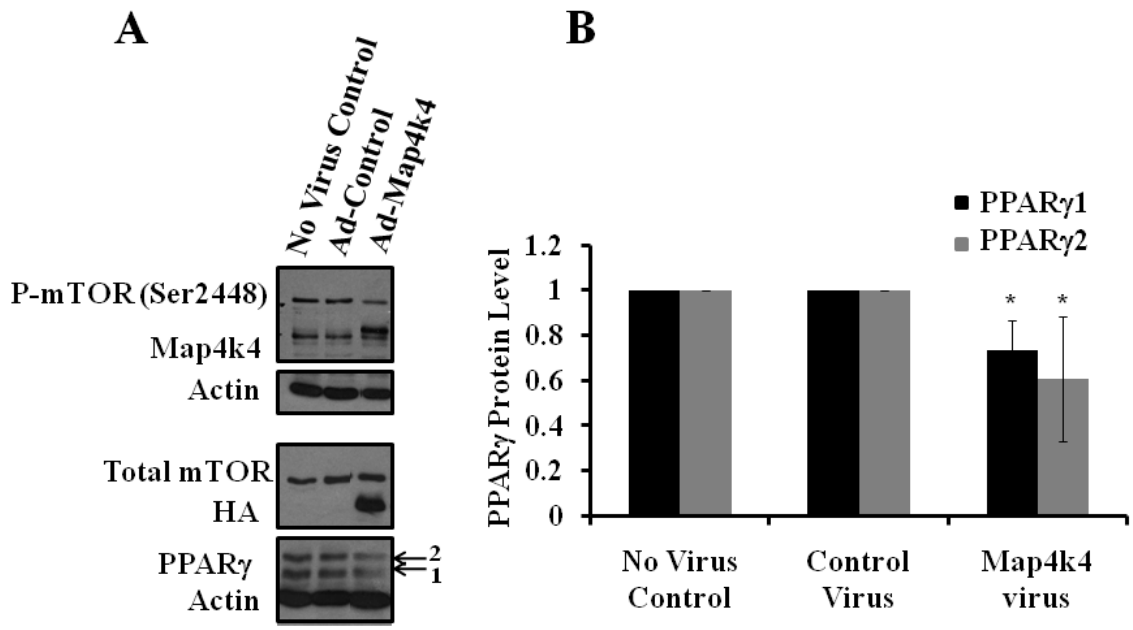


FIGURE 2.12. Adenovirus-mediated overexpression of *Map4k4* decreases PPARγ protein level as well as mTOR (Ser-2448) phosphorylation in 3T3-L1 adipocytes. 4 days post-differentiation induction, 3T3-L1 adipocyteswere infected with 40µl ($1.4X10^{12}$ particles/ml) of either HA control (*Ad-control*) or 3HA-*Map4k4*-HA (*Ad-Map4k4*) adenovirus. 72 h post-infection, cell lysates were examined for PPARγ protein levels as well as *Map4k4*, HA, phospho-mTOR (Ser-2448), and total mTOR by Western blot (A) (representative blots) and densitometry (B) analysis (n=4). *, p<0.05 when compared with control adenovirus infected samples by ANOVA.

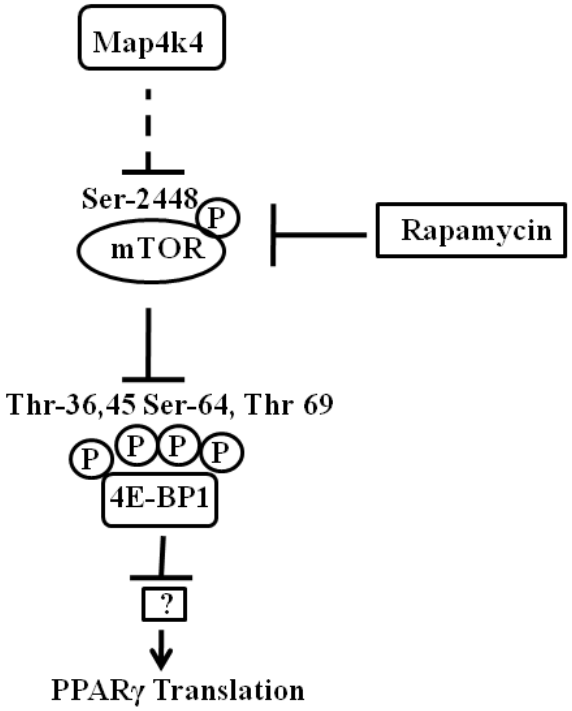


FIGURE 2.13. Model for *Map4k4*-mediated PPARγ protein regulation. Our data support the following hypothesis. Silencing *Map4k4* in 3T3-L1 adipocytes using siRNA enhances mTOR activity, as indicated by an increase in phosphorylation at Ser-2448. This in turn inhibits 4E-BP1 activity by enhancing phosphorylation at Thr-36, Thr-45, Ser-64, and Thr-69. Phosphorylation of 4E-BP1 renders it inactive leading to its dissociation from the translational initiation factor eIF4E. Thus eIF4E is activated, and cap-dependent translational initiation, responsible for the increase in PPARγ protein levels, is enhanced. However, 4E-BP1 silencing alone does not mimic *Map4k4* depletion but blocks the *Map4k4* effect, indicating it plays an additional role in regulation of PPARγ protein expression.

Table 2.1.

Primer	Sequence
Map4K4-F	CATCTCCAGGGAAATCCTCAGG
Map4K4-R	TTCTGTAGTCGTAAGTGGCGTCTG
36B4-F	TCCAGGCTTTGGGCATCA
36B4-R	CTTTATCAGCTGCACATCACTCAGA
PPARγ1-F	GACTACCCTTTACTGAAATTACC
PPARγ2-F	ATGGGTGAAACTCTGGGAG
PPARγ1&2-R	GTGGTCTTCCATCACGGAGA

DISCUSSION

mTOR plays a central role in mediating responses to many stimuli such as nutrients, hormones, and stress that result in the regulation of multiple cellular processes involved in cell growth and metabolism, including amino acid and protein biosynthesis and glucose homeostasis [57]. Treatment with rapamycin, a specific inhibitor of mTOR, has been shown to prevent adipocyte differentiation and lipid accumulation [64]. Although mTOR-mediated regulation of adipogenesis is not clearly understood, one mechanism could be through regulation of PPARγ, because its activity was attenuated by rapamycin treatment [64]. mTOR is also known as an important regulator of protein synthesis by mediating activation or inhibition of several downstream translation initiation and elongation factors directly or indirectly through changing their phosphorylation state [124]. The role of mTOR in regulating PPARγ protein synthesis has not previously been addressed. In this study, we show that *Map4k4*, a Ste20 serine/threonine protein kinase, regulates PPARγ protein expression in 3T3-L1 adipocytes, by a post-transcriptional mechanism via mTOR-mediated regulation of the 4E-BP1 initiation factor. The specificity of the effect of *Map4k4* silencing to up-regulate protein expression is not limited to PPARγ. Fig. 2.4A shows that there is a small but significant effect on incorporation of labeled amino acid into total protein in response to *Map4k4* depletion, indicating that many proteins are up-regulated in addition to PPARγ. These may include proteins affected through direct mTOR-dependent modulation of translation (*e.g.* PPARγ), as well as proteins downstream of such transcription factors. Our previously published data [324] also showed a significant increase in protein

expression of adipogenic genes like CCAAT/enhancer-binding protein α and CAAT/enhancer- binding protein β and Glut4 (insulin-responsive facilitated glucose transporter isoform 4), upon siRNAmediated *Map4k4* gene silencing. We have analyzed the mRNA levels of some additional genes known to be transcriptionally controlled by PPARγ, and we find that several are indeed also increased by *Map4k4* knockdown. These include phosphoenolpyruvate carboxykinase, acetyl-CoA acyltransferase, and monoglyceride lipase (data not shown). However, other PPARγ-responsive and adipocyte-specific genes such as aP2 (or Fabp4, fatty acid-binding protein 4), adipsin, adiponectin, and leptin, which we analyzed by reverse transcription- PCR as well as immunoblotting, are not apparently affected by *Map4k4* silencing in mature adipocytes (data not shown). The basis of this differential effect of *Map4k4* knockdown on some PPARγ-responsive genes and not others is interesting but unknown. Our overall findings are summarized in Fig. 2.13. Inhibition of *Map4k4* expression results in an enhanced phosphorylation of mTOR as well as 4E-BP1. Inactivation by phosphorylation of 4E-BP1 results in an increase in PPARγ protein synthesis (Fig. 2.13). Active mTOR phosphorylates 4E-BP1 leading to the dissociation of the eIF4e-4E-BP1 complex [124]. Therefore, stimulating mTOR activity could result in enhanced protein synthesis. Our finding of enhanced mTOR (Ser-2448) phosphorylation (Fig. 2.4B) upon *Map4k4* depletion could mean increased mTOR kinase activity, but whether phosphorylation of mTOR at Ser- 2448 reflects its kinase activity is debatable [353]. However, 4E-BP1 was not protected from dephosphorylation upon rapamycin treatment in *Map4k4*-depleted conditions (Fig. 2.10A) supporting the idea that knockdown of *Map4k4* enhances kinase

activity of mTOR in the absence of the inhibitor. How this elevated activity of mTOR occurs in *Map4k4* knockdown conditions remains to be answered. The effect of *Map4k4* gene silencing on activation of mTOR to regulate 4E-BP1 appears to be downstream to Akt in the insulin signaling pathway, as Akt phosphorylation is not affected upon *Map4k4* depletion [324]. Additionally, mTOR-mediated 4E-BP1 regulation appears to be distinct from S6K1 (the other well known mTOR substrate) regulation, because *Map4k4* gene silencing did not affect basal phosphorylation of S6K1 (Fig. 2.6). These results are consistent with published data showing outputs from mTOR to 4E-BP1 and S6K1 to be distinct [117].

Further work is required to address the question of how activation of mTOR is achieved upon *Map4k4* gene silencing. Additionally, upon depletion of *Map4k4*, the increase in basal mTOR phosphorylation at Ser-2448, as well as enhanced 4E-BP1 phosphorylation, suggests the presence of an additional player upstream of mTOR. Alternatively, a component could be functioning in a complex with mTOR to regulate 4E-BP1 phosphorylation and mTOR kinase activity thereby enhancing PPARγ protein synthesis. Our result shows a decrease in PPARγ protein level concomitant with diminished mTOR phosphorylation at Ser-2448 upon adenovirus-mediated transgenic expression of *Map4k4*. This result supports the hypothesis that *Map4k4* is a negative regulator of PPARγ protein translation by suppressing phosphorylation of mTOR. However, a detailed analysis of the molecular mechanism involving the regulation of mTOR phosphorylation by *Map4k4* either directly, by means of downstream substrates, or by interacting partners will be necessary to better understand PPARγ translational

regulation. In summary, this study shows that *Map4k4* regulation of PPARγ protein is a post-transcriptional mechanism that is coupled to mTOR signaling through regulation of 4E-BP1. Furthermore, 4E-BP1 is necessary for enhancement in the PPARγ protein upon *Map4k4* depletion. Addressing this novel role of 4E-BP1 in PPARγ protein regulation may reveal new insights into post-transcriptional regulation of gene expression. Considering the central role played by PPARγ in regulating whole body metabolism and insulin sensitivity, its regulation by *Map4k4* may have important physiological implications.

Limitations and Future Perspectives

The results presented in this chapter have provided evidence for translational regulation of PPARγ protein expression by Map4k4. Additionally, the increase in PPARγ protein levels observed in the MAP4K4 silenced condition is due to enhanced mTOR activity towards 4E-BP1, resulting in a modest but significant increase in protein synthesis. Although these results are intriguing, several questions remain to be addressed. The observation that despite mTOR activation, p70S6K1 activity as evidenced by its phosphorylation state is not altered by MAP4K4 gene silencing, is very interesting.

Differential regulation of mTOR substrates in the MAP4K4 knockdown condition could be due to changes in substrate binding site availability on Raptor. mTOR exists as two distinct complexes that is determined by the interacting proteins. Hence, it can be hypothesized that, mTORC1 could further exist as two distinct complexes by binding to an unknown protein ‗A' as summarized in Figure XYZ. Since Map4k4 is a protein kinase, I speculate that it could possibly function as a regulator of this unknown protein by phosphorylation, either directly or indirectly through a kinase cascade. In the MAP4K4 knockdown condition the unphosphorylated protein-A blocks p70S6K1 binding site on Raptor and thus inhibiting its phosphorylation and activation by mTOR. Studies to identify distinct mTOR and/or Raptor binding partners in MAP4K4 gene silencing condition, as well as immuno-precipitation studies to analyze Raptor binding capacity of p70S6K1 and 4E-BP1 in the MAP4K4 knockdown condition could provide insights into the differential regulation of mTOR substrates.

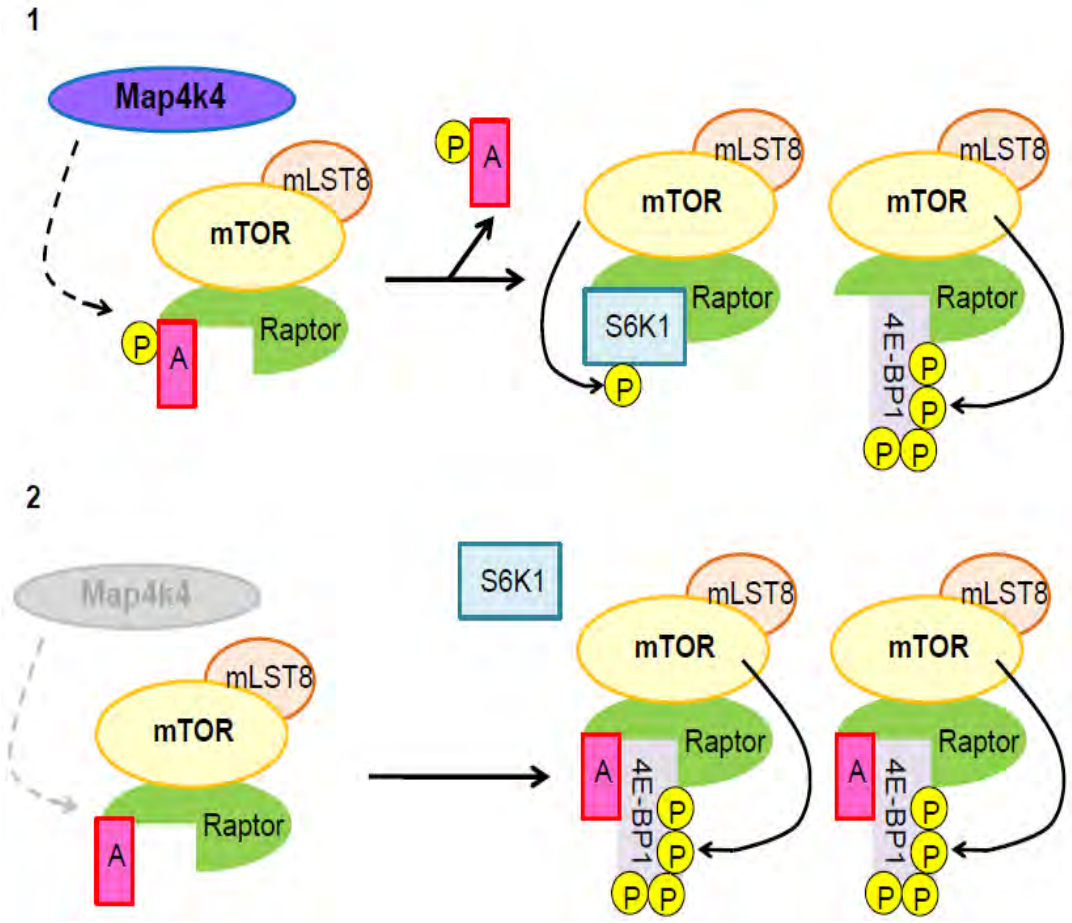


FIGURE 2.14. Map4k4 mediated differential regulation of mTOR substrates p70S6K1 and 4E-BP1. (1) In a cell mTORC1 binds to both S6K1 and 4E-BP1 equally. Map4k4 could function to phosphorylate an unknown protein A bound to Raptor, thus facilitating S6K1 binding to Raptor and its subsequent mTOR mediated phosphorylation and activation. (2) In the Map4k4 silenced condition the unphosphorylated protein A is still bound to Raptor and blocks S6K1-Raptor interaction and hence prevents its phosphorylation and activation by mTOR. In these both conditions 4E-BP1 phosphorylation is not inhibited. Since S6K1 no longer competes for the Raptor binding site, 4E-BP1 can bind to Raptor unhindered and thus results in the observed increase in its phosphorylation.

4E-BP1 is a negative regulator of protein synthesis. Silencing of this eIF4E-binding protein should mimic MAP4K4 knockdown effect towards PPARγ protein expression. Surprisingly, siRNA mediated 4E-BP1 gene silencing did not increase PPARγ protein levels. Studies utilizing transgenic expression of phosphorylation site mutants of 4E-BP1 could shed light on the role of 4E-BP1 in the regulation of PPARγ protein levels. Furthermore, MAP4K4 silencing mediated increase in PPARγ protein levels was abolished in the absence of 4E-BP1&2. This result suggests that 4E-BP1&2 could be functioning as a positive regulator of PPARγ protein levels, although only in the MAP4K4 knockdown condition but not in basal conditions. A decrease in PPARγ mRNA level was observed both upon 4E-BP1&2 gene silencing as well as in combination with MAP4K4 siRNA. However, upon differentiation induction, 4E-BP1&2 double knockout MEFs exhibited increased PPARγ mRNA levels [121]. This discrepancy in our observations and the published data could be in the timing of differentiation induction and 4E-BP1&2 knockdown. Whereas, the MEFs were 4E-BP1&2 deficient even before differentiation was induced, while in our studies siRNA mediated 4E-BP1&2 gene silencing was performed in differentiated 3T3-L1 adipocytes. Detailed analysis is needed to thoroughly understand the role of 4E-BP1&2 in the regulation of PPARγ mRNA and protein expression.

An additional area of investigation remaining is the mechanism by which MAP4K4 regulates mTOR activity. Akt is the upstream activator of mTOR. Previously published data demonstrated that Akt phosphorylation was not regulated by MAP4K4 [324]. Hence, MAP4K4 could be acting downstream to Akt to regulate mTOR function.

Furthermore, published literature shows that TSC1/TSC2 as well as Rheb-mediated mTOR regulation results in modulation of both p70S6K1 as well as 4E-BP1 activity. Our results show that upon MAP4K4 gene silencing whereas, mTOR-mediated 4E-BP1 phosphorylation and hence, its inactivation, is functional, p70S6K1 is not stimulated. Hence, MAP4k4 is a possible novel regulator of mTOR differential activity towards its substrates. Since TNFα stimulation augments MAP4K4 expression and insulin is a potent stimulator of mTOR activity, we also want to determine if MAP4K4 regulates mTOR-mediated expression of PPARγ in response to TNFα and insulin. Conceivably MAP4K4 may regulate mTOR activity and PPARγ synthesis in obesity and associated insulin resistance. By answering these questions, we might achieve better insight into the regulation of the insulin-signaling pathway in the adipocytes that is impaired in insulin resistance.

CHAPTER III: Adipose-Specific Knockdown of MAP4K4 Results in Obese Mice with Enhanced Insulin Sensitivity

Contributions: All experiments were performed by me except for Figure 3.1, where the ShRNA construct was generated by Nana Hagan and Myriam Aouadi. Data for Figures 3.1 C&D and Figure 3.2 were generated by Myriam Aouadi. Glucose tolerance test (GTT) shown in Figure 3.11A was performed by me in collaboration with Myriam Aouadi and Greg Tesz. All mouse dissections were performed by me in collaboration with Myriam Aouadi, Greg Tesz, Sarah Nicoloro, Mengxi Wang and Matthieu Prot. Data shown in Figure (3.4), (3.5), (3.7), (3.8), (3.9 A, B, & C), (3.10), (3.11 B) were performed in collaboration with UMass Mouse Phenotyping Center. The transgenic animals used for experiments in this chapter were generated by UMass Transgenic Core Facility. Tissue sections used for histological studies were generated by DERC Morphology Core Facility at UMass. I did all the statistical analysis in this chapter.

Abstract

In an RNAi based screen, the Ste20 protein kinase ortholog Map4k4 was identified as an inhibitor of PPARγ expression and triglyceride accumulation in 3T3-L1 adipocytes. The effect of Map4k4 on PPARγ appears to be mediated through regulation of protein synthesis rather than transcription or protein degradation. To investigate the role of Map4k4 suppression on adipose tissue function and whole-body metabolism, we produced transgenic mice that express an shRNA that attenuates Map4k4 expression selectively in adipose tissue (MAP4K4 - KD). The KD mice displayed significantly more adipose tissue with enhanced PPARγ protein levels than control mice. This increased adiposity in the KD mice was not due to changes in energy expenditure, physical activity or caloric intake compared to control mice. Importantly, the KD mice exhibited lowered fasting glycemia and were insulin sensitive. This could be attributed to the reduced hepatic glucose production observed in the KD mice under basal (in both standard diet (SD) & high fat diet (HFD)) and hyperinsulinemic clamp (in HFD) conditions. Remarkably, adipocytes from the KD mice exhibited striking hypertrophy with increased triglyceride storage. Thus, Map4k4 deficiency in adipocyte improves adipose tissue function to store triglyceride, with beneficial effects on liver glucose production and whole body glucose homeostasis.

Introduction

Obesity, the accumulation of excess body fat, is a medical condition associated with insulin resistance, a phenotype of impaired insulin sensitivity in peripheral tissues [209]. Adipose tissue is a dynamic metabolic and endocrine organ that regulates energy homeostasis [327, 354] which responds to energy demands by storing excess energy as triglycerides and releasing free fatty acids and glycerol when needed. Impaired adipose lipid storage capacity leads to ectopic lipid deposition in liver and muscle resulting in insulin resistance [355, 356], raising the hypothesis that diminished adipose function in states of nutritional excess may contribute to insulin resistance. Impaired metabolic function is reflected in the deregulated endocrine secretions of the adipose tissue. Obesity associated inflammation that is characterized by increased circulating concentrations of pro-inflammatory cytokines [357] and enhanced macrophage recruitment to adipose tissues [246] also contributes to insulin resistance.

In both humans and rodents, liver plays an important role in glucose and lipid homeostasis [28, 358]. Under fasting conditions, hepatic glucose production provides fuel for the brain and red blood cells. Glucose is released by the liver initially from glycogen breakdown, a process called glycogenolysis, followed by gluconeogenesis where glucose is produced from lactate and pyruvate [359-361]. Additionally, β-oxidation of fatty acids provides alternate fuel source for the brain and heart [362]. Upon feeding, insulin suppresses glycogenolysis, gluconeogenesis as well as β-oxidation. In states of metabolic deregulation, as in obesity-associated insulin resistance, the ability of insulin to inhibit

hepatic glucose production is hampered [363]. Several studies have demonstrated that increase in circulating concentrations of free fatty acids [364], as well as adipokines induce hepatic insulin resistance [365].

Adipose tissue is a major source of pro-inflammatory cytokines and lipid mediators in the obesity-associated inflammatory state [364-366]. Thiazolidinediones (TZDs) are a class of medication used to treat type II diabetes that act by (i) modifying adipocyte differentiation, (ii) decreasing levels of leptin, interleukins, [16] lowering plasma free fatty acids, (iv) as well as normalizing hepatic insulin resistance. Most of these effects are believed to be in part due to TZDs‘ function to act as ligands and upregulate peroxisome proliferator-activated receptor-γ (PPARγ) activity. PPARγ, a nuclear hormone receptor and a transcription factor, is required for adipogenesis and is considered a master regulator of adipocyte function [327-329]. Furthermore, PPARγ regulates glucose homeostasis and lipid metabolism in mature adipocytes [330].

Map4k4, a serine/threonine protein kinase that belongs to a large group of protein kinases related to *Saccharomyces cerevisiae* Ste20, has been identified as a negative regulator of PPARγ protein synthesis [367]. Silencing Map4k4 in 3T3-L1 adipocytes improved insulin-stimulated glucose uptake and adipogenesis [324]. Remarkably, TNFα acutely increases the expression [315], as well as stimulates the activity (unpublished data) of Map4k4. Studies using human skeletal muscle explants have shown that TNFα-induced insulin resistance requires Map4k4 [321]. Furthermore, Map4k4 silencing in human and rat primary beta cells prevented the ability of TNFα to inhibit IRS2 as well as

glucose stimulated insulin secretion [322]. Interestingly, in murine macrophages it has been demonstrated that Map4k4 expression is required for LPS stimulated expression and secretion of TNFα and IL-1β [323]. A recent study demonstrated a correlation between an increase in Map4k4 expression and decreased differentiation capacity of preadipocytes in the obese human subcutaneous adipose tissue [368]. Together, these studies highlight the important role played by Map4k4 in different tissues that could potentially mediate obesity-induced inflammation and insulin resistance. However, the role of Map4k4 in regulating adipocyte function *in vivo* is not known.

To address this in detail, we embarked on studying a mouse model with reduced Map4k4 expression in adipocytes. So as to circumvent the embryonic lethality of mice lacking Map4k4 [317], and to quickly generate transgenic mice, in the present study we utilized pSico lentiviral vector mediated expression of shRNA [369] against Map4k4. Here we describe the phenotype of mice that are deficient in Map4k4 specifically in the adipose tissue using an aP2 driven Cre-lox based approach.

Experimental Procedures

Generation of Plasmids - Oligos coding for the various shRNAs were annealed and cloned into *Hpa*I–*Xho*I-digested pSico. The following MAP4K4 target region was chosen: GCTGTCTGGTGAAGAATTA. All constructs were verified by DNA sequencing.

Cell transfection - 3T3-L1 preadipocytes were maintained in DMEM 10 % FBS and 1 % penicillin/streptomycin. Cells were transfected by lipofectamin with 1 ug of pSico, MAP4K4-pSico, or MAP4K4-pSico and Cre. Cells were harvested 48 hours after transfection.

Generation of Transgenic Mice - The standard protocol involves the injection of DNA into the pronucleus of 129 strain fertilized eggs (0.5-day-post-coital embryos). These injected eggs are then implanted into the oviduct of a pseudopregnant foster mother. The injected DNA integrates randomly into the genome in a fraction of the implanted embryos. 3 positive founders were genotype by PCR and back-cross 7 times to C57BL6/J mice. Transgenic mice were bred with C57BL/6J mice carrying an AP2 promoter driven Cre recombinase expression. (This is a generous gift from Dr. Roger. Davis, University of Massachusetts Medical School, Worcester, MA). Mice were bred to create the following genotypes: ShRNA-Map4k4 (C), ShRNA-Map4k4-AP2-Cre (KD), AP2-Cre (Cre), Wild Type (Wt).

pSico primers for genotyping:

5‘-CCCGTATGGCTTTCATTTTCTCC-3‘**;** 5‘-AAGGAAGGTCCGCTGGATTGAG-3‘

Cre primers for genotyping:

5‘-GAACGCTAGAGCCTGTTTTGCACGTT-3‘

5‘ GCATTACCGGTCGATGCAACGAGT-3‘

Phenotypic Evaluation of Mice - Mice were housed on a 12h light/dark schedule and have free access to water and food. The study period between 8-16 weeks of age involved standard chow diet fed male C (n=5), KD (n=7); high fat diet (Harlan-Teklad, TD.99249) fed male C (n=6), KD (n=9). Study period between 16-26 weeks of age involved, high fat diet (Harlan-Teklad, TD.93075) fed male C (n=4), KD (n=5). Study involving 18 month old male mice fed standard chow diet C (n=3), KD (n=5). Body weight measurements were done weekly for studies using 8-16 and 16-24 week age mice; body weight of 18 month old mice was measured once before sacrificing the animals. Body composition and energy balance were assessed using Proton Magnetic Resonance Spectroscopy (^{1}H-MRS) (Echo Medical System) and metabolic cages (TSE Systems) respectively. Blood samples were obtained by tail-cut method. Whole blood was collected and the plasma was isolated for analysis of glucose, insulin, adiponectin, leptin and cytokines.

Glucose Tolerance Test - Intraperitoneal glucose tolerance test was conducted on 23 weeks old (after 7 weeks of high fat diet feeding) C and KD mice, as well as at 25 week old (after 9 weeks of high fat diet feeding) C mice, using 1000 mg/kg D-Glucose in water, as previously described [370].

Hyperinsulinimic Euglycemic Clamp Study - This study was performed as previously described [371]. Briefly, Surgery was performed to cannulate jugular vein at ~5 days before the clamp experiments. Following overnight fast, a 2-hr hyperinsulinemic-euglycemic clamp was perfomed in conscious mice with primed-

continuous infusion of human insulin (2.5 mU/kg/min) and a variable infusion of 20% glucose to maintain euglycemia. ^{3}H-gluocose was infused during the clamp to measure glucose turnover, and ^{14}C-2-deoxyglucose was bolus injected at 75 min of clamp to measure glucose uptake in individual organs. Tissues were taken at the end of clamps for biochemical and molecular analysis.

Assays of plasma or tissue - Plasma glucose concentration was measured with Accu-check® active Insulin, leptin and adiponectin were measured using Luminex. Tissue triglycerides were extracted as previously described previously [372] and triglyceride content determined using Triglyceride Determination Kit (Sigma TR0100).

Preadipocyte Isolation - Preadipocytes from adult tissue were isolated from sub-cutaneous fat pads and maintained in DMEM 10 % FBS and 1 % penicillin/streptomycin. Two days post-confluent cells were treated 48h with the adipocyte differentiation cocktail containing 5µg/ml insulin, 0.25µM dexamethasone and 0.5mM 3-isobutyl-1-methylxanthine (IBMX). The medium was then replaced for 6 days by DMEM 10 % FBS and 1 % penicillin/streptomycin.

Histology - Adipose tissue samples were obtained at the end of the study period from all groups of mice. Samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut and subsequently stained using H&E according to standard protocol. Liver tissue samples were obtained at the end of the study period from all groups of animals. Samples were fixed in OCT, on dry ice. Sections were cut and subsequently stained using H&E according to standard protocol. Histology sections

and staining were done by Pathology Department, University of Massachusetts Medical School, Worcester, MA.

Western Blot Analysis - Tissues samples were homogenized in RIPA buffer (composition). Western blot analysis was used to assess protein levels of PPARγ, actin, tubulin; protein and phosphorylation levels of Akt. Preadipocytes that were differentiated in culture were homogenized in HEPES buffer. Intensity of the bands on the immunoblots was plotted as graphs, expressed in terms of arbitrary densitometry units.

Quantitavie RT-PCR - RNA isolation was performed according to the Trizol Reagent Protocol from (Invitrogen, Carlsbad CA). The cDNA was synthesized from 1 µg of total RNA using iScript cDNA Synthesis Kit according to the manufacturer‘s instructions from (Bio-Rad, Hercules CA). For real time PCR, synthesized cDNA forward and reverse primers along with the iQ SYBR Green Supermix (Bio-Rad, Hercules CA) were run on the MyIQ Realtime PCR System (Bio-Rad). The ribosomal mRNA, 36B4 was used as an internal loading control.

Northern Blot Analysis - For the small RNA Northern blotting, 15 µg of total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions, and was resolved on a 15% denaturing polyacrylamide gel, transferred to a nylon membrane, and was cross linked. The membrane was hybridized overnight to a ^{32}P 5′-labeled DNA probe corresponding to the sense strand of the MAP4K4 shRNA (GCTGTCTGGTGAAGAATTA). Hybridization and washes were performed at 42°C.

Calculations and statistical analyses - The characteristics of the outcomes were evaluated by visual inspection of histograms generated using GrapPad Prism 5 or Microsoft EXCEL, and the observed effects were evaluated by either student ttest or multifactorial analysis of variance [79]. The data are presented as the means ± S.E.

Results

Generation and Validation of Adipose-Specific Map4k4 Knockdown Mice. shRNA-pSico vector construct - The U6 promoter has been widely used to drive the expression of shRNAs and a U6-based lentiviral vector for the generation of transgenic mice has been recently described [369]. To create a conditional U6 promoter, a cytomegalovirus (CMV)-enhanced GFP stop/reporter cassette was inserted between two loxP sites so that after Cre-mediated recombination the cassette would be excised, generating a functional U6 promoter (Fig. 3.1A). Importantly, the CMV-GFP cassette marks transfected cells and loss of GFP expression indicates successful Cre-mediated recombination. The ability of pSico vector to conditionally silence endogenous genes was demonstrated by insertion of a hairpin designed to inhibit expression of MAP4K4 (Fig. 3.1B). The resulting plasmid was named pSico-MAP4K4. The construct was then tested in 3T3-L1 preadipocytes. The empty vector was used as a control (Mock). High-efficiency transduction by these vectors was achieved as indicated by GFP expression in MAP4K4-pSico transfected cells. As shown in Fig. 3.1C, after transfection with a Cre-expressing plasmid, recombination with concomitant loss of GFP fluorescence was observed for the pSico-MAP4K4 vector. Consistent with the decrease in GFP expression,

Cre-mediated recombination resulted in a reduction of MAP4K4 expression in cells transfected with MAP4K4-pSico and Cre compared to the control cells transfected only with MAP4K4-pSico (Fig. 3.1D).

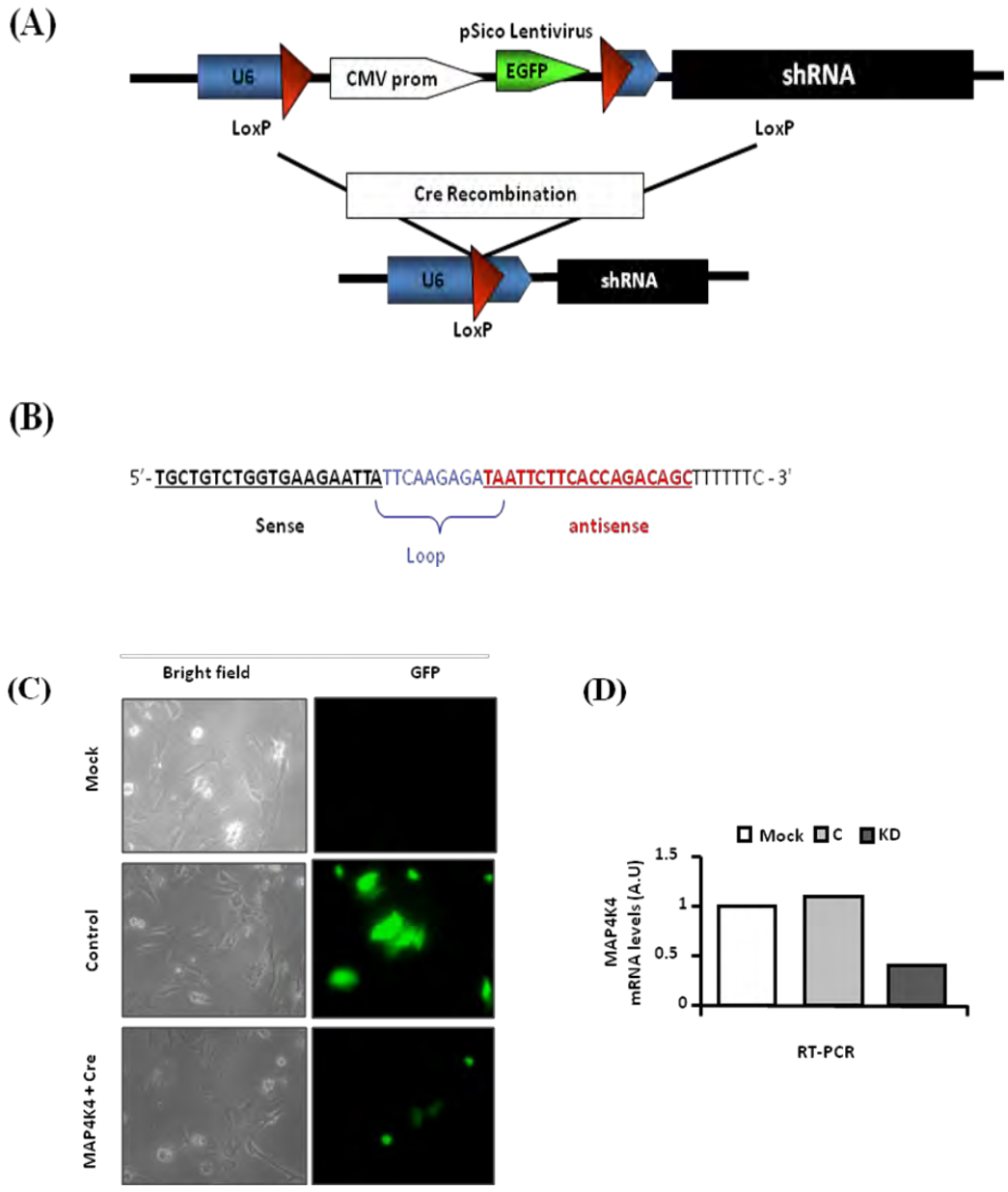


FIGURE 3.1. Generation of shRNA and gene silencing of MAP4K4 in 3T3-L1 cells. (A) Schematic representation of pSico before and after Cre-mediated recombination. (B) Nucleotide sequence of MAP4K4 shRNA. (C) 3T3-L1 preadipocytes were transfected with the empty pSico plasmid (Mock), MAP4K4-shRNA containing pSico (MAP4K4-pSico) or MAP4K4-pSico and a Cre expressing plasmid. Cells were analyzed by fluorescence microscopy to detect GFP. Similar cell density and identical exposure time was used for all images. (D) Real-time PCR showing MAP4K4 knock-down in cells co-transfected with MAP4K4-pSico and a Cre expressing plasmid.

Conditional RNAi in Mice - One motivation for using a conditional U6 cassette into a lentiviral vector was to rapidly generate conditional knock-down mice. To avoid any side effects of the GFP expression in mice, only a small DNA fragment missing the LTRs and the 3‘ UTR polyA tail necessary for GFP expression was injected in the eggs. The DNA fragment was injected in eggs at 1 cell stage that were then injected into pseudo-pregnant females at 2 cell stage. The genomic DNA was extracted from the obtained mice and subjected to PCR for genotyping. The mice were apparently normal and fertile, indicating that the presence of the nonexpressing pSico-MAP4K4 shRNA before Cre activation did not affect MAP4K4 expression and was compatible with normal mouse development. To achieve tissue-specific activation of the MAP4K4 shRNA, MAP4K4-pSico mice (C mice) were crossed to aP2-Cre transgenic mice that express Cre under the control of aP2 promoter, which is specifically expressed in the adipose tissue. Genotyping of the offspring was done by PCR for the detection of the transgene (MAP4K4-pSico) and the Cre (Fig. 3.2 A). To detect MAP4K4 siRNA expression, a northern-blot was performed on RNA extracted from white adipose tissue (WAT) and a control tissue, muscle. MAP4K4-siRNAs were detected in WAT from MAP4K4-pSico/Cre (KD mice), whereas no MAP4K4-siRNA was observed in WAT from C mice in the absence of Cre expression, confirming the complete suppression of U6 promoter activity by the loxP-CMV-EGFP-loxP cassette (Fig. 3.2 B). Furthermore, no MAP4K4 siRNA was detected in muscle from both KD and C mice, confirming that MAP4K4 shRNA was specifically expressed in adipose tissue. Consistent with functional MAP4K4-siRNA expression by the MAP4K4-pSico vector, Cre-mediated recombination

resulted in a significant reduction of MAP4K4 mRNA in WAT of KD mice. MAP4K4 expression was also measured in in brown adipose tissue (BAT), liver and muscle. As expected, MAP4K4 expression was reduced in BAT, whereas no difference was observed in liver or muscle (Fig. 3.2 C). Although the aP2-Cre transgenic mice used in our studies express Cre recombinase specifically in the adipocyte, since macrophages also express aP2, we sought to confirm the specificity of Cre recombinase expression. We measured the expression of MAP4K4 in macrophages isolated from the adipose tissue as well as from PECs. As expected, MAP4K4 expression did not change in the KD mice as compared to the control animals, both in the adipose derived macrophages as well as in the PECs (Fig 3.3 A & B). These results establish that MAP4K4 was specifically silenced in adipose tissue of KD mice.

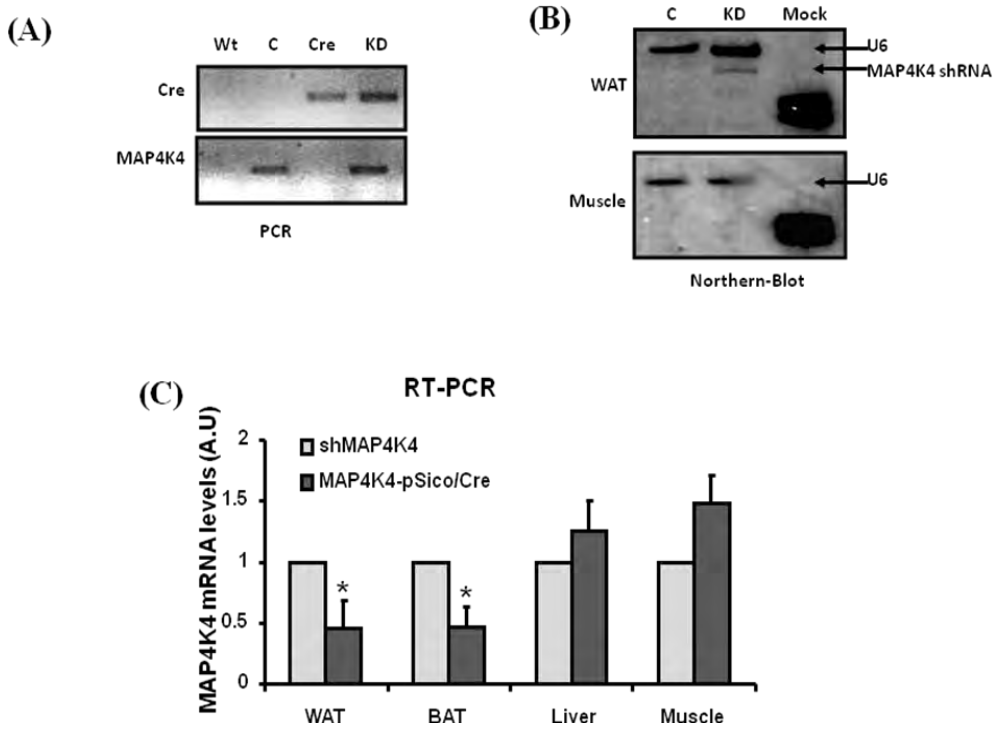


FIGURE 3.2. Conditional knockdown of MAP4K4 in transgenic mice. (A) PCR detection of Cre recombinase and MAP4K4-pSico in genomic DNA extracted from the tail of mice derived from a cross between MAP4K4-pSico and aP2-Cre mice. (B) Northern blot analysis using RNA extracted from white adipose tissue (WAT) and muscle from the indicated genotype. (C) Knock-down of MAP4K4 in the WAT and BAT. Knockdown‚ KD', compared to control ‚C' mice. (n=4). *p<0.05 by Student's t-test analysis.

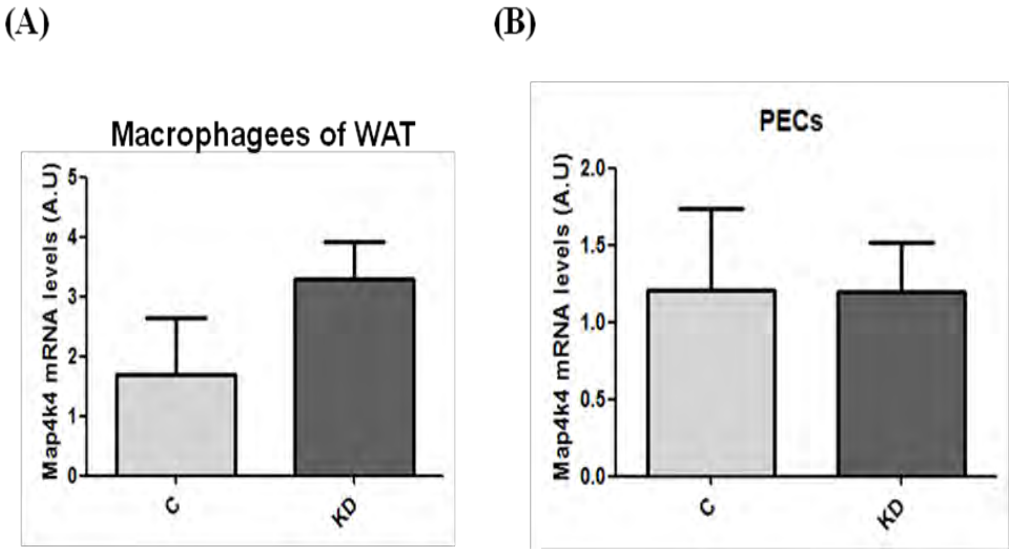


FIGURE 3.3. Specificity of Map4k4 gene silencing. Map4k4 mRNA levels were measured by real time qPCR in (A) Macrophages isolated from adipose tissue and (B) PECs.

Map4k4 Deficiency in Adipose Tissue Enhances Adiposity - As a first analysis of the effect of adipose-specific Map4k4 gene silencing, we monitored the body weight of KD and control littermates over 8 weeks between 8-16 weeks of age. On a standard chow diet, there was no significant difference in the total body weight between KD and control animals (Fig. 3.4 A). To further analyze the body composition of KD mice, fat mass was examined. Even though there was no difference in the total body weight at 16 weeks of age, KD animals exhibited an increase in fat mass on a standard chow diet feeding that was measured using proton magnetic resonance spectroscopy (Fig. 3.4 B). The observed increase in fat mass was not due to increased food intake or decreased physical activity (Fig. 3.5 A & B).

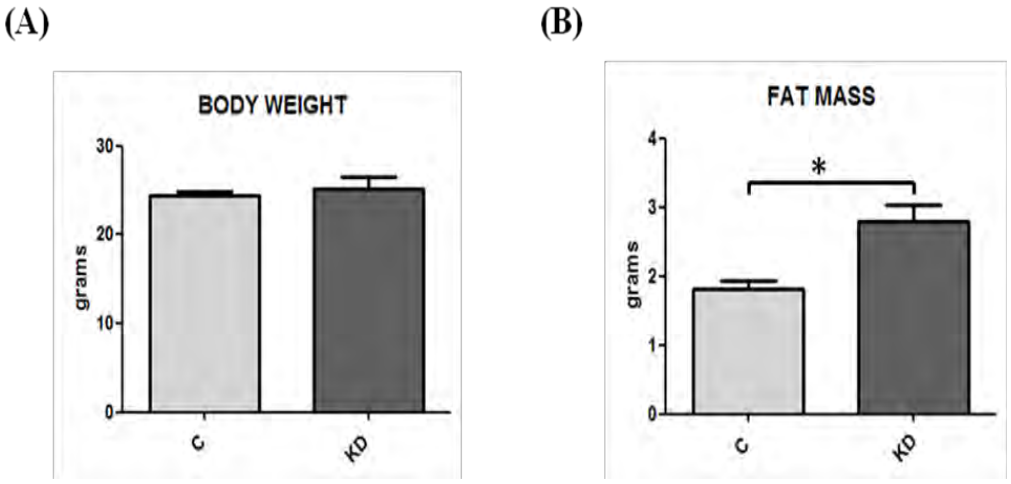


FIGURE 3.4. MAP4K4 silencing in adipose tissue enhances adiposity. (A) Body weight of 16 week old male mice (n=5-7). (B) Increase in fat mass in 16 week old male mice fed with standard diet (n=5-7)**.** *$p<0.05$ by Student's t-test analysis.

Contribution of Brown Adipose Tissue to the Map4k4 Knockdown Phenotype - We have observed a significant decrease in the expression of Map4k4 in the BAT as shown in Fig. 3.2C, and an increase in total fat mass as shown in Fig 3.3B. Since brown adipose tissue is a major tissue responsible for thermogenesis and energy expenditure in mice, one hypothesis for the increase in adiposity by MAP4K4 silencing could be a decrease in energy expenditure. Therefore, we compared energy expenditure, oxygen consumption and carbon dioxide release in the 16 week old KD and control littermates. We observed no differences in any of these parameters (Fig. 3.5 C, D & E). This result suggested that MAP4K4 might not play a role in regulation of energy expenditure in the young adipose-specific Map4k4 knockdown animals.

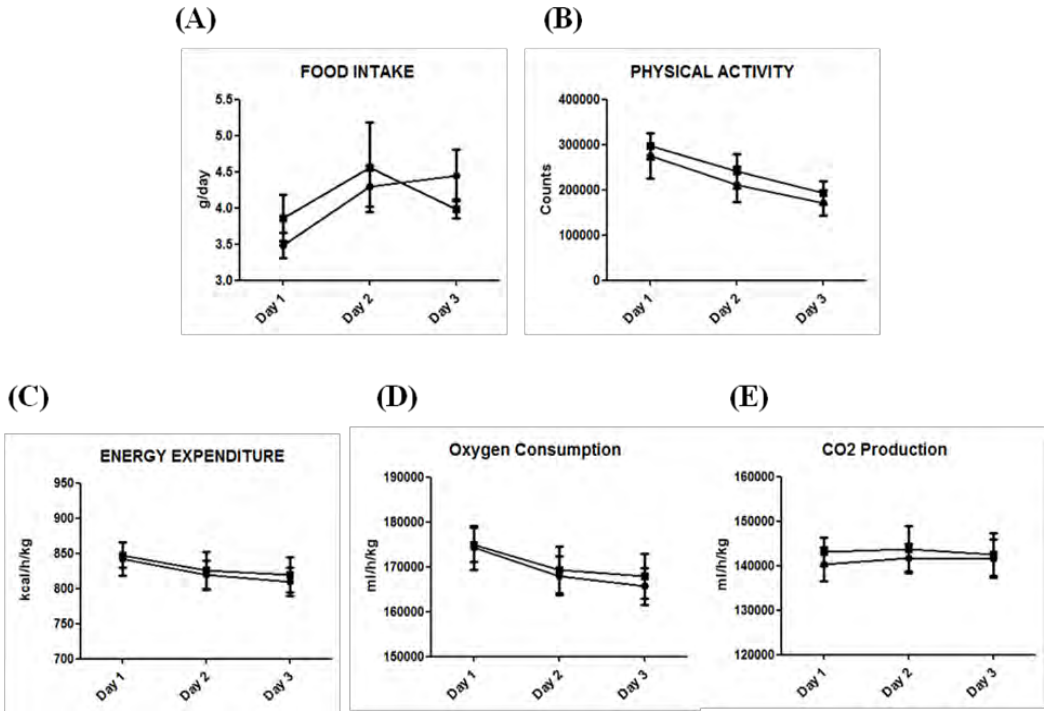


FIGURE 3.5. Metabolic Parameters. (A) Food intake (B) Physical Activity (C) Energy expenditure (D) Oxygen consumption and (E) Carbon dioxide production were measured in 16 week old KD and C mice fed a standard diet.

Map4k4 Knockdown in the Adipose Tissue Enhances PPARγ Protein Expression - Our previously published data showed that Map4k4 gene silencing enhances PPARγ protein levels in 3T3-L1 adipocytes, by a translational mechanism [324, 367]. To investigate if KD animals exhibit a similar increase in PPARγ protein levels, subcutaneous stromal vascular fraction-derived preadipocytes were cultured to mature adipocytes and PPARγ protein as well as mRNA levels were measured. As expected, PPARγ-1 as well as PPARγ-2 protein levels were significantly increased in the KD mice as compared to the control animals (Fig. 3.6 A), without any change in the mRNA expression (Fig. 3.6 B). Immunoblot analysis of lysates from the whole adipose tissue revealed a similar increase in the PPARγ protein levels in the KD mice (Fig. 3.6 C). These results suggest that the increase in fat mass in the KD animals could be due to increased PPARγ protein levels.

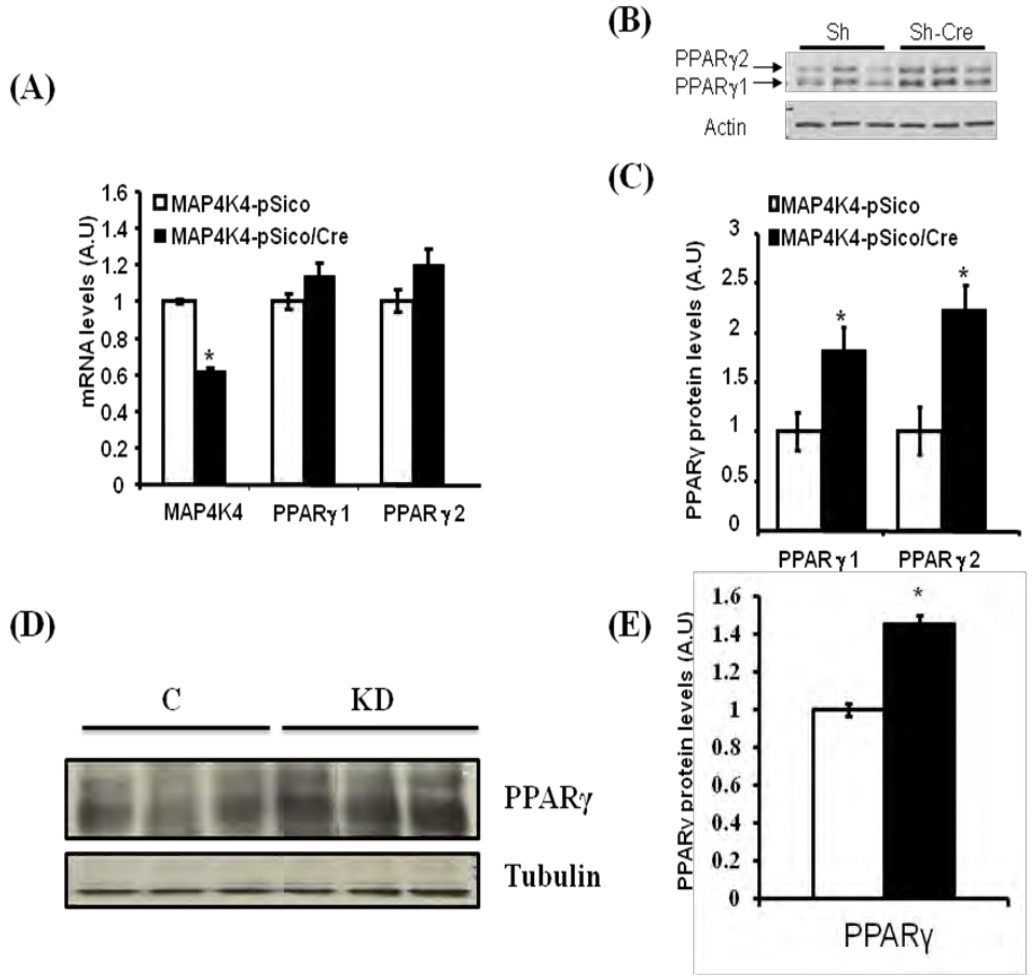


FIGURE 3.6. MAP4K4 regulates PPARγ synthesis in adipocytes. Adult preadipocytes from subcutaneous adipose tissue were differentiated into adipocyte. PPARγ mRNA levels were determined by (A) real time PCR, normalized to 36B4. (B) Representative immunoblot of PPARγ in adipocytes, each lane represents an individual mouse. This experiment was performed with cells from 3-6 different mice and is expressed +/- s.e.m. (C) Densitometry mean PPARγ values as compared to actin +/- s.e.m. of westernblot. (D) Representative immunoblot of PPARγ in mouse subcutaneous adipose tissue, each lane represents individual mouse. (E) Densitometry mean PPARγ values as compared to tubulin +/- s.e.m. of westernblot. *$p<0.05$ by Student's t-test analysis.

Adipose Specific Map4k4 Gene Silencing Regulates Whole body Insulin Sensitivity - Increase in fat mass is usually associated with deregulated insulin sensitivity. To examine the consequences of enhanced adiposity in the KD animals, as a first test, basal serum glucose level was examined. Despite an increase in the fat mass, KD mice exhibited a significant decrease in basal glycemia (Fig. 3.7 A), indicating improved glucose metabolism. Liver plays a major role in maintaining constant serum glucose levels. Indeed, hepatic glucose production was found to be significantly decreased in the KD animals (Fig. 3.7 B). Additionally, whole body glycolysis was elevated in the adipose specific MAP4K4 knockdown animals (Fig. 3.7 C), showing that the animals have improved peripheral insulin sensitivity. Thus, indicating that selective silencing of Map4k4 in the adipocytes leads to enhanced whole body basal metabolic parameters.

To further access insulin sensitivity of the KD mice, hyperinsulinemic euglycemic clamp studies were performed. Glucose infusion rate, glucose turnover and glycogen synthesis was not altered in the KD animals (Fig. 3.8 A, B & C). The liver responded maximally to insulin and hence there was no difference in the clamp hepatic glucose production and hepatic insulin action in the KD animals as compared to the control mice (data not shown).

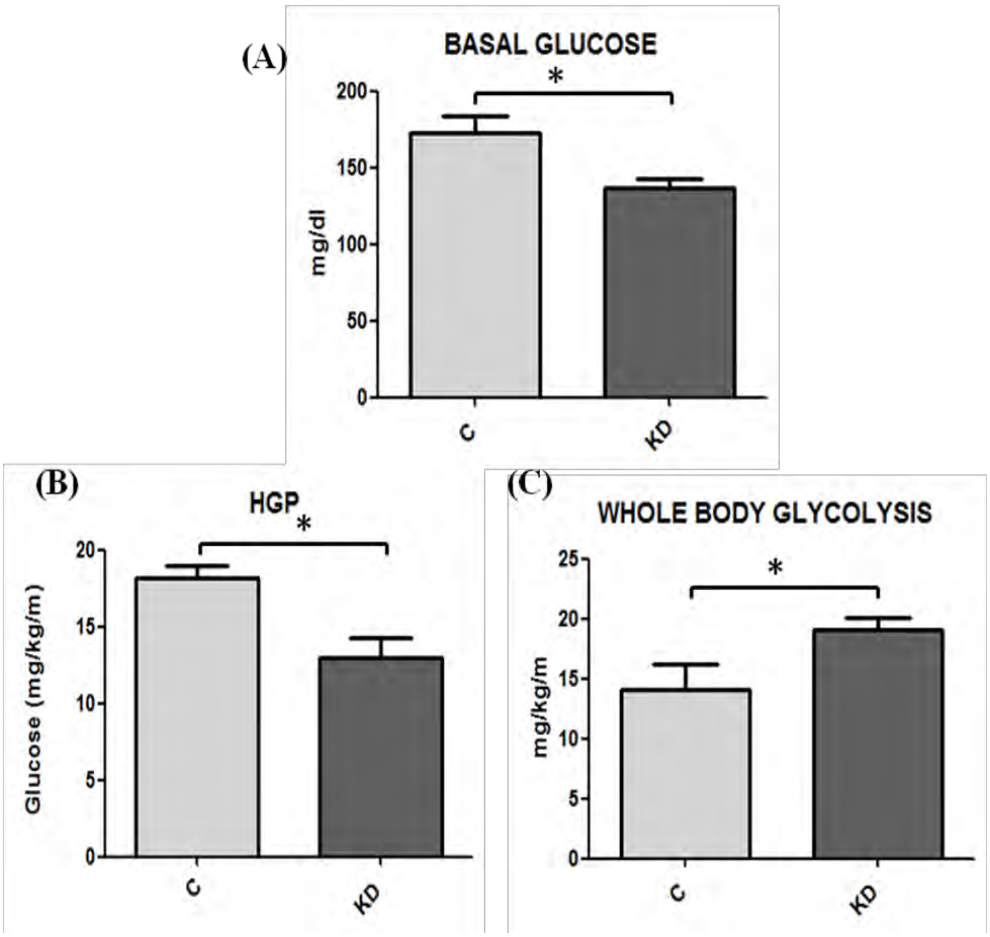


FIGURE 3.7. MAP4K4 knock-down mice exhibit enhanced whole body insulin sensitivity. (A) Basal plasma glucose level of standard diet fed 16 week old mice (n=5-7). (B) Basal hepatic glucose output (C) whole body glycolysis. *$p<0.05$ by Student's t-test analysis.

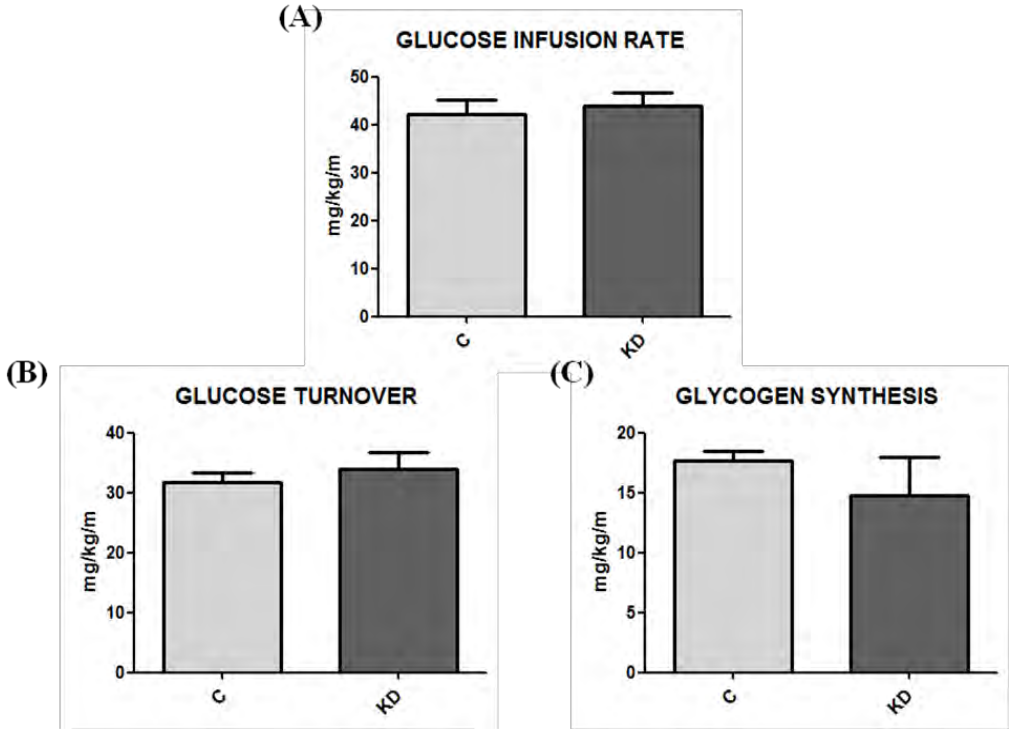
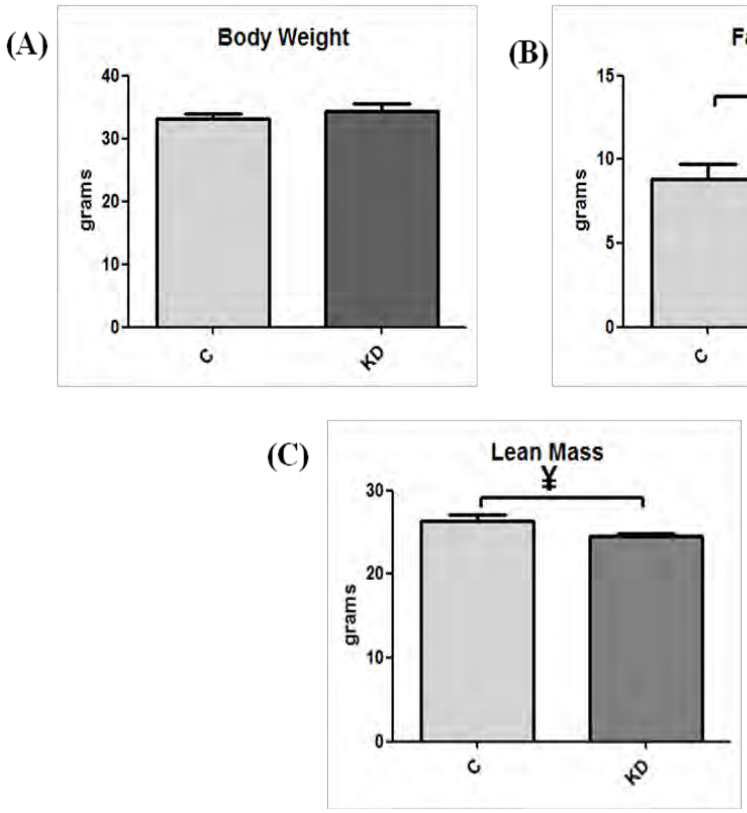


FIGURE 3.8. Glucose metabolism in SD fed condition. Graphical representation of (A) GIR (glucose infusion rate) (B) Glucose turnover and (C) Glycogen synthesis measured during hyperinsulinimic euglycemic clamp Study in 16 week old KD and C animals.

Enhanced Adiposity with Decreased Lean Mass in the Adipose Specific Map4k4 Knockdown Animals upon High Fat Feeding - Since the adipose-specific Map4k4 knockdown animals exhibited enhanced basal metabolic parameters and normal insulin sensitivity despite increased fat mass, we challenged these animals with high fat diet to examine if they retain insulin sensitivity upon diet-induced obesity. When challenged with a high fat diet (HFD) during 8-16 weeks of age, KD mice did not show any significant change in total body weight (Fig. 3.9 A), but there was a trend toward increased fat mass (Fig. 3.9 B). Additionally, KD animals had a significant decrease in lean mass (Fig. 3.9 C). However, during 16-24 weeks of age, upon high fat feeding, KD animals gained weight more rapidly than the control animals and this increase in weight reached statistical significance as early as 3 weeks of HFD. After 8 weeks of HFD, the increase in fat mass in the 24 week old KD animals is reflected in the gained body weight that was 60% of their starting weight (Fig. 3.9 D). This increase in total body weight is partly due to a significant increase in the weight of subcutaneous WAT (Fig. 3.9 E), with no change in the weight of epididymal WAT or BAT (Fig. 3.9 F & G).


(A)
Body Weight
grams
40
30
20
10
0
C
KD
(B)
Fat Mass
‡
grams
15
10
5
0
C
KD
(C)
Lean Mass
¥
grams
30
20
10
0
C
KD

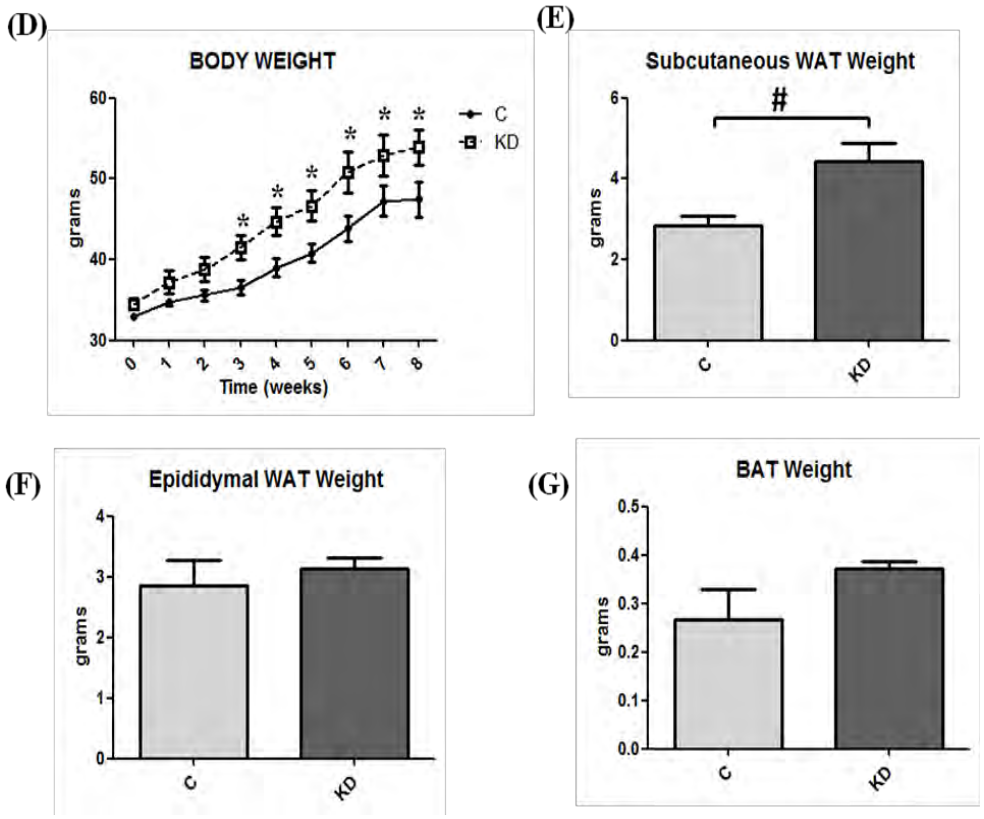


FIGURE 3.9. MAP4K4 silencing in adipose tissue increases high fat diet induced obesity. (A) Body weight of male mice at 16 weeks of age after 8 weeks of high fat diet (n=6-9). (B) Fat mass and (C) lean mass measured using ^{1}H-Magnetic Resonance Spectroscopy. (D) Development of obesity in male mice at 24 weeks of age after 8 weeks of high-fat diet (n=4-5). (E) Subcutaneous (F) epididymal and (G) brown fat pad absolute weights (n=4-5). *p<0.001, #p<0.02, ¥p<0.05, ǂp=0.065 by Student's t-test analysis.

Map4k4 Deficient Adipose Tissue Regulates Hepatic Insulin Sensitivity - Map4k4 knockdown animals in the HFD fed condition exhibited a trend towards decreased serum glucose level, a primary indicator of insulin sensitivity (Fig. 3.10 A). To further investigate the effect of DIO on glucose tolerance, a GTT was performed. Although the KD mice were significantly obese, this did not worsen their glucose clearance capacity as compared to the control littermates (data not shown). In fact, the KD animals were more glucose tolerant than the control animals when matched for weight (Fig. 3.11 A). Although glucose tolerance directly correlates to insulin sensitivity, small changes contributed by individual metabolic organs cannot be measured. To examine in detail the effect of high fat diet on individual metabolic tissues, a hyperinsulinemic euglycemic clamp study was performed. When weight matched, KD animals exhibited enhanced glucose tolerance as compared to control mice, and as the body weight of the 16 week old KD and control animals on HFD fed condition was comparable, the clamp study was performed in the 16 week old animals. Whole body glycolysis, glucose infusion rate and hepatic insulin action did not change in the KD animals (Fig. 3.10 B, C & D). Even though these results might suggest that the KD animals and the control animals could be equally insulin resistant, the KD mice exhibited a trend towards decreased basal liver glucose output. Strikingly, insulin stimulated hepatic glucose production was significantly decreased in the KD mice at the same degree of obesity as the control littermates. Whereas the control animals exhibited only a 40% decrease, the KD animals exhibited a 57% reduction in insulin stimulated hepatic glucose output (Fig. 3.11 B). Finally, the KD animals exhibited an increase in Akt phosphorylation (Thr308) in the

liver during the clamp analysis (Fig. 3.11 C), indicating enhanced insulin signaling. These results together show that adipose specific Map4k4 gene silencing protects from high fat diet induced insulin resistance that is due to improved hepatic insulin sensitivity.

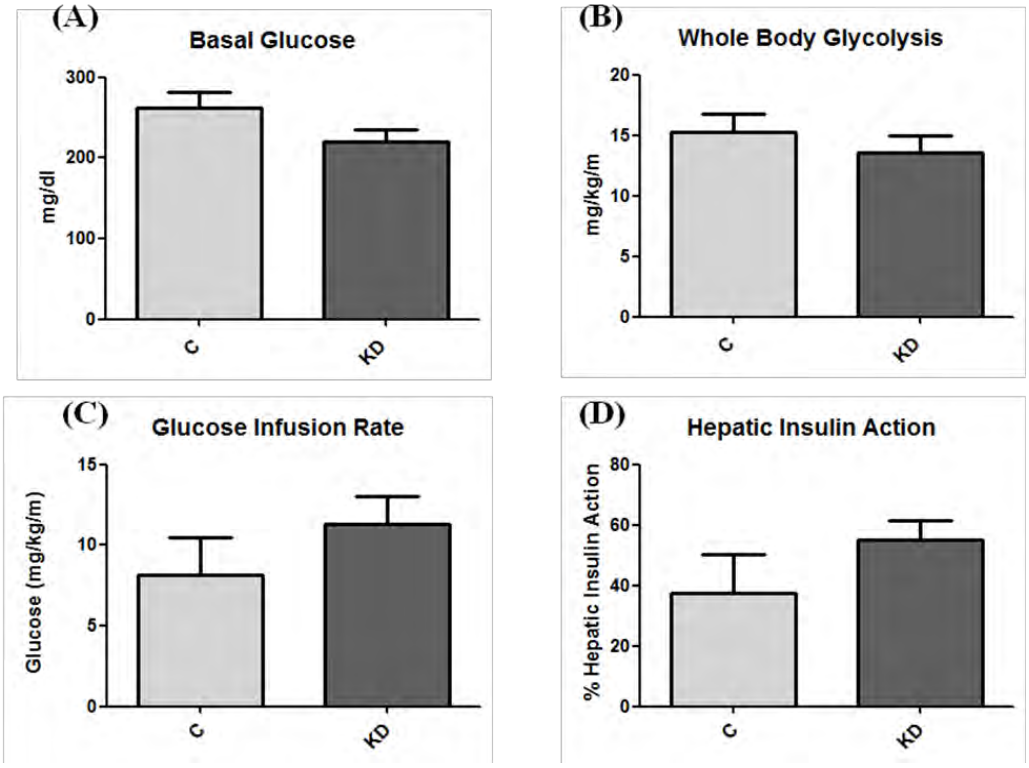


FIGURE 3.10. Graphical representation of Metabolic Parameters. (A) Basal glycemia (fed). (B) Whole body glycolysis, (C) glucose infusion rate and (D) hepatic insulin action measured during hyperinsulinimic euglycemic clamp study in 16 week old KD and C animals at the end of 8 week of HFD feeding.

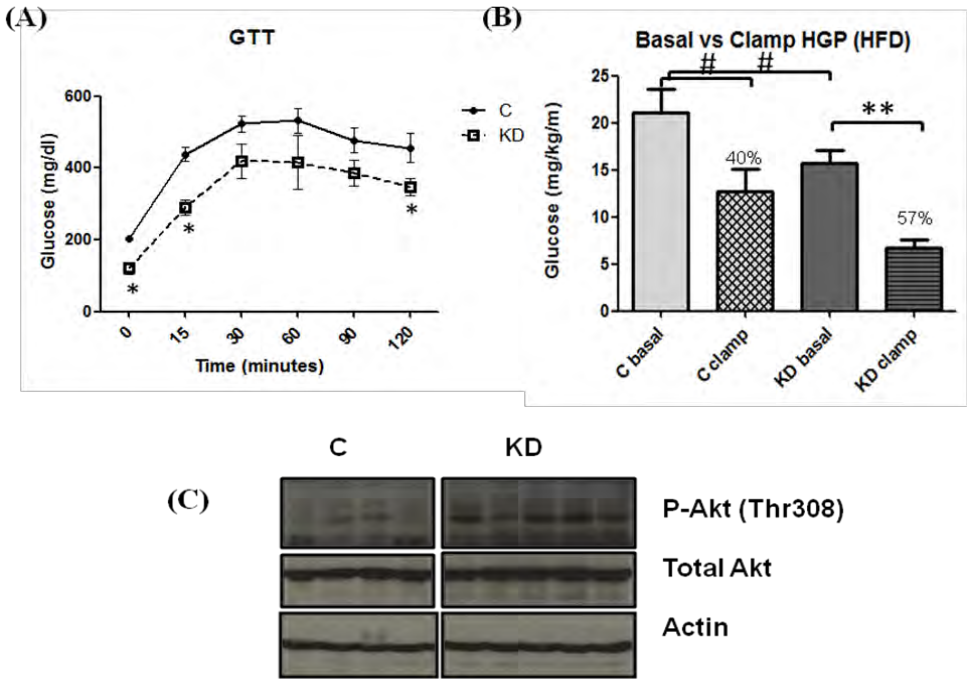


FIGURE 3.11. MAP4K4 deficiency in adipose tissue causes increased hepatic insulin sensitivity. (A) Glucose Tolerance tests (GTT) were performed after injection of 1g/kg of glucose in overnight fasted, weight-matched mice fed a high-fat diet from the age of 16 weeks for 8 weeks (KD mice) for 10 weeks (C mice) (n=4-5). (B) Basal hepatic glucose production (HGP) and insulin stimulated HGP during clamp in male mice fed a high-fat diet from 8 weeks of age for 8 weeks. (C) Representative immunoblot of P-Akt (Thr308), total Akt and tubulin in the liver, after the clamp study. **p<0.001,*p<0.01, #p=0.05 by Student‘s t-test analysis.

Role of Adipose Cell Size in the KD Mouse Phenotype - Adipose-specific Map4k4 gene silencing resulted in an increase in fat mass in the KD animals in the SD fed condition. However, at the same degree of obesity in a HFD fed condition, the KD mice exhibited an improved whole body metabolism and glucose homeostasis as a result of enhanced hepatic insulin signaling. To look more closely into the role played by Map4k4 silenced adipose tissue, in regulating liver function, adipocyte morphology was examined in the KD animals. Notably, adipocyte cell size was found to be significantly increased in the KD animals as compared to the control animals; however this was only in the subcutaneous adipose tissue and not in the epididymal adipose tissue (Fig. 3.12 A), as measured from the adipose tissue histological sections. This correlates with the differential degree of MAP4K4 knockdown observed in these two adipose depots (Fig. 3.12 B). This effect on adipocyte size is supported by our recent publication [367] where, an increase in cell size of 3T3-L1 adipocytes, upon siRNA mediated Map4k4 gene silencing was noted. Sherman and colleagues [373] have concluded from their recent studies using human obese subjects that, small cell size of subcutaneous adipocytes represents immature cells that have impaired triglyceride storage capacity. Extrapolating this to our observation of increase in adipocyte cell size in the subcutaneous adipose tissue of Map4k4 knockdown animals could suggest that, these larger adipocytes have a better lipid storage capacity.

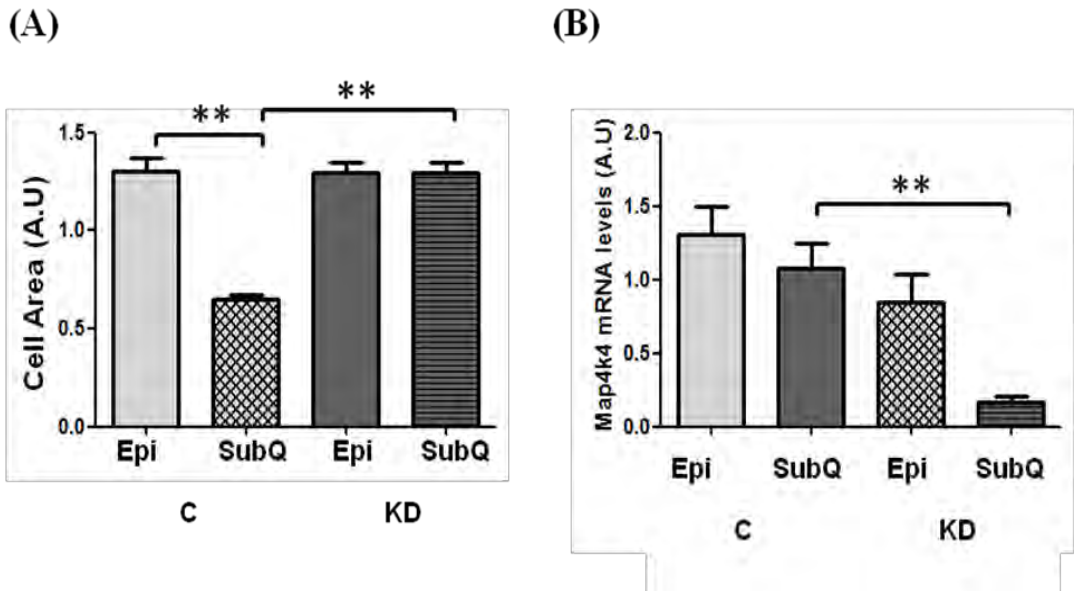


FIGURE 3.12. MAP4K4 knockdown results in adipocyte hypertrophy. (A) Graphical representation of adipocyte cell size represented as cell area measured using ImageJ program from histological sections of epididymal and subcutaneous adipose tissue from 8 week old male mice fed a high-fat diet for 8 weeks. (B) Map4k4 knockdown in epididymal and subcutaneous WAT. (n=6). **p<0.0001 by Student‘s t-test analysis.

To address this, total triglyceride content of adipose tissue was measured. As expected, triglyceride levels were enhanced in the KD animals (Fig. 3.13 A). This is consistent with the previously published data from our lab where, in 3T3-L1 adipocytes, in an siRNA mediated Map4k4 gene silencing condition, increase in triglyceride content was observed [324].

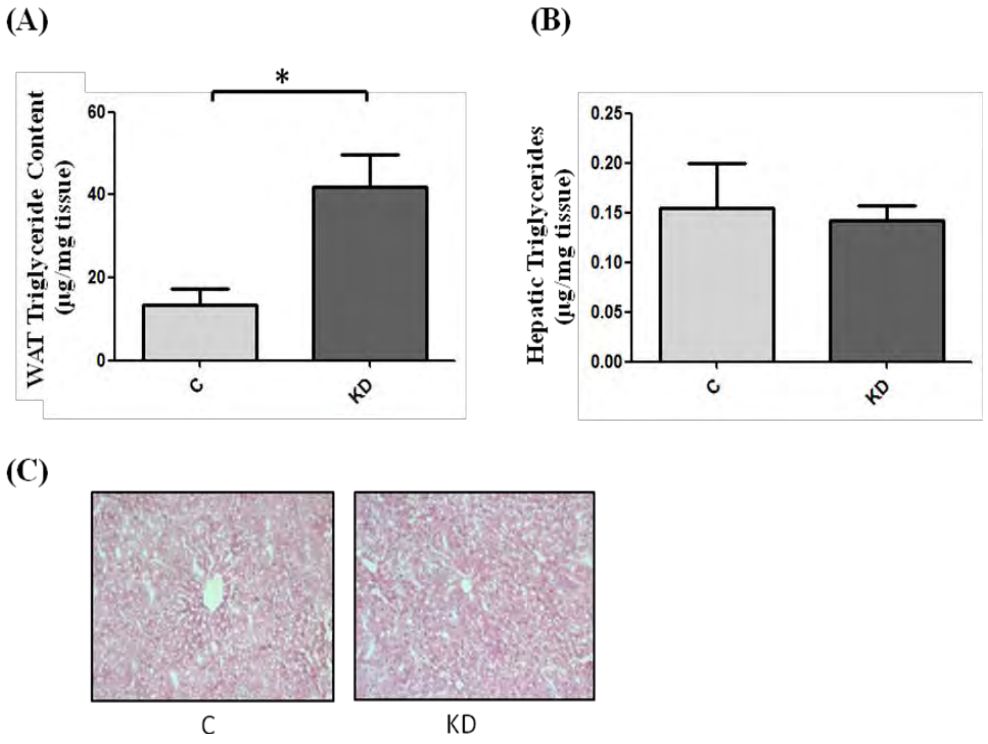


FIGURE 3.13. Adipose tissue and liver triglyceride content. Total tissue lipid was extracted and triglyceride content measured by calorimetric analysis in (A) white adipose tissue and (B) liver, in male mice fed on high fat diet for 8 weeks starting at 8 weeks of age (n=6). (C) Representative histological sections of liver stained with hematoxylin and eosin.

Since, Map4k4 gene silencing in the adipose tissue regulates hepatic insulin sensitivity, additionally, KD animals exhibited an increase in adipocyte cell size that was accompanied by an increase in the TG content, it is logical to see if these changes would result in decreased ectopic lipid deposition in the KD mice, particularly in the liver in the high fat fed condition. Surprisingly, KD animals and the control mice had a similar degree of hepatic lipid deposition as visualized by liver histology sections, as well as similar TG content in the liver (Fig. 3.13 B & C). These results show that, even with a similar degree of hepatic lipid deposition in the high fat fed condition, adipose tissue specific Map4k4 gene silencing protects the animal from high fat diet induced insulin resistance.

Discussion

Here, we described the generation and phenotypic characterization of conditional Map4k4 knockdown mice. Knockdown of Map4k4 specifically in adipose tissue (KD) results in mice that have enhanced fat mass. KD mice also exhibit an overall better metabolic profile than control mice including enhanced glucose tolerance and improved hepatic insulin sensitivity was due to an increase in hepatic insulin signaling, despite diet-induced obesity. Furthermore, we present evidence that the increase in fat mass is a consequence of improved adipose lipid storage capacity as reflected by an increase in cell size and elevated levels of adipose tissue triglyceride content. These results suggest that

Map4k4 in adipose tissue plays an important role in the control of both adipose metabolism as well as whole-body glucose homeostasis.

Similar to adipose-specific Map4k4 knockdown, whole-body knockout of collagen VI, results in mice that are obese due to expansion of individual adipocytes and is associated with significant improvements in whole body glucose homeostasis [374]. Furthermore, the authors demonstrated that treatment with a PPARγ agonist reduced the expression levels of a majority of collagens in adipose tissue. Additionally, Xu and coworkers [375] have shown that PPARγ interacts with CIITA-RFX5 complex to repress type I collagen gene expression. Although the effect of adipose-specific Map4k4 knockdown on collagen expression needs to be determined, the above mentioned observations raise the possibility that collagen expression could be down-regulated in the Map4k4 deficient adipose tissue through a PPARγ-dependent mechanism, thus resulting in adipocyte hypertrophy.

Adipose-specific PPARγ knockout mice exhibited elevated levels of plasma FFAs and TG as well as decreased levels of leptin and adiponectin, accompanied by increased hepatic glucogenesis and insulin resistance . Additionally, PPARγ2 in the adipose has been shown to prevent lipotoxicity by promoting adipose tissue expansion in conditions of excess nutritional availability [376]. Furthermore, PPARγ activation in adipocytes was shown to be sufficient for systemic insulin sensitization [377]. Most of our observations in the KD animals are consistent with these studies of a PPARγ centric regulation of adipose tissue function as well as whole body glucose homeostasis. However, contrary to

the observations made in the above mentioned studies, KD mice exhibited (i) a decrease in basal hepatic glucose production, (ii) adipocyte hypertrophy, [16] and no change in plasma levels of insulin and leptin (Fig. 3.14 A & B), as compared to the control animals. PPARγ also regulates the expression of adiponectin. To this end, adiponectin transgenic mice exhibited morbid obesity, accompanied by reduced triglyceride levels in the liver, leading to improved insulin sensitivity [378]; on the contrary, KD animals had similar levels of TG in the liver and did not show any change in the circulating levels of adiponectin (Fig. 3.14 C). However, eventhough, PPARγ is essential to maintain normal adipose function and whole body insulin sensitivity, the elevated PPARγ protein levels observed in the adipose specific Map4k4 knockdown animals might not be sufficient to explain the observed whole body phenotype.

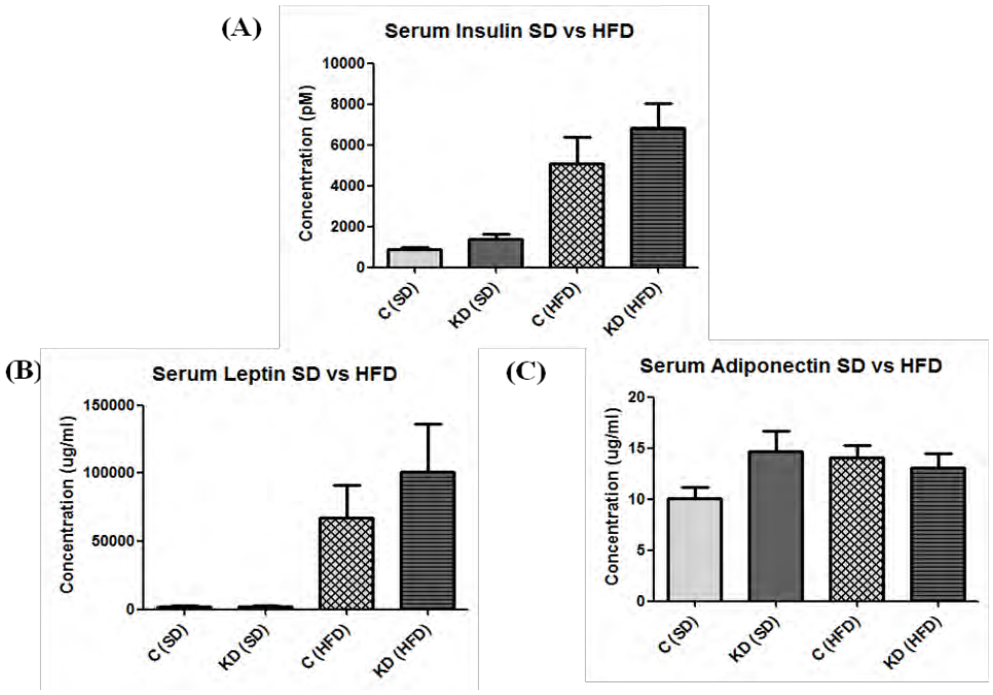


FIGURE 3.14. Circulating hormone levels. Graphical representation of (A) Insulin (B) Leptin and (C) Adiponectin concentration in the plasma of SD and HFD (8 weeks) fed 16 week old KD and C mice.

The mechanism by which Map4k4 knockdown in the adipose tissue regulates liver insulin sensitivity remains to be elucidated. Li et al. [379] demonstrated that both lipogenesis and gluconeogenesis depend on the insulin receptor-PI3K-Akt axis for their regulation. Brown and coworkers [380] have demonstrated that in obesity-induced hepatic insulin resistance, while the ability of insulin to stimulate lipogenesis is sustained, insulin‘s ability to inhibit gluconeogenesis is impaired. Interestingly, the HFD fed KD animals display decreased hepatic glucose output, which is a reflection of diminished gluconeogenesis. However, they display a similar degree of liver TG content, indicating stimulation of lipogenesis as a result of InsR-PI3K-Akt axis activation that supports a normal, insulin-sensitive liver function. This is confirmed by the observed increase in Akt phosphorylation in the KD mouse liver during the clamp study.

The increase in fat mass observed in obese conditions is correlated with chronic systemic inflammation [246, 381] that is characterized by the increased production of cytokines including TNFα, IL-1, IL-6 and resistin [382]. Loss of IL-6 has been shown to improve hepatic insulin action in obese mice [383]. Additionally, IL-1β induced insulin resistance in HepG2 and in primary rat hepatocytes [384]. However, though LPS-induced TNFα and IL-1β production was shown to be inhibited upon Map4k4 silencing in macrophages [323], plasma levels of these cytokines did not change in the KD mice fed a HFD. Remarkably, IL-1α and IL-6 plasma concentrations were significantly diminished in the KD animals (Fig. 3.15). Therefore, improved hepatic insulin sensitivity in the KD animals could be as a result of decreased levels of IL-1α and IL-6. IL-6 is synthesized and secreted by adipocytes [385, 386], however, it is not known whether adipocytes

secrete IL-1α, however, BAT is known to be a source of IL-1α [387]. Furthermore, macrophages do secrete both IL-6 and IL-1α. Moreover, altering the activation state of adipose resident macrophages has been show to change not only the inflammatory status of the adipose tissue, but also alter the serum cytokine profile [388]. However, changes in IL-1α and IL-6 were observed only in the 16-24 week but not in the 8-16 week study period. Therefore, a more detailed analysis is needed to determine the role of inflammatory cytokines in the regulation of liver function, in the KD mice.

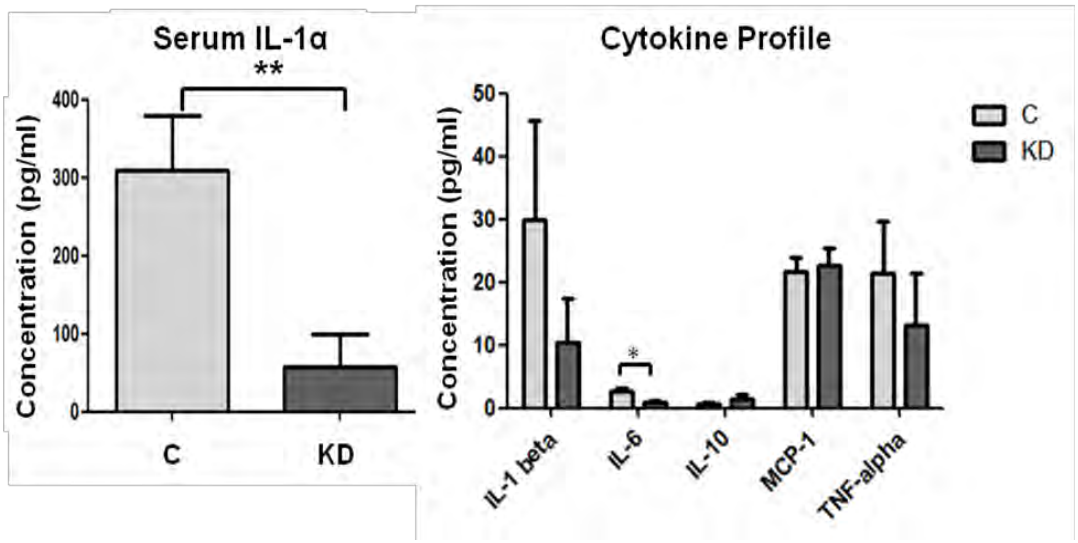


FIGURE 3.15. Graphical representation of plasma cytokine levels. Serum levels of cytokines in KD and C animals fed on HFD for 8 weeks starting at 16 weeks of age.

Enhanced adipose tissue inflammation that results in deregulated metabolic functions is a consequence of chronic stimulation of adipose tissue macrophages (ATMs). Increased macrophage recruitment to adipose is observed in obesity (39, 40), as a response to enhanced basal lipolysis [254]. Although the increase in fat mass was not outwardly visible in younger KD animals, there was visible obesity as the animals aged (Fig. 3.16 A) that resulted in a significant increase of total body weight (Fig. 3.16 B). This contributed to an increase in the weight of all the fat depots examined epididymal, subcutaneous (auxiliary & inguinal) as well as brown adipose tissue (Fig. 3.16 C, D & E). Additionally, adipocyte cell size was observed to be enhanced in the 18 month old KD mice Supplementary Fig. 3.16 F). Furthermore, fasting glycemia was comparable in the KD and control animals (Fig. 3.16 G) suggesting insulin sensitivity despite obesity. In the overnight fasted 18 month old KD mice, ATGL protein levels as well as P-HSL levels were significantly reduced (Fig. 3.16 H), indicating a decrease in basal lipolysis. Furthermore, the number of macrophages in the adipose tissue of the KD mice was lower, suggesting diminished recruitment of ATMs (Fig. 3.16 I). However, the macrophage number did not differ in the younger animals fed a HFD (data not shown), suggesting a nutritional status-dependent differential regulation of macrophage recruitment. One may hypothesize that adipose tissue-specific Map4k4 knockdown provides a microenvironment that is conducive to alternate activation of ATMs; the data showing a decrease in serum levels of IL-1α and IL-6 along with no change in the adipose tissue macrophage content in the KD mice on HFD fed condition supports this hypothesis.

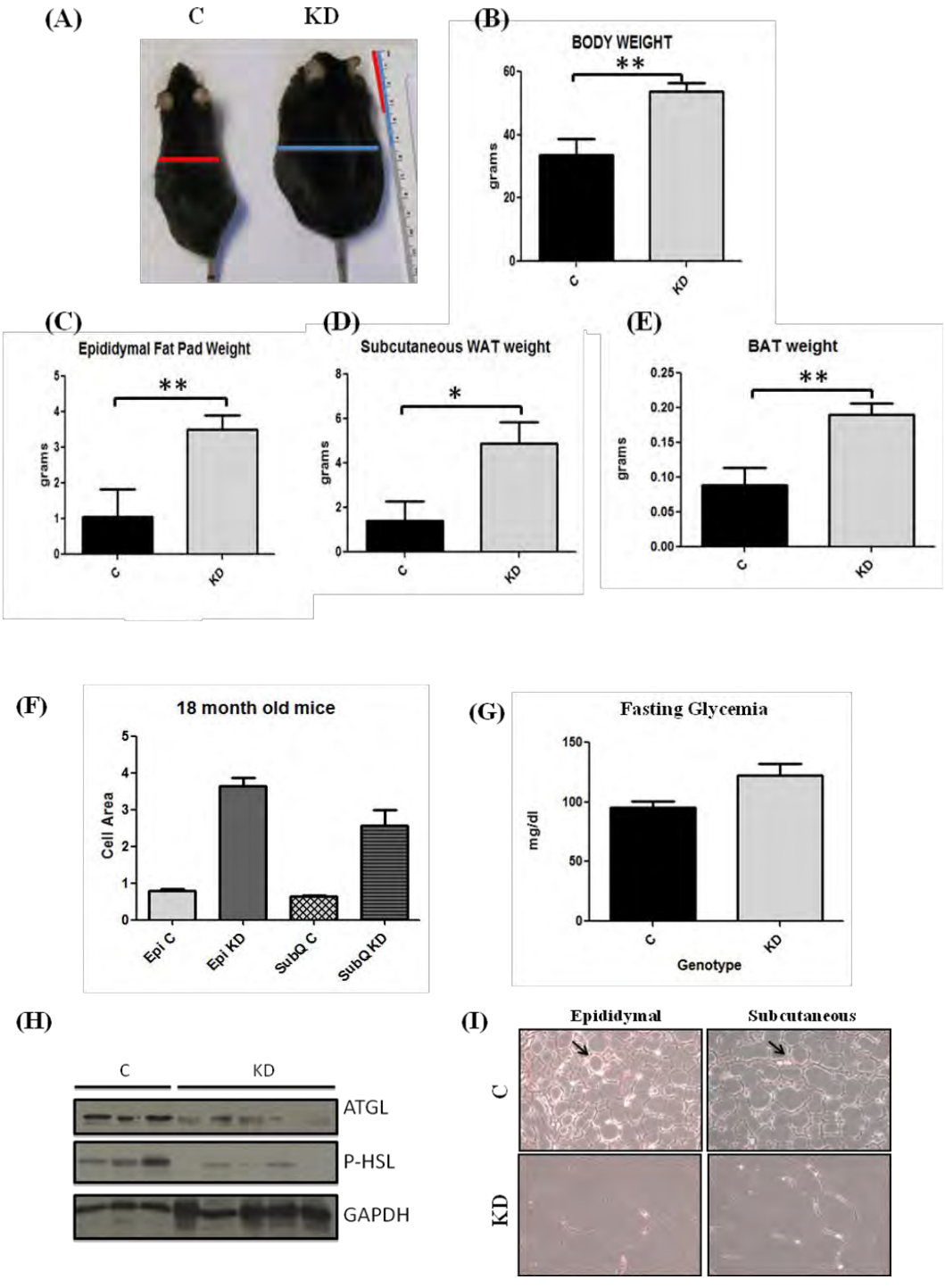

(A)
C
KD
(B)
BODY WEIGHT
**
grams
60
40
20
0
C
KD
(C)
Epididymal Fat Pad Weight
**
grams
5
4
3
2
1
0
C
KD
(D)
Subcutaneous WAT weight
*
grams
8
6
4
2
0
C
KD
(E)
BAT weight
**
grams
0.25
0.20
0.15
0.10
0.05
0.00
C
KD
(F)
18 month old mice
Cell Area
5
4
3
2
1
0
Epi C
Epi KD
SubQ C
SubQ KD
(G)
Fasting Glycemia
mg/dl
150
100
50
0
C
KD
Genotype
(H)
C
KD
ATGL
P-HSL
GAPDH
(I)
Epididymal
Subcutaneous
C
KD

FIGURE 3.16. Enhanced Adiposity in 18 month old mice. (A) Representative picture depicting visible obesity in 18 month old male KD mice fed a standard diet. (B) Body weight (C) epididymal (D) subcutaneous and (E) brown adipose tissue fat pad absolute weights. (F) Epididymal and subcutaneous adipocyte cell area (G) Basal glycemia (fasting) (H) Immunoblot showing ATGL, P-HSL protein levels in Epididymal adipose tissue lysates. GAPDH is the loading control (I) Representative pictures of epididymal and subcutaneous adipose tissue sections stained for macrophage cell surface protein F4/80, detected using Immunofluorescence microscopy with Alexafluor-647. Arrows show macrophages forming crown like structures. (n=3-5). **p=0.01, *p<0.05 by Student‘s t-test analysis**.**

To summarize (Fig. 3.17), inhibition of Map4k4 expression in the adipose tissue results in enhanced adiposity leading to improved lipid storage capacity that could be in part due to an increase in PPARγ protein levels. Therefore, altering adipocyte fat sequestration & synthesis potential, as well as PPARγ function, could favor an alternately activated inflammatory state of the adipose tissue. Thus, the changed adipose tissue environment augments insulin sensitivity in the liver, which modulates whole body glucose homeostasis.

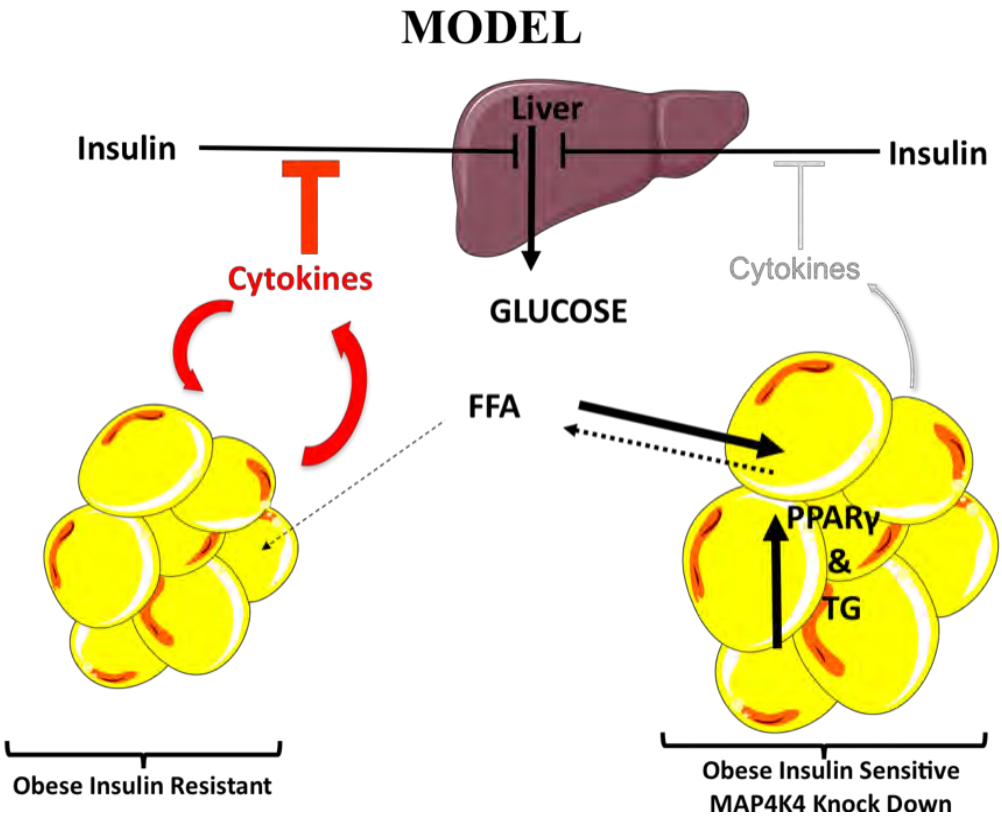


FIGURE 3.17. Map4k4 in the adipose regulates liver insulin sensitivity. Silencing Map4k4 in the adipose tissue results in adipocyte hypertrophy leading to enhanced lipid storage capacity. This possibly leads to a change in the adipose tissue milieu, resulting in a decreased synthesis and secretion of pro-inflammatory cytokines by the adipocytes and/or macrophages. This altered adipose tissue function leads to an increase in hepatic insulin sensitivity, thus regulating whole body metabolism.

Briefly, we have shown that shRNA mediated gene silencing using the pSico lenti-viral system is a quick and efficient way of generating knockdown mouse models. Furthermore, the adipose-specific Map4k4 knockdown animal has proven to be a good model system to study the role of Map4k4 in regulation of adipose tissue function. Although much work needs to be done to completely understand the intriguing phenotype presented by the adipose-specific Map4k4 knockdown animals, it may be concluded that, silencing Map4k4 in the adipocytes results in an insulin-sensitive obese state.

Limitations and Future Perspectives

The results presented in this chapter are intriguing; however several questions remain to be addressed to completely understand the role of MAP4K4 in regulation of adipose function that in turn modulates the whole body metabolic function. First, studies are required to determine the molecular link between adipose tissue and the liver in the adipose-specific MAP4K4 knockdown animals based on the observed suppression of hepatic glucose output. Although silencing a gene in the adipose tissue that results in decreased hepatic glucose production has been previously observed in adipose-specific JNK1 knockout animals; the observation of improved hepatic insulin sensitivity accompanied by diminished glucose output despite enhanced adiposity in the adipose-specific MAP4K4 knockdown condition, is novel. Additionally, the observed decrease in plasma levels of IL-1α in the KD mice (Figure 3.15) is very interesting. Furthermore, Vaartjes and coworkers demonstrated that IL-1α could acutely promote hepatic glucose release, in freshly isolated rat hepatocytes [389]. Therefore, I hypothesize that adipose-

specific MAP4K4 regulates hepatic glucose production through regulation of IL-1α. The decrease in the plasma levels of IL-1alpha observed in the KD animals could be due to disruption of IL-1α gene due to MAP4K4 transgene integration. However, this might not be the case since the control animals also have the MAP4K4 transgene integrated into their genome although they do not express the shRNA.

It is very alluring to speculate that MAP4K4 in the adipose tissue could plausibly be regulating the secretion of IL-1α by two mechanisms, as summarized in Figure 3.18. Adipocytes themselves could be synthesizing and secreting IL-1α, although it is not known if white adipocytes secrete this cytokine. Alternately, MAP4K4 in the adipocytes could be regulating the synthesis and secretion of an unknown protein/factor-X that in turn regulates the expression and secretion of IL-1α by the endothelial cells, since endothelial cells are known to secrete this pro-inflammatory cytokine. Preliminary studies involving siRNA mediated MAP4K4 gene silencing in the adipocytes, as well as co-culture of 3T3-L1 adipocytes with endothelial cells to study changes in the IL-1α secretion into the culture media could provide insight into the tissue specific contribution of the cytokine.

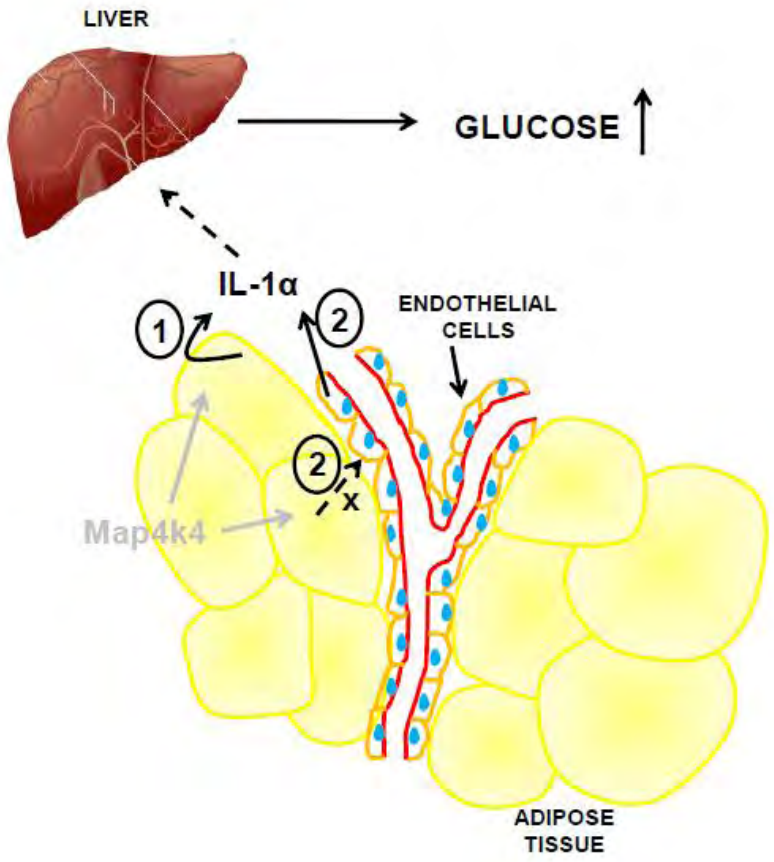


FIGURE 3.18. Hypothetical model for Map4k4 mediated IL-1α regulation. Mechanism (1) Map4k4 regulates adipocyte expression (mRNA and/or protein) of IL-1α. Mechanism (2) Map4k4 in the adipocytes regulates the expression of an unknown factor X. Factor X secreted by the adipocytes regulates the synthesis and secretion of IL-1α by the endothelial cells. Thus released IL-1α from the adipose tissue stimulates hepatic gluconeogenesis resulting in elevated plasma glucose levels.

Second, experiments are required to address if the mTOR signaling pathway similarly regulates PPARγ expression in the adipose tissue of the KD animals, as was observed in the cell culture studies in Chapter II. MAP4K4 knockdown in 3T3-L1 aidpocytes resulted in an increase in PPARγ protein levels. Consistent with this, an increase in PPARγ protein levels was observed in the KD animals. Additionally, adipocyte cell size was significantly enhanced in the MAP4K4 knockdown condition in the cell culture system; similar observation of enhanced adipocyte cell size was made in the KD mice. Together these observations provide strong evidence of a role for mTOR in regulation of adipocyte size and PPARγ protein expression, in the context of MAP4K4 knockdown. The increase in PPARγ protein levels is due to enhanced mTOR activity towards 4E-BP1 but not p70S6K1. Studies directed towards addressing the differential regulation of p70S6K1 and 4E-BP1 in the adipocytes of adipose-specific MAP4K4 knockdown animals is needed. The increase in adipocyte cell size as well as enhanced triglyceride content in the KD mice could possibly be due to elevated mTOR activity towards 4E-BP1 but not p70S6K1 as summarized in Figure 3.19. Since p70S6K1 has been demonstrated in the literature to be a negative regulator of IRS and hence insulin signaling, it can be hypothesized that, in the absence of MAP4K4, elevated mTORC1 activity results in differential regulation of mTORC1 downstream substrates providing conditions for constitutive activation of insulin signaling pathway towards mTORC1 to regulate PPARγ, adipocyte cell size as well as lipogenesis, in the adipose-specific MAP4K4 knockdown animals. It would be interesting to see whether these properties of

adipocytes in the KD animals are inhibited upon rapamycin treatment thus providing evidence for role of mTORC1 in MAP4K4 cellular functions.

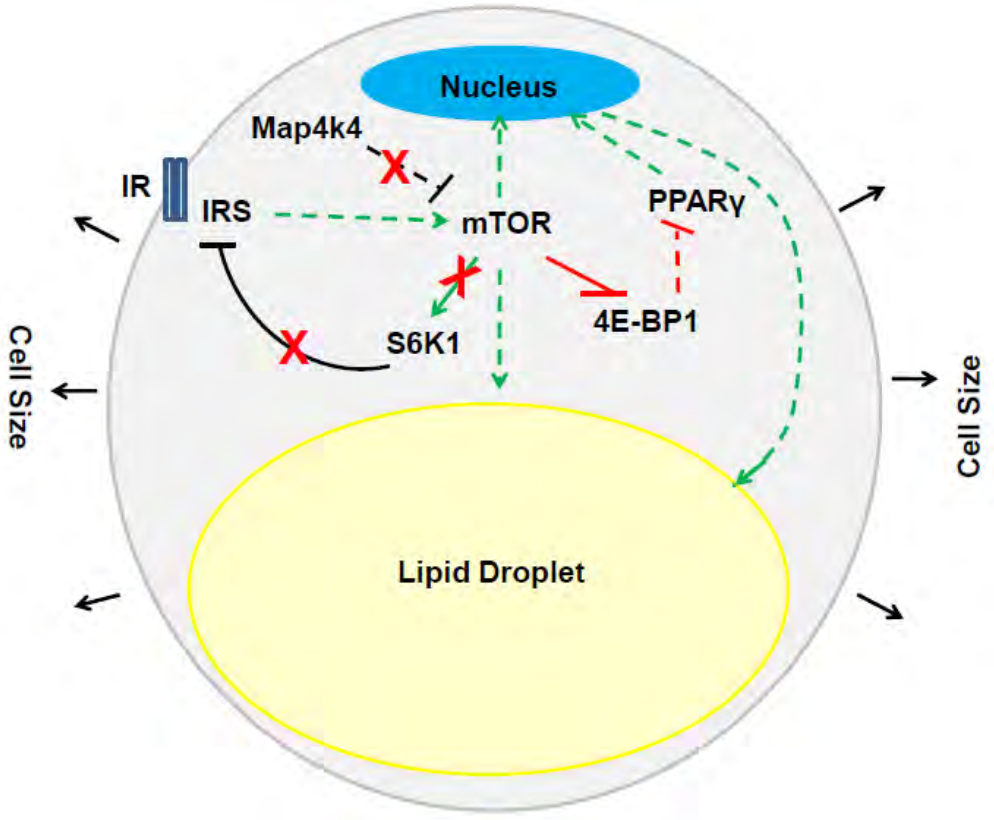


FIGURE 3.19. Map4k4 regulates p70S6K1. Map4k4 in adipocytes plays an important role in inducing insulin resistance by promoting mTORC1 activity towards S6K1 and thus resulting in the activation of the feedback inhibition of IRS. Silencing Map4k4 results in loss of this signaling to IRS by S6K1 thus resulting in constitutive mTORC1 activity towards 4E-BP1 thus, preventing its inhibition of PPARγ protein translation. mTORC1 activation also leads to increase in cell size.

Since, MAP4K4 knockdown in 3T3-L1 adipocytes resulted in differential regulation of 4E-BP1 and p70S6K1 despite mTOR activation, and the improved adipocyte function in the KD animals could be attributed to the sustained insulin signaling to mTORC1 due to absence of p70S6K1 mediated negative feedback inhibition of IRS. Although this hypothesis still needs to be tested in the KD mice, it would be interesting to see if MAP4K4 gene silencing in hepatocytes would exhibit improved hepatic insulin signaling to mTORC1 and a differential regulation of the two downstream targets and hence an improved whole body glucose metabolism similar to adipose-specific MAP4K4 knockdown. Since the improved hepatic insulin sensitivity in the KD animals was due to elevated insulin signaling that was reflected in enhanced Akt phosphorylation, it can be hypothesized (summarized in Figure 3.20) that upon MAP4K4 gene silencing in the liver, absence of p70S6K1 negative feedback inhibition to IRS would result in sustained Akt activation and hence leading to decreased glucose output from the liver. It remains to be seen whether liver specific MAP4K4 gene ablation would be sufficient to improve hepatic insulin sensitivity and thus regulate whole body glucose homeostasis.

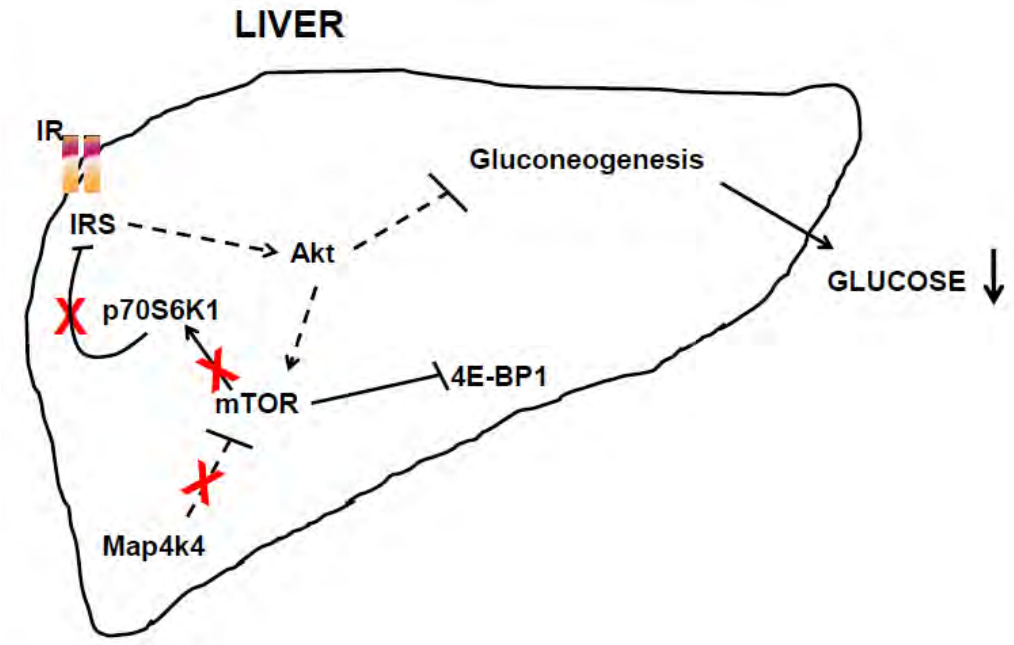


FIGURE 3.20. Hypothetical model for Map4k4 function in the liver. Silencing Map4k4 in the liver prevents mTORC1 mediated p70S6K1 activation and hence results in loss of feedback inhibition of IRS leading to constitutive activation of the insulin signaling pathway towards Akt. Activated Akt inhibits gluconeogenesis and thus results in decreased plasma glucose levels contributing to improved whole body glucose homeostasis.

Third, detailed studies are warranted to understand the role of lipolysis in the observed phenotype of adipose-specific MAP4K4 knockdown animals. The KD animals exhibited decreased lipolysis as evidenced by diminished expression of ATGL and HSL phosphorylation at 18 months of age. If the KD animals exhibit this at younger age, needs to be studied. Kosteli and colleagues have recently shown that macrophages are recruited to the adipose tissue in response to enhanced lipolysis, so as to sequester the released free fatty acids. Map4k4 in the adipocytes could be a positive regulator of lipolysis, thus resulting in macrophage recruitment and/or their activation. Furthermore, the activated macrophages would release pro-inflammatory cytokines to regulate hepatic function, as summarized in Figure 3.21. Adipose tissue explants from the control and KD animals could be used to study fasting induced lipolysis. Furthermore, fasting induced lipolysis mediated macrophage recruitment and the activation state of these ATMs would be studied using FACS analysis.

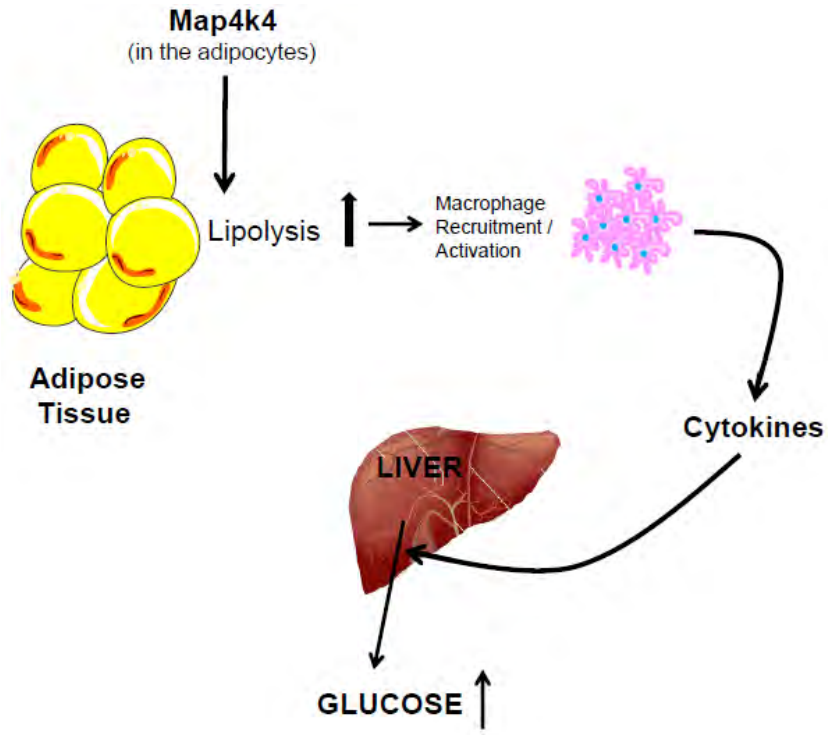


FIGURE 3.21. Hypothetical model for role of Map4k4 stimulated adipocyte lipolysis in regulating macrophage recruitment and thus hepatic gluconeogenesis. Map4k4 in the adipocytes independently or through inhibition of mTORC1 activity stimulates lipolysis. Resulting increase in FFAs recruits macrophages to the adipose tissue. Activated macrophages secrete pro-inflammatory cytokines that stimulates hepatic gluconeogeneis and results in elevated plasma glucose levels.

Fourth, the discrepancy in the degree of MAP4K4 knockdown in the subcutaneous versus epididymal adipose tissue of the mice needs to be addressed. Studies in the animal models have suggested that expression of aP2 is greater in subcutaneous adipocytes than in epididymal adipocytes and hence the expression of MAP4K4 shRNA could be elevated in the subcutaneous adipose tissue thus resulting in better MAP4K4 knockdown. Additionally, it is possible that the improved function of the subcutaneous adipocytes in the MAP4K4 knockdown mice prevents the expression of the shRNA in the gonadal fat, as subcutaneous adipose tissue develops earlier in the life of an animal. To address this, adipose tissue expression of MAP4K4 shRNA needs to be monitored throughout postnatal development. We are currently in the process of confirming the observed phenotype in an additional mouse founder line, as all the adipose-specific MAP4K4 knockdown animals used in the experiments described in this chapter were generated from one mouse founder. Furthermore, we plan to generate MAP4K4 tissue-specific knockdown animals utilizing a different sequence of shRNA. Although MAP4K4 gene silencing was not observed in PECs and adipose derived macrophages, we need to confirm the specificity of aP2-Cre expression using bone marrow transplant studies. By answering all these questions, we might achieve better insight into the regulation of adipocyte function that is impaired in insulin resistance.

CHAPTER IV: Discussion

Adipose tissue is the normal repository for excess energy in the form of triglycerides. Obesity-associated insulin resistance may be characterized by a state of dyslipidemia, where ectopic accumulation of lipid in liver and muscle is observed. This is accompanied by a chronic state of inflammation which leads to co-morbidities including type 2 diabetes. To this end, transgenic expression of the insulin responsive glucose transporter in mice resulted in improved adipose tissue function, increased adipose insulin sensitivity, and prevention of diet-induced obesity [390].

Studies from our laboratory demonstrated enhanced GLUT4 expression and insulin stimulated glucose uptake in the MAP4K4 ablated 3T3-L1 adipocytes [324]. Moreover, TNFα treatment enhanced MAP4K4 expression [315]. Additionally, TNFα-mediated inhibition of GLUT4 expression was prevented by silencing MAP4K4 in cultured adipocytes [324]. Thus, the objectives for this thesis were to better understand the role of MAP4K4 in adipose function, utilizing cell culture as well as mouse model systems.

PPARγ is necessary for adipogenesis and is considered the master regulator of adipocyte function. Thiazolidinediones (TZDs), comprising troglitazone, rosiglitazone, and pioglitazone are a class of medications used in the treatment of type 2 diabetes that function as PPARγ agonists to improve adipose function, thus contributing to improved whole body insulin sensitivity [391, 392]. However, troglitazone has been withdrawn as a drug due to incidence of hepatitis and potential liver failure, rosiglitazone has been

implicated in increased risk of coronary heart disease and heart attacks, and there is a possible association of pioglitazone and bladder cancer, necessitates the discovery of new drugs to treat insulin resistance and type 2 diabetes. In light of the previous studies that identified MAP4K4 as a negative regulator of adipogenesis and PPARγ expression, which provided adequate impetus for developing innovative therapeutics, identification of the signaling pathway utilized by MAP4K4 to regulate PPARγ expression was needed. Thus in Chapter II, I identified a pathway by which MAP4K4 regulates PPARγ expression. Since studies from several laboratories have provided evidence for not only the important role played by PPARγ in adipocyte function, but also its role in regulating whole body insulin sensitivity, I wanted to determine if MAP4K4 regulated adipocyte function *in vivo*. Thus, in Chapter III, I demonstrated that MAP4K4 regulates adipose tissue PPARγ expression in mice and also plays a significant role in whole body glucose homeostasis. To achieve adipocyte-specific MAP4K4 gene ablation *in vivo*, a shRNA construct was employed, that was expressed in an aP2-driven Cre-recombinase-dependent manner. Together, these studies add considerably to understanding the role of MAP4K4 in adipose function.

Importantly, the results from 3T3-L1 cell culture studies established Map4k4 as a negative regulator of PPARγ protein expression and adipocyte cell size. Interestingly, adipose-specific MAP4K4 gene silencing in mice promoted an increase in PPARγ protein levels, TAG accumulation as well as enhanced adipocyte cell size thus confirming the observations made in the cell culture model system.

Regulation of PPARγ Expression by MAP4K4 in Adipocytes

In Chapter II of this study, the regulation of PPARγ expression by MAP4K4 in adipocytes was characterized. This study was important for determining the mechanism by which MAP4K4 regulates adipocyte biology. As mentioned earlier, MAP4K4 was identified as a negative regulator of PPARγ and adipogenic gene expression [324]. How MAP4K4 regulated PPARγ expression, was not known. Hence these studies provided new insights into the mechanisms by which MAP4K4 regulates adipocyte function.

The finding that mTOR phosphorylation at Ser2448 was enhanced when MAP4K4 was silenced, as shown in Figure 2.3B, identified MAP4K4 as an important player in the mTOR-mediated insulin signaling pathway regulation. The ser2448 site on mTOR is considered the Akt mediated activation site, thus suggesting that MAP4K4 could be regulating Akt function that subsequently could modulate mTOR activity. However, in the previous studies, Akt phosphorylation was not observed to change in the MAP4K4 knockdown conditions in 3T3-L1 adipocytes [324]. Hence, MAP4K4 could be regulating mTOR at a step downstream to Akt. Although an increase in mTOR Ser2448 phosphorylation is an indicator of increased mTOR activity it is not direct evidence. The increase in phosphorylation of 4E-BP1 on the priming sites thr36/45, as well as on ser64 and thr69 is definite proof of increased mTOR activity. However, the possibility of a 4E-BP1 phosphatase being inactive in the MAP4K4 knockdown condition cannot be ruled out. Thus, performing an mTOR in vitro kinase assay would be of interest.

Interestingly, MAP4K4 silencing mediated an increase in PPARγ protein levels due to increase in protein synthesis, since neither PPARγ mRNA expression (Figure 2.1 C), nor the protein degradation changed (Figure 2.2 A). These results show evidence for PPARγ translational regulation for the first time in the literature. However, as these are indirect evidence, determining the increase in PPARγ protein level by utilization of an [^{35}S]met incorporation study to specifically observe an increase in incorporation of radioactive amino acid into PPARγ protein would be unequivocal evidence.

Although 4E-BP1 and p70S6K1 are two downstream substrates of mTOR, the differential regulation of these two substrates in the MAP4K4 silencing conditions is surprising (Figure 2.6). The observation of no change in the phosphorylation status of p70S6K1 upon MAP4K4 knockdown was unexpected. However, studies show that a higher mTOR activity is required for the phosphorylation and activation of p70S6K1, while only a small increase in the activity in mTOR is sufficient to have an effect on 4E-BP1 phosphorylation. Furthermore, since 4E-BP1 and p70S6K1 compete for the binding site on Raptor, if 4E-BP1 is favored as a substrate for mTOR in the MAP4K4 knockdown condition, then p70S6K1 would be inactive. It would be interesting to study if a differential binding capacity of the two substrates exists in the MAP4K4 silenced state.

Rapamycin is an mTOR specific inhibitor. As shown in Figure 2.10 A, rapamycin treatment clearly inhibited mTOR activity that is reflected by the decreased 4E-BP1 phosphorylation. The MAP4K4 silencing-mediated increases in 4E-BP1 phosphorylation as well as PPARγ protein levels was prevented by rapamycin, clearly showing that this

was a specific effect of inhibition of mTOR activity. Furthermore, adenovirus mediated MAP4K4 overexpression decreased mTOR Ser2448 phosphorylation and also resulted in diminished PPARγ protein levels (Figure 2.12 A). Thus, the data presented in this body of work has provided evidence for MAP4K4 mediated translational regulation of PPARγ through an mTOR/4E-BP1-dependent mechanism.

Adipose MAP4K4 Regulates Hepatic Insulin Sensitivity

Adipocyte-specific MAP4K4 gene silencing was achieved by expression of a lentiviral-based shRNA construct against MAP4K4 and recombination with cre recombinase driven by the aP2 promoter. An important discovery in these studies was that adipose MAP4K4 regulated liver insulin sensitivity. Silencing of MAP4K4 in the adipose tissue resulted in decreased hepatic glucose output (Figure 3.11B) during the clamp study, which is possibly due to increased activation of insulin signaling pathway, as seen in elevated Akt phosphorylation (Figure 3.11C). These results show that the liver retains insulin sensitivity in the adipose-specific MAP4K4 knockdown (KD) conditions, even when challenged with high fat diet.

The KD animals exhibit enhanced adiposity even when fed a chow diet (Figure 3.4B), and surprisingly, this was accompanied by decreased glycemia (Figure 3.5A). This decrease in serum glucose levels could have been due to decreased basal hepatic glucose production as well as increased whole body glycolysis (Figure 3.7 B & C). Furthermore, MAP4K4 gene silencing resulted in increased PPARγ protein expression (Figure 3.6 A & D) as was previously observed in 3T3-L1 adipocytes. Surprisingly, despite improved

insulin sensitivity, the liver of the KD mice still had a similar amount of lipid deposition as the control mice, in the high fat fed condition (Figure 3.13 B & C). These results suggest that hepatic lipid content might not be an indicator of hepatic insulin resistance.

Previous studies using adipose-specific transgenic expression of PPARγ have demonstrated that PPARγ activation in the adipocytes is sufficient to maintain whole body insulin sensitivity. However, in the transgenic mice, the basal hepatic glucose output did not change. Furthermore, neither the body weight nor the % of WAT varied. Moreover, the authors have shown that adipocyte cell size, as well as serum leptin levels were significantly decreased [377]. In contrast to these mice, the KD animals display decreased basal hepatic glucose output, and increased total body weight (Figure 3.9 D) as well as enhanced subcutaneous WAT mass (Figure 3.9 E). The increase in adipose function in the KD mice was reflected by increased adipocyte size (Figure 3.12 A) and elevated triglyceride content in the adipose tissue (Figure 3.13 A). However, circulating levels of leptin in KD mice did not change (Figure 3.14 B). These observations in the KD mice are different from the PPARγ transgenic animal data, and suggests that the phenotypic effects observed in the adipose specific MAP4K4 knockdown mice is not purely due to elevated PPARγ expression. The studies from the KD animals provide evidence for PPARγ dependent as well as independent phenotypes in the adipose tissue of MAP4K4 KD animals.

Studies using adipose-specific JNK1 knockout mice demonstrated that, ablation of JNK1 in the adipose tissue decreased IL-6 synthesis, which was responsible for

improved liver insulin sensitivity after high fat diet. However, these animals exhibited elevated basal glucose levels, despite similar body weight and adiposity as the control animals [393]. Though serum levels of IL-6 was observed to be decreased in the adipose-specific MAP4K4 knockdown (KD) animals IL-1α concentrations were also significantly reduced (Figure 3.15). Furthermore, the KD mice exhibited an increase in PPARγ protein levels, decreased basal glucose levels despite enhanced body weight and adiposity compared to the control animals. Thus the phenotype exhibited by the adipose-specific MAP4K4 knockdown animals appears to be unique.

mTOR is central to growth and cell size regulation. In Chapter II I have demonstrated that MAP4K4 regulated PPARγ protein translation in an mTOR dependent manner. These studies provided clear evidence for mTOR signaling pathway in mediating the effects of MAP4K4. The increase in adipocyte cell size in the KD mice is a clear indicator of elevated mTOR activity. Furthermore, Chakrabarti and colleagues have demonstrated that mTOR promotes fat storage by suppressing lipolysis and stimulating lipogenesis [394]. In accordance with this, WAT triglyceride content in the KD mice was elevated (Figure 3.9A), a fasting induced lipolysis appears to also be decreased, as evidenced by decreased ATGL and phospho-HSL protein levels (Figure 3.16 H). However, it remains to be determined whether MAP4K4 knockdown regulates mTOR activity in the knockdown mouse model.

Adipose-specific S6K1 knockout mouse model demonstrated that, despite elevated glucose and free fatty acid levels upon high fat feeding, the animals remain

insulin sensitive, owing to the absence of the negative feedback to IRS [111]. Since p70S6K1 phosphorylation did not change in the MAP4K4 knockdown condition in 3T3-L1 adipocytes, as shown in Chapter II, silencing MAP4K4 in the adipose tissue could possibly regulate S6K1 similarly. The increase in adipocyte cell size and triglyceride content in MAP4K4 KD mice are indicators of increased mTOR activity as well as improved insulin sensitivity, which could be the reflection of diminished IRS feedback inhibition due to the absence of S6K1 activation in these mice. However, studies need to be performed to address if S6K1 activity is not changed, and whether IRS feedback inhibition is prevented.

Collectively, the observations made in the 3T3-L1 adipocyte cell culture system as well as mouse model provide evidence for an important role played by MAP4K4 in the adipocyte to regulate whole body insulin sensitivity as summarized in the Figure 4. Adipose-specific MAP4K4 gene silencing resulted in increased cell size, protein translation, lipogenesis, decreased lipolysis and cytokine & chemokine synthesis and secretion. It would be naïve to assign a single mechanism or one effector molecule responsible for mediating all the above-mentioned MAP4K4 downstream functions. Since MAP4K4 is a serine/threonine protein kinase kinase kinase kinase, several downstream protein kinases would be involved in mediating the signaling cascade that in turn regulate several target proteins. However, the observations point towards mTORC1 that modulates several adipocyte functions, as the effector molecule responsible for MAP4K4 cellular effects. MAP4K4 functions as a negative regulator of mTORC1 activity and PPARγ protein expression and hence inhibits cell size, lipogenesis and lipid

storage. Furthermore, MAP4K4 could be activating lipolysis, chemokine and cytokine synthesis & secretion through mTOR inhibiton or independently, resulting in elevated macrophage recruitment and/or activation that further increase adipose tissue local as well as systemic pro-inflammatory cytokines levels. Systemic low grade inflammation would lead to decreased hepatic insulin signaling and sensitivity resulting in increased hepatic gluconeogenesis and elevated plasma glucose levels leading to whole body insulin resistance.

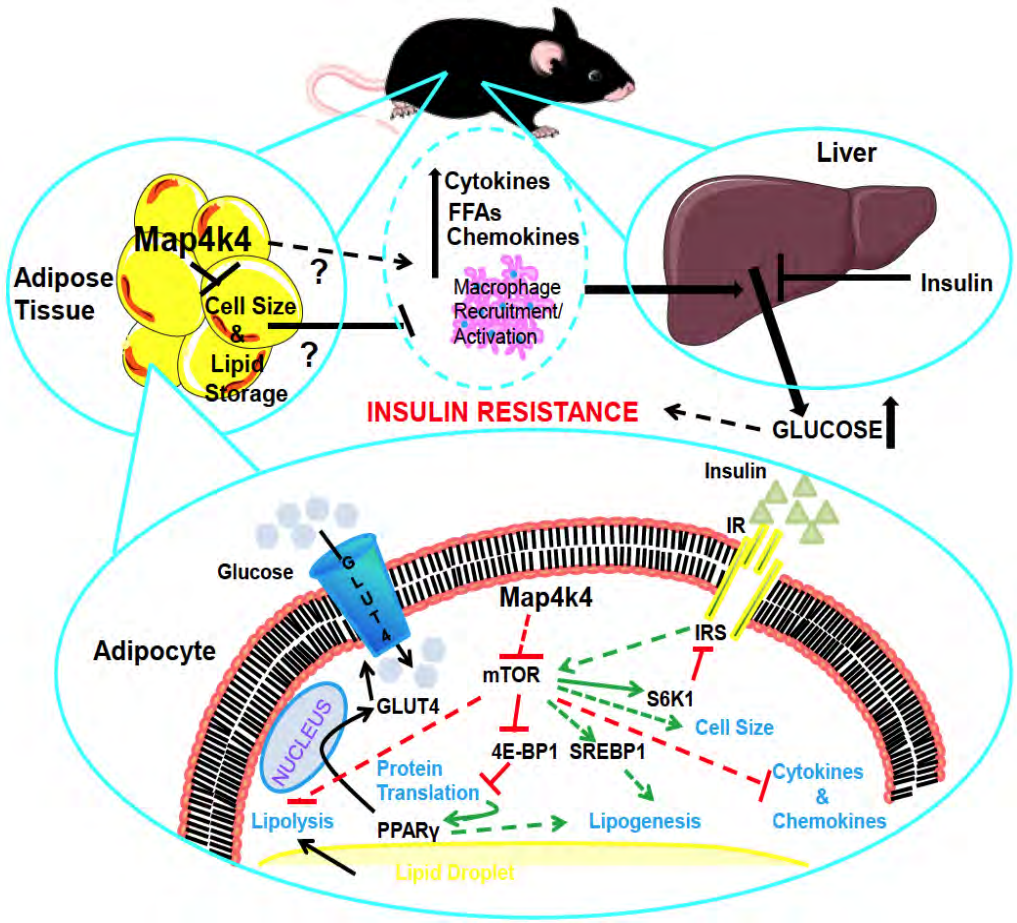


FIGURE 4. Role of adipocyte Map4k4 in inducing insulin resistance – a hypothetical model. At molecular level, Map4k4 functions as a negative regulator of mTORC1. mTOR plays an important role in mediating insulin‘s cellular effects by activating p70S6K1 and inhibiting 4E-BP1 by phosphorylation. Inactivation of 4E-BP1 promotes PPARγ protein synthesis. mTOR also regulates SREBP1 activity and its protein synthesis. Map4k4 inhibits mTOR activity towards 4E-BP1 but promotes p70S6K1 activation, and hence contributes to insulin resistance due to activation of feedback inhibition of IRS. At a cellular level, Map4k4 inhibits mTOR functions of promoting protein translation, lipogenesis, cell size and glucose uptake and inhibition of lipolysis and cytokine & chemokine synthesis. At the level of whole organism, Map4k4 in the adipocyte prevents adipose functions of lipid storage and cell growth while promoting macrophage recruitment and activation due to elevated FFAs and cytokine & chemokine levels that together lead to insulin resistance in the adipose tissue. Inflamed adipose tissue promotes systemic low-grade inflammation that decreases hepatic insulin sensitivity that further contributes to insulin resistance.

Although MAP4K4 silencing improves insulin sensitivity, it also results in adiposity and hence MAP4K4 might not be of interest as a potential therapeutic target of obesity induced insulin resistance, due to aesthetic considerations. However, our mouse model with adipose-specific MAP4K4 gene silencing would be of immense utility and would be a great tool to better understand the differences in insulin sensitivity exhibited by obese individuals, as all obese people are not insulin resistant.

Together, the data presented in Chapter II and Chapter III provides evidence of a definitive role for MAP4K4 in regulation of adipose function. Thus, I have demonstrated that MAP4K4 negatively regulates PPARγ protein translation, adipocyte TAG accumulation and cell size as well as provided evidence implicating adipose tissue MAP4K4 in modulating whole body insulin sensitivity.

References

1. Reaven, G.M., *Banting lecture 1988. Role of insulin resistance in human disease.* Diabetes, 1988. **37**(12): p. 1595-607.
2. Snijder, M.B., et al., *Independent and opposite associations of waist and hip circumferences with diabetes, hypertension and dyslipidemia: the AusDiab Study.* Int J Obes Relat Metab Disord, 2004. **28**(3): p. 402-9.
3. Van Pelt, R.E., et al., *Lower-body adiposity and metabolic protection in postmenopausal women.* J Clin Endocrinol Metab, 2005. **90**(8): p. 4573-8.
4. Snijder, M.B., et al., *Trunk fat and leg fat have independent and opposite associations with fasting and postload glucose levels: the Hoorn study.* Diabetes Care, 2004. **27**(2): p. 372-7.
5. Abella, A., et al., *Cdk4 promotes adipogenesis through PPARgamma activation.* Cell Metab, 2005. **2**(4): p. 239-49.
6. Banerjee, S.S., et al., *The Kruppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-gamma expression and adipogenesis.* J Biol Chem, 2003. **278**(4): p. 2581-4.
7. MacDougald, O.A. and M.D. Lane, *Transcriptional regulation of gene expression during adipocyte differentiation.* Annu Rev Biochem, 1995. **64**: p. 345-73.
8. Darlington, G.J., S.E. Ross, and O.A. MacDougald, *The role of C/EBP genes in adipocyte differentiation.* J Biol Chem, 1998. **273**(46): p. 30057-60.
9. Cao, Z., R.M. Umek, and S.L. McKnight, *Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells.* Genes Dev, 1991. **5**(9): p. 1538-52.
10. Wu, Z., et al., *Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity.* Mol Cell, 1999. **3**(2): p. 151-8.
11. MacDougald, O.A. and S. Mandrup, *Adipogenesis: forces that tip the scales.* Trends Endocrinol Metab, 2002. **13**(1): p. 5-11.
12. Sarruf, D.A., et al., *Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor gamma.* Mol Cell Biol, 2005. **25**(22): p. 9985-95.
13. Fu, M., et al., *Cyclin D1 inhibits peroxisome proliferator-activated receptor gamma-mediated adipogenesis through histone deacetylase recruitment.* J Biol Chem, 2005. **280**(17): p. 16934-41.
14. Mori, T., et al., *Role of Kruppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis.* J Biol Chem, 2005. **280**(13): p. 12867-75.
15. Wu, J., et al., *The KLF2 transcription factor does not affect the formation of preadipocytes but inhibits their differentiation into adipocytes.* Biochemistry, 2005. **44**(33): p. 11098-105.
16. Kanazawa, A., et al., *Single nucleotide polymorphisms in the gene encoding Kruppel-like factor 7 are associated with type 2 diabetes.* Diabetologia, 2005. **48**(7): p. 1315-22.

17. Gray, S., et al., *The Kruppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4.* J Biol Chem, 2002. **277**(37): p. 34322-8.
18. Ge, K., et al., *Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis.* Nature, 2002. **417**(6888): p. 563-7.
19. Qi, C., et al., *Transcriptional coactivator PRIP, the peroxisome proliferator-activated receptor gamma (PPARgamma)-interacting protein, is required for PPARgamma-mediated adipogenesis.* J Biol Chem, 2003. **278**(28): p. 25281-4.
20. Yu, C., et al., *The nuclear receptor corepressors NCoR and SMRT decrease peroxisome proliferator-activated receptor gamma transcriptional activity and repress 3T3-L1 adipogenesis.* J Biol Chem, 2005. **280**(14): p. 13600-5.
21. Zuo, Y., L. Qiang, and S.R. Farmer, *Activation of CCAAT/enhancer-binding protein (C/EBP) alpha expression by C/EBP beta during adipogenesis requires a peroxisome proliferator-activated receptor-gamma-associated repression of HDAC1 at the C/ebp alpha gene promoter.* J Biol Chem, 2006. **281**(12): p. 7960-7.
22. Sakaue, H., et al., *Role of MAPK phosphatase-1 (MKP-1) in adipocyte differentiation.* J Biol Chem, 2004. **279**(38): p. 39951-7.
23. Bost, F., et al., *The extracellular signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis.* Diabetes, 2005. **54**(2): p. 402-11.
24. Engelman, J.A., M.P. Lisanti, and P.E. Scherer, *Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis.* J Biol Chem, 1998. **273**(48): p. 32111-20.
25. Engelman, J.A., et al., *Constitutively active mitogen-activated protein kinase kinase 6 (MKK6) or salicylate induces spontaneous 3T3-L1 adipogenesis.* J Biol Chem, 1999. **274**(50): p. 35630-8.
26. Batchvarova, N., X.Z. Wang, and D. Ron, *Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153).* EMBO J, 1995. **14**(19): p. 4654-61.
27. Ballotti, R., et al., *Activation and regulation of the insulin receptor kinase.* Diabete Metab, 1992. **18**(1 Pt 2): p. 98-103.
28. Saltiel, A.R. and C.R. Kahn, *Insulin signalling and the regulation of glucose and lipid metabolism.* Nature, 2001. **414**(6865): p. 799-806.
29. Lizcano, J.M. and D.R. Alessi, *The insulin signalling pathway.* Curr Biol, 2002. **12**(7): p. R236-8.
30. White, M.F., *IRS proteins and the common path to diabetes.* Am J Physiol Endocrinol Metab, 2002. **283**(3): p. E413-22.
31. Noguchi, T., et al., *Tyrosine phosphorylation of p62(Dok) induced by cell adhesion and insulin: possible role in cell migration.* EMBO J, 1999. **18**(7): p. 1748-60.
32. Kotani, K., P. Wilden, and T.S. Pillay, *SH2-Balpha is an insulin-receptor adapter protein and substrate that interacts with the activation loop of the insulin-receptor kinase.* Biochem J, 1998. **335 (Pt 1)**: p. 103-9.

33. Gual, P., Y. Le Marchand-Brustel, and J.F. Tanti, *Positive and negative regulation of insulin signaling through IRS-1 phosphorylation.* Biochimie, 2005. **87**(1): p. 99-109.
34. Taniguchi, C.M., B. Emanuelli, and C.R. Kahn, *Critical nodes in signalling pathways: insights into insulin action.* Nat Rev Mol Cell Biol, 2006. **7**(2): p. 85-96.
35. van Slegtenhorst, M., et al., *Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products.* Hum Mol Genet, 1998. **7**(6): p. 1053-7.
36. Shepherd, P.R., D.J. Withers, and K. Siddle, *Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling.* Biochem J, 1998. **333 (Pt 3)**: p. 471-90.
37. Rhee, S.G., *Regulation of phosphoinositide-specific phospholipase C.* Annu Rev Biochem, 2001. **70**: p. 281-312.
38. Vanhaesebroeck, B. and D.R. Alessi, *The PI3K-PDK1 connection: more than just a road to PKB.* Biochem J, 2000. **346 Pt 3**: p. 561-76.
39. Alessi, D.R., et al., *Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.* Curr Biol, 1997. **7**(4): p. 261-9.
40. Sarbassov, D.D., et al., *Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex.* Science, 2005. **307**(5712): p. 1098-101.
41. Kumar, A., et al., *Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism.* Diabetes. **59**(6): p. 1397-406.
42. Kumar, A., et al., *Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity.* Mol Cell Biol, 2008. **28**(1): p. 61-70.
43. Sekulic, A., et al., *A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells.* Cancer Res, 2000. **60**(13): p. 3504-13.
44. Skolnik, E.Y., et al., *The function of GRB2 in linking the insulin receptor to Ras signaling pathways.* Science, 1993. **260**(5116): p. 1953-5.
45. Sasaoka, T. and M. Kobayashi, *The functional significance of Shc in insulin signaling as a substrate of the insulin receptor.* Endocr J, 2000. **47**(4): p. 373-81.
46. Tzatsos, A. and K.V. Kandror, *Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation.* Mol Cell Biol, 2006. **26**(1): p. 63-76.
47. Davis, R.J., *Transcriptional regulation by MAP kinases.* Mol Reprod Dev, 1995. **42**(4): p. 459-67.
48. Orton, R.J., et al., *Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway.* Biochem J, 2005. **392**(Pt 2): p. 249-61.
49. Marais, R., J. Wynne, and R. Treisman, *The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain.* Cell, 1993. **73**(2): p. 381-93.
50. Chang, P.Y., et al., *Insulin stimulation of mitogen-activated protein kinase, p90rsk, and p70 S6 kinase in skeletal muscle of normal and insulin-resistant mice.*

Implications for the regulation of glycogen synthase. J Biol Chem, 1995. **270**(50): p. 29928-35.
51. Taniguchi, C.M., et al., *The p85alpha regulatory subunit of phosphoinositide 3-kinase potentiates c-Jun N-terminal kinase-mediated insulin resistance.* Mol Cell Biol, 2007. **27**(8): p. 2830-40.
52. Aubert, J., N. Belmonte, and C. Dani, *Role of pathways for signal transducers and activators of transcription, and mitogen-activated protein kinase in adipocyte differentiation.* Cell Mol Life Sci, 1999. **56**(5-6): p. 538-42.
53. Sabers, C.J., et al., *Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells.* J Biol Chem, 1995. **270**(2): p. 815-22.
54. Sabatini, D.M., et al., *RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs.* Cell, 1994. **78**(1): p. 35-43.
55. Chiu, M.I., H. Katz, and V. Berlin, *RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex.* Proc Natl Acad Sci U S A, 1994. **91**(26): p. 12574-8.
56. Fingar, D.C. and J. Blenis, *Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression.* Oncogene, 2004. **23**(18): p. 3151-71.
57. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR signaling in growth and metabolism.* Cell, 2006. **124**(3): p. 471-84.
58. Huang, S. and P.J. Houghton, *Targeting mTOR signaling for cancer therapy.* Curr Opin Pharmacol, 2003. **3**(4): p. 371-7.
59. Martin, P.M. and A.E. Sutherland, *Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway.* Dev Biol, 2001. **240**(1): p. 182-93.
60. Murakami, M., et al., *mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells.* Mol Cell Biol, 2004. **24**(15): p. 6710-8.
61. Gangloff, Y.G., et al., *Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development.* Mol Cell Biol, 2004. **24**(21): p. 9508-16.
62. Yeh, W.C., B.E. Bierer, and S.L. McKnight, *Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells.* Proc Natl Acad Sci U S A, 1995. **92**(24): p. 11086-90.
63. Gagnon, A., S. Lau, and A. Sorisky, *Rapamycin-sensitive phase of 3T3-L1 preadipocyte differentiation after clonal expansion.* J Cell Physiol, 2001. **189**(1): p. 14-22.
64. Kim, J.E. and J. Chen, *regulation of peroxisome proliferator-activated receptor-gamma activity by mammalian target of rapamycin and amino acids in adipogenesis.* Diabetes, 2004. **53**(11): p. 2748-56.
65. Giraud, J., et al., *Nutrient-dependent and insulin-stimulated phosphorylation of insulin receptor substrate-1 on serine 302 correlates with increased insulin signaling.* J Biol Chem, 2004. **279**(5): p. 3447-54.

66. Loewith, R., et al., *Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control.* Mol Cell, 2002. **10**(3): p. 457-68.
67. Wullschleger, S., et al., *Molecular organization of target of rapamycin complex 2.* J Biol Chem, 2005. **280**(35): p. 30697-704.
68. Hara, K., et al., *Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action.* Cell, 2002. **110**(2): p. 177-89.
69. Kim, D.H., et al., *GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR.* Mol Cell, 2003. **11**(4): p. 895-904.
70. Kim, D.H., et al., *mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery.* Cell, 2002. **110**(2): p. 163-75.
71. Sancak, Y., et al., *PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase.* Mol Cell, 2007. **25**(6): p. 903-15.
72. Sarbassov, D.D., et al., *Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton.* Curr Biol, 2004. **14**(14): p. 1296-302.
73. Jacinto, E., et al., *Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive.* Nat Cell Biol, 2004. **6**(11): p. 1122-8.
74. Jacinto, E., et al., *SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity.* Cell, 2006. **127**(1): p. 125-37.
75. Foster, K.G., et al., *Regulation of mTOR complex 1 (mTORC1) by raptor Ser863 and multisite phosphorylation.* J Biol Chem. **285**(1): p. 80-94.
76. Carriere, A., et al., *ERK1/2 phosphorylate raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1).* J Biol Chem. **286**(1): p. 567-77.
77. Gwinn, D.M., et al., *AMPK phosphorylation of raptor mediates a metabolic checkpoint.* Mol Cell, 2008. **30**(2): p. 214-26.
78. Polak, P., et al., *Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration.* Cell Metab, 2008. **8**(5): p. 399-410.
79. Bentzinger, C.F., et al., *Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy.* Cell Metab, 2008. **8**(5): p. 411-24.
80. Vander Haar, E., et al., *Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40.* Nat Cell Biol, 2007. **9**(3): p. 316-23.
81. Wang, L., et al., *PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding.* J Biol Chem, 2007. **282**(27): p. 20036-44.
82. Kovacina, K.S., et al., *Identification of a proline-rich Akt substrate as a 14-3-3 binding partner.* J Biol Chem, 2003. **278**(12): p. 10189-94.
83. Kandt, R.S., et al., *Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease.* Nat Genet, 1992. **2**(1): p. 37-41.
84. Wienecke, R., A. Konig, and J.E. DeClue, *Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity.* J Biol Chem, 1995. **270**(27): p. 16409-14.

85. Tee, A.R., et al., *Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb.* Curr Biol, 2003. **13**(15): p. 1259-68.
86. Inoki, K., et al., *Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling.* Genes Dev, 2003. **17**(15): p. 1829-34.
87. Zhang, Y., et al., *Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.* Nat Cell Biol, 2003. **5**(6): p. 578-81.
88. Zhang, H.H., et al., *Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway.* PLoS One, 2009. **4**(7): p. e6189.
89. Tee, A.R., R. Anjum, and J. Blenis, *Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin.* J Biol Chem, 2003. **278**(39): p. 37288-96.
90. Roux, P.P., et al., *Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13489-94.
91. Lee, D.F., et al., *IKKbeta suppression of TSC1 function links the mTOR pathway with insulin resistance.* Int J Mol Med, 2008. **22**(5): p. 633-8.
92. Zacharek, S.J., Y. Xiong, and S.D. Shumway, *Negative regulation of TSC1-TSC2 by mammalian D-type cyclins.* Cancer Res, 2005. **65**(24): p. 11354-60.
93. Corradetti, M.N., et al., *Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome.* Genes Dev, 2004. **18**(13): p. 1533-8.
94. Inoki, K., et al., *TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth.* Cell, 2006. **126**(5): p. 955-68.
95. Lee, M.N., et al., *Glycolytic flux signals to mTOR through glyceraldehyde-3-phosphate dehydrogenase-mediated regulation of Rheb.* Mol Cell Biol, 2009. **29**(14): p. 3991-4001.
96. Findlay, G.M., et al., *A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling.* Biochem J, 2007. **403**(1): p. 13-20.
97. Kim, E., *Mechanisms of amino acid sensing in mTOR signaling pathway.* Nutr Res Pract, 2009. **3**(1): p. 64-71.
98. Sancak, Y., et al., *The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1.* Science, 2008. **320**(5882): p. 1496-501.
99. Yonezawa, K., et al., *Kinase activities associated with mTOR.* Curr Top Microbiol Immunol, 2004. **279**: p. 271-82.
100. Acosta-Jaquez, H.A., et al., *Site-specific mTOR phosphorylation promotes mTORC1-mediated signaling and cell growth.* Mol Cell Biol, 2009. **29**(15): p. 4308-24.
101. Chung, J., et al., *Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases.* Cell, 1992. **69**(7): p. 1227-36.

102. Miron, M., P. Lasko, and N. Sonenberg, *Signaling from Akt to FRAP/TOR targets both 4E-BP and S6K in Drosophila melanogaster.* Mol Cell Biol, 2003. **23**(24): p. 9117-26.
103. von Manteuffel, S.R., et al., *The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70s6k.* Mol Cell Biol, 1997. **17**(9): p. 5426-36.
104. Gingras, A.C., B. Raught, and N. Sonenberg, *Regulation of translation initiation by FRAP/mTOR.* Genes Dev, 2001. **15**(7): p. 807-26.
105. Dufner, A. and G. Thomas, *Ribosomal S6 kinase signaling and the control of translation.* Exp Cell Res, 1999. **253**(1): p. 100-9.
106. Hannan, K.M., G. Thomas, and R.B. Pearson, *Activation of S6K1 (p70 ribosomal protein S6 kinase 1) requires an initial calcium-dependent priming event involving formation of a high-molecular-mass signalling complex.* Biochem J, 2003. **370**(Pt 2): p. 469-77.
107. Keshwani, M.M., X. Gao, and T.K. Harris, *Mechanism of PDK1-catalyzed Thr-229 phosphorylation of the S6K1 protein kinase.* J Biol Chem, 2009. **284**(34): p. 22611-24.
108. Romanelli, A., V.C. Dreisbach, and J. Blenis, *Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1.* J Biol Chem, 2002. **277**(43): p. 40281-9.
109. Saitoh, M., et al., *Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site.* J Biol Chem, 2002. **277**(22): p. 20104-12.
110. Selman, C., et al., *Ribosomal protein S6 kinase 1 signaling regulates mammalian life span.* Science, 2009. **326**(5949): p. 140-4.
111. Um, S.H., et al., *Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity.* Nature, 2004. **431**(7005): p. 200-5.
112. Jefferies, H.B., et al., *Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k.* EMBO J, 1997. **16**(12): p. 3693-704.
113. Beretta, L., et al., *Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation.* EMBO J, 1996. **15**(3): p. 658-64.
114. Nojima, H., et al., *The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif.* J Biol Chem, 2003. **278**(18): p. 15461-4.
115. Beugnet, A., X. Wang, and C.G. Proud, *Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1.* J Biol Chem, 2003. **278**(42): p. 40717-22.
116. Gingras, A.C., et al., *Hierarchical phosphorylation of the translation inhibitor 4E-BP1.* Genes Dev, 2001. **15**(21): p. 2852-64.
117. Wang, X., et al., *Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins.* Mol Cell Biol, 2005. **25**(7): p. 2558-72.

118. Lee, V.H., et al., *Analysis of the regulatory motifs in eukaryotic initiation factor 4E-binding protein 1.* FEBS J, 2008. **275**(9): p. 2185-99.
119. Gingras, A.C., et al., *Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism.* Genes Dev, 1999. **13**(11): p. 1422-37.
120. Tsukiyama-Kohara, K., et al., *Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1.* Nat Med, 2001. **7**(10): p. 1128-32.
121. Le Bacquer, O., et al., *Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2.* J Clin Invest, 2007. **117**(2): p. 387-96.
122. Lorenz, M.C. and J. Heitman, *TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin.* J Biol Chem, 1995. **270**(46): p. 27531-7.
123. Fingar, D.C., et al., *Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E.* Genes Dev, 2002. **16**(12): p. 1472-87.
124. Wang, X. and C.G. Proud, *The mTOR pathway in the control of protein synthesis.* Physiology (Bethesda), 2006. **21**: p. 362-9.
125. Pende, M., et al., *S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway.* Mol Cell Biol, 2004. **24**(8): p. 3112-24.
126. Ruvinsky, I., et al., *Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis.* Genes Dev, 2005. **19**(18): p. 2199-211.
127. Wang, L. and C.G. Proud, *Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes.* Circ Res, 2002. **91**(9): p. 821-9.
128. Wang, L., X. Wang, and C.G. Proud, *Activation of mRNA translation in rat cardiac myocytes by insulin involves multiple rapamycin-sensitive steps.* Am J Physiol Heart Circ Physiol, 2000. **278**(4): p. H1056-68.
129. Bell, G.I., et al., *Molecular biology of mammalian glucose transporters.* Diabetes Care, 1990. **13**(3): p. 198-208.
130. Iozzo, P., et al., *Insulin stimulates liver glucose uptake in humans: an 18F-FDG PET Study.* J Nucl Med, 2003. **44**(5): p. 682-9.
131. Katz, E.B., et al., *Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4.* Nature, 1995. **377**(6545): p. 151-5.
132. Zisman, A., et al., *Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance.* Nat Med, 2000. **6**(8): p. 924-8.
133. Kim, J.K., et al., *Glucose toxicity and the development of diabetes in mice with muscle-specific inactivation of GLUT4.* J Clin Invest, 2001. **108**(1): p. 153-60.
134. Stenbit, A.E., et al., *GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes.* Nat Med, 1997. **3**(10): p. 1096-101.

135. Tsao, T.S., et al., *Prevention of insulin resistance and diabetes in mice heterozygous for GLUT4 ablation by transgenic complementation of GLUT4 in skeletal muscle.* Diabetes, 1999. **48**(4): p. 775-82.
136. Bryant, N.J., R. Govers, and D.E. James, *Regulated transport of the glucose transporter GLUT4.* Nat Rev Mol Cell Biol, 2002. **3**(4): p. 267-77.
137. Cheatham, B., et al., *Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation.* Mol Cell Biol, 1994. **14**(7): p. 4902-11.
138. Kane, S., et al., *A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain.* J Biol Chem, 2002. **277**(25): p. 22115-8.
139. Zerial, M. and H. McBride, *Rab proteins as membrane organizers.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 107-17.
140. Eguez, L., et al., *Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein.* Cell Metab, 2005. **2**(4): p. 263-72.
141. Larance, M., et al., *Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking.* J Biol Chem, 2005. **280**(45): p. 37803-13.
142. Gonzalez, E. and T.E. McGraw, *Insulin signaling diverges into Akt-dependent and -independent signals to regulate the recruitment/docking and the fusion of GLUT4 vesicles to the plasma membrane.* Mol Biol Cell, 2006. **17**(10): p. 4484-93.
143. Chen, S., et al., *Mice with AS160/TBC1D4-Thr649Ala knockin mutation are glucose intolerant with reduced insulin sensitivity and altered GLUT4 trafficking.* Cell Metab. **13**(1): p. 68-79.
144. Zhou, Q.L., et al., *A novel pleckstrin homology domain-containing protein enhances insulin-stimulated Akt phosphorylation and GLUT4 translocation in adipocytes.* J Biol Chem. **285**(36): p. 27581-9.
145. Fujishiro, M., et al., *MKK6/3 and p38 MAPK pathway activation is not necessary for insulin-induced glucose uptake but regulates glucose transporter expression.* J Biol Chem, 2001. **276**(23): p. 19800-6.
146. Henriksen, E.J., *Dysregulation of glycogen synthase kinase-3 in skeletal muscle and the etiology of insulin resistance and type 2 diabetes.* Curr Diabetes Rev. **6**(5): p. 285-93.
147. Eldar-Finkelman, H., et al., *Increased glycogen synthase kinase-3 activity in diabetes- and obesity-prone C57BL/6J mice.* Diabetes, 1999. **48**(8): p. 1662-6.
148. MacAulay, K., et al., *Glycogen synthase kinase 3alpha-specific regulation of murine hepatic glycogen metabolism.* Cell Metab, 2007. **6**(4): p. 329-37.
149. de Figueiredo, L.F., et al., *Can sugars be produced from fatty acids? A test case for pathway analysis tools.* Bioinformatics, 2009. **25**(1): p. 152-8.
150. Kondrashov, F.A., et al., *Evolution of glyoxylate cycle enzymes in Metazoa: evidence of multiple horizontal transfer events and pseudogene formation.* Biol Direct, 2006. **1**: p. 31.

151. Granner, D., et al., *Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin.* Nature, 1983. **305**(5934): p. 549-51.
152. Dickens, M., et al., *Central role for phosphatidylinositide 3-kinase in the repression of glucose-6-phosphatase gene transcription by insulin.* J Biol Chem, 1998. **273**(32): p. 20144-9.
153. Streeper, R.S., et al., *A multicomponent insulin response sequence mediates a strong repression of mouse glucose-6-phosphatase gene transcription by insulin.* J Biol Chem, 1997. **272**(18): p. 11698-701.
154. Lange, A.J., et al., *Isolation of a cDNA for the catalytic subunit of rat liver glucose-6-phosphatase: regulation of gene expression in FAO hepatoma cells by insulin, dexamethasone and cAMP.* Biochem Biophys Res Commun, 1994. **201**(1): p. 302-9.
155. Granner, D.K., K. Sasaki, and D. Chu, *Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. The dominant role of insulin.* Ann N Y Acad Sci, 1986. **478**: p. 175-90.
156. Schmoll, D., et al., *Identification of a cAMP response element within the glucose-6-phosphatase hydrolytic subunit gene promoter which is involved in the transcriptional regulation by cAMP and glucocorticoids in H4IIE hepatoma cells.* Biochem J, 1999. **338 (Pt 2)**: p. 457-63.
157. Lamers, W.H., R.W. Hanson, and H.M. Meisner, *cAMP stimulates transcription of the gene for cytosolic phosphoenolpyruvate carboxykinase in rat liver nuclei.* Proc Natl Acad Sci U S A, 1982. **79**(17): p. 5137-41.
158. Agati, J.M., D. Yeagley, and P.G. Quinn, *Assessment of the roles of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, protein kinase B, and protein kinase C in insulin inhibition of cAMP-induced phosphoenolpyruvate carboxykinase gene transcription.* J Biol Chem, 1998. **273**(30): p. 18751-9.
159. Schmoll, D., et al., *Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity.* J Biol Chem, 2000. **275**(46): p. 36324-33.
160. Sutherland, C., R.M. O'Brien, and D.K. Granner, *Phosphatidylinositol 3-kinase, but not p70/p85 ribosomal S6 protein kinase, is required for the regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by insulin. Dissociation of signaling pathways for insulin and phorbol ester regulation of PEPCK gene expression.* J Biol Chem, 1995. **270**(26): p. 15501-6.
161. Miyake, K., et al., *Hyperinsulinemia, glucose intolerance, and dyslipidemia induced by acute inhibition of phosphoinositide 3-kinase signaling in the liver.* J Clin Invest, 2002. **110**(10): p. 1483-91.
162. Liao, J., et al., *Activation of protein kinase B/Akt is sufficient to repress the glucocorticoid and cAMP induction of phosphoenolpyruvate carboxykinase gene.* J Biol Chem, 1998. **273**(42): p. 27320-4.
163. Ogg, S., et al., *The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans.* Nature, 1997. **389**(6654): p. 994-9.

164. Barthel, A., et al., *Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells.* Biochem Biophys Res Commun, 2001. **285**(4): p. 897-902.
165. Biggs, W.H., 3rd, et al., *Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1.* Proc Natl Acad Sci U S A, 1999. **96**(13): p. 7421-6.
166. Brunet, A., et al., *Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.* Cell, 1999. **96**(6): p. 857-68.
167. Guo, S., et al., *Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPbeta.* J Biol Chem, 2001. **276**(11): p. 8516-23.
168. Kops, G.J., et al., *Direct control of the Forkhead transcription factor AFX by protein kinase B.* Nature, 1999. **398**(6728): p. 630-4.
169. Rena, G., et al., *Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B.* J Biol Chem, 1999. **274**(24): p. 17179-83.
170. Takaishi, H., et al., *Regulation of nuclear translocation of forkhead transcription factor AFX by protein kinase B.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 11836-41.
171. Nakae, J., et al., *The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression.* J Clin Invest, 2001. **108**(9): p. 1359-67.
172. Nakae, J., et al., *Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1.* Nat Genet, 2002. **32**(2): p. 245-53.
173. Connaughton, S., et al., *Regulation of pyruvate dehydrogenase kinase isoform 4 (PDK4) gene expression by glucocorticoids and insulin.* Mol Cell Endocrinol. **315**(1-2): p. 159-67.
174. Lochhead, P.A., et al., *5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase.* Diabetes, 2000. **49**(6): p. 896-903.
175. Park, M.J., et al., *Transcriptional repression of the gluconeogenic gene PEPCK by the orphan nuclear receptor SHP through inhibitory interaction with C/EBPalpha.* Biochem J, 2007. **402**(3): p. 567-74.
176. Kim, Y.D., et al., *Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP.* Diabetes, 2008. **57**(2): p. 306-14.
177. Musi, N., et al., *Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes.* Diabetes, 2002. **51**(7): p. 2074-81.
178. Sul, H.S., et al., *Regulation of the fatty acid synthase promoter by insulin.* J Nutr, 2000. **130**(2S Suppl): p. 315S-320S.

179. Shimano, H., et al., *Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes.* J Biol Chem, 1999. **274**(50): p. 35832-9.
180. Shimomura, I., et al., *Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy.* Genes Dev, 1998. **12**(20): p. 3182-94.
181. Yamamoto, T., et al., *Protein kinase Cbeta mediates hepatic induction of sterol-regulatory element binding protein-1c by insulin.* J Lipid Res. **51**(7): p. 1859-70.
182. Yahagi, N., et al., *A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids.* J Biol Chem, 1999. **274**(50): p. 35840-4.
183. Xu, J., et al., *Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats.* J Biol Chem, 1999. **274**(33): p. 23577-83.
184. Mater, M.K., et al., *Sterol response element-binding protein 1c (SREBP1c) is involved in the polyunsaturated fatty acid suppression of hepatic S14 gene transcription.* J Biol Chem, 1999. **274**(46): p. 32725-32.
185. Kim, H.J., M. Takahashi, and O. Ezaki, *Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs.* J Biol Chem, 1999. **274**(36): p. 25892-8.
186. Matsumoto, M., et al., *Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism.* J Clin Invest, 2006. **116**(9): p. 2464-72.
187. Kersten, S., et al., *Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting.* J Clin Invest, 1999. **103**(11): p. 1489-98.
188. Yoshikawa, T., et al., *Cross-talk between peroxisome proliferator-activated receptor (PPAR) alpha and liver X receptor (LXR) in nutritional regulation of fatty acid metabolism. I. PPARs suppress sterol regulatory element binding protein-1c promoter through inhibition of LXR signaling.* Mol Endocrinol, 2003. **17**(7): p. 1240-54.
189. Li, S., M.S. Brown, and J.L. Goldstein, *Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis.* Proc Natl Acad Sci U S A. **107**(8): p. 3441-6.
190. Kalderon, B., et al., *Fatty acid cycling in the fasting rat.* Am J Physiol Endocrinol Metab, 2000. **279**(1): p. E221-7.
191. Slavin, B.G., J.M. Ong, and P.A. Kern, *Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes.* J Lipid Res, 1994. **35**(9): p. 1535-41.
192. Londos, C., et al., *Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells.* Semin Cell Dev Biol, 1999. **10**(1): p. 51-8.

193. Schwartz, J.P. and R.L. Jungas, *Studies on the hormone-sensitive lipase of adipose tissue.* J Lipid Res, 1971. **12**(5): p. 553-62.
194. Haemmerle, G., et al., *Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis.* J Biol Chem, 2002. **277**(7): p. 4806-15.
195. Wang, S.P., et al., *The adipose tissue phenotype of hormone-sensitive lipase deficiency in mice.* Obes Res, 2001. **9**(2): p. 119-28.
196. Voshol, P.J., et al., *Increased hepatic insulin sensitivity together with decreased hepatic triglyceride stores in hormone-sensitive lipase-deficient mice.* Endocrinology, 2003. **144**(8): p. 3456-62.
197. Haemmerle, G., et al., *Hormone-sensitive lipase deficiency in mice changes the plasma lipid profile by affecting the tissue-specific expression pattern of lipoprotein lipase in adipose tissue and muscle.* J Biol Chem, 2002. **277**(15): p. 12946-52.
198. Fredrikson, G., H. Tornqvist, and P. Belfrage, *Hormone-sensitive lipase and monoacylglycerol lipase are both required for complete degradation of adipocyte triacylglycerol.* Biochim Biophys Acta, 1986. **876**(2): p. 288-93.
199. Villena, J.A., et al., *Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis.* J Biol Chem, 2004. **279**(45): p. 47066-75.
200. Ahmadian, M., et al., *Adipose overexpression of desnutrin promotes fatty acid use and attenuates diet-induced obesity.* Diabetes, 2009. **58**(4): p. 855-66.
201. Tansey, J.T., et al., *Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6494-9.
202. Souza, S.C., et al., *Overexpression of perilipin A and B blocks the ability of tumor necrosis factor alpha to increase lipolysis in 3T3-L1 adipocytes.* J Biol Chem, 1998. **273**(38): p. 24665-9.
203. Miyoshi, H., et al., *Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms.* J Biol Chem, 2006. **281**(23): p. 15837-44.
204. Holm, C., et al., *Molecular mechanisms regulating hormone-sensitive lipase and lipolysis.* Annu Rev Nutr, 2000. **20**: p. 365-93.
205. Hibuse, T., et al., *Aquaporin 7 deficiency is associated with development of obesity through activation of adipose glycerol kinase.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 10993-8.
206. Guan, H.P., et al., *Corepressors selectively control the transcriptional activity of PPARgamma in adipocytes.* Genes Dev, 2005. **19**(4): p. 453-61.
207. Mazzucotelli, A., et al., *The transcriptional coactivator peroxisome proliferator activated receptor (PPAR)gamma coactivator-1 alpha and the nuclear receptor PPAR alpha control the expression of glycerol kinase and metabolism genes independently of PPAR gamma activation in human white adipocytes.* Diabetes, 2007. **56**(10): p. 2467-75.

208. Shakur, Y., et al., *Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family.* Prog Nucleic Acid Res Mol Biol, 2001. **66**: p. 241-77.
209. Guilherme, A., et al., *Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 367-77.
210. Kahn, B.B. and J.S. Flier, *Obesity and insulin resistance.* J Clin Invest, 2000. **106**(4): p. 473-81.
211. Boura-Halfon, S. and Y. Zick, *Phosphorylation of IRS proteins, insulin action, and insulin resistance.* Am J Physiol Endocrinol Metab, 2009. **296**(4): p. E581-91.
212. Gao, Z., et al., *Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes.* Mol Endocrinol, 2004. **18**(8): p. 2024-34.
213. Morino, K., et al., *Muscle-specific IRS-1 Ser->Ala transgenic mice are protected from fat-induced insulin resistance in skeletal muscle.* Diabetes, 2008. **57**(10): p. 2644-51.
214. Copps, K.D., et al., *Irs1 serine 307 promotes insulin sensitivity in mice.* Cell Metab, 2010. **11**(1): p. 84-92.
215. Tremblay, F., et al., *Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance.* Proc Natl Acad Sci U S A, 2007. **104**(35): p. 14056-61.
216. Zhang, J., et al., *S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2.* J Biol Chem, 2008. **283**(51): p. 35375-82.
217. Hu, E., P. Liang, and B.M. Spiegelman, *AdipoQ is a novel adipose-specific gene dysregulated in obesity.* J Biol Chem, 1996. **271**(18): p. 10697-703.
218. Shapiro, L. and P.E. Scherer, *The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor.* Curr Biol, 1998. **8**(6): p. 335-8.
219. Richards, A.A., et al., *Adiponectin multimerization is dependent on conserved lysines in the collagenous domain: evidence for regulation of multimerization by alterations in posttranslational modifications.* Mol Endocrinol, 2006. **20**(7): p. 1673-87.
220. Ouchi, N., et al., *Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin.* Circulation, 1999. **100**(25): p. 2473-6.
221. Matsubara, M., S. Maruoka, and S. Katayose, *Inverse relationship between plasma adiponectin and leptin concentrations in normal-weight and obese women.* Eur J Endocrinol, 2002. **147**(2): p. 173-80.
222. Jurimae, J., et al., *Plasma adiponectin and insulin sensitivity in overweight and normal-weight middle-aged premenopausal women.* Metabolism, 2009. **58**(5): p. 638-43.
223. Yamauchi, T., et al., *The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity.* Nat Med, 2001. **7**(8): p. 941-6.

224. Nawrocki, A.R., et al., *Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists.* J Biol Chem, 2006. **281**(5): p. 2654-60.
225. Wu, X., et al., *Adiponectin suppresses IkappaB kinase activation induced by tumor necrosis factor-alpha or high glucose in endothelial cells: role of cAMP and AMP kinase signaling.* Am J Physiol Endocrinol Metab, 2007. **293**(6): p. E1836-44.
226. Sun, Y. and H.F. Lodish, *Adiponectin deficiency promotes tumor growth in mice by reducing macrophage infiltration.* PLoS One, 2010. **5**(8): p. e11987.
227. Kadowaki, T. and T. Yamauchi, *Adiponectin and adiponectin receptors.* Endocr Rev, 2005. **26**(3): p. 439-51.
228. Harvey, J. and M.L. Ashford, *Leptin in the CNS: much more than a satiety signal.* Neuropharmacology, 2003. **44**(7): p. 845-54.
229. Friedman, J.M. and J.L. Halaas, *Leptin and the regulation of body weight in mammals.* Nature, 1998. **395**(6704): p. 763-70.
230. Elmquist, J.K., C.F. Elias, and C.B. Saper, *From lesions to leptin: hypothalamic control of food intake and body weight.* Neuron, 1999. **22**(2): p. 221-32.
231. Myers, D.D., et al., *Assessing the risk of transmission of three infectious agents among mice housed in a negatively pressurized caging system.* Contemp Top Lab Anim Sci, 2003. **42**(6): p. 16-21.
232. Ronti, T., G. Lupattelli, and E. Mannarino, *The endocrine function of adipose tissue: an update.* Clin Endocrinol (Oxf), 2006. **64**(4): p. 355-65.
233. Seufert, J., *Leptin effects on pancreatic beta-cell gene expression and function.* Diabetes, 2004. **53 Suppl 1**: p. S152-8.
234. Prodi, E. and S. Obici, *Minireview: the brain as a molecular target for diabetic therapy.* Endocrinology, 2006. **147**(6): p. 2664-9.
235. Flier, J.S., *Obesity wars: molecular progress confronts an expanding epidemic.* Cell, 2004. **116**(2): p. 337-50.
236. Munzberg, H. and M.G. Myers, Jr., *Molecular and anatomical determinants of central leptin resistance.* Nat Neurosci, 2005. **8**(5): p. 566-70.
237. El-Haschimi, K., et al., *Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity.* J Clin Invest, 2000. **105**(12): p. 1827-32.
238. Bates, S.H., et al., *Roles for leptin receptor/STAT3-dependent and -independent signals in the regulation of glucose homeostasis.* Cell Metab, 2005. **1**(3): p. 169-78.
239. Kahn, B.B., et al., *AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism.* Cell Metab, 2005. **1**(1): p. 15-25.
240. Minokoshi, Y., et al., *Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase.* Nature, 2002. **415**(6869): p. 339-43.
241. Unger, R.H., *Lipotoxic diseases.* Annu Rev Med, 2002. **53**: p. 319-36.
242. Kim, J.K., et al., *PKC-theta knockout mice are protected from fat-induced insulin resistance.* J Clin Invest, 2004. **114**(6): p. 823-7.
243. Boden, G. and X. Chen, *Effects of fat on glucose uptake and utilization in patients with non-insulin-dependent diabetes.* J Clin Invest, 1995. **96**(3): p. 1261-8.

244. Samuel, V.T., et al., *Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease.* J Biol Chem, 2004. **279**(31): p. 32345-53.
245. Samuel, V.T., et al., *Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease.* J Clin Invest, 2007. **117**(3): p. 739-45.
246. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.
247. Ferrante, A.W., Jr., *Obesity-induced inflammation: a metabolic dialogue in the language of inflammation.* J Intern Med, 2007. **262**(4): p. 408-14.
248. Sartipy, P. and D.J. Loskutoff, *Monocyte chemoattractant protein 1 in obesity and insulin resistance.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 7265-70.
249. Kanda, H., et al., *MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity.* J Clin Invest, 2006. **116**(6): p. 1494-505.
250. Inouye, K.E., et al., *Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue.* Diabetes, 2007. **56**(9): p. 2242-50.
251. Spalding, K.L., et al., *Dynamics of fat cell turnover in humans.* Nature, 2008. **453**(7196): p. 783-7.
252. Marin, P., et al., *The morphology and metabolism of intraabdominal adipose tissue in men.* Metabolism, 1992. **41**(11): p. 1242-8.
253. Hoffstedt, J., et al., *Variation in adrenergic regulation of lipolysis between omental and subcutaneous adipocytes from obese and non-obese men.* J Lipid Res, 1997. **38**(4): p. 795-804.
254. Kosteli, A., et al., *Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue.* J Clin Invest, 2010. **120**(10): p. 3466-79.
255. Lumeng, C.N., J.L. Bodzin, and A.R. Saltiel, *Obesity induces a phenotypic switch in adipose tissue macrophage polarization.* J Clin Invest, 2007. **117**(1): p. 175-84.
256. Odegaard, J.I., et al., *Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance.* Nature, 2007. **447**(7148): p. 1116-20.
257. Peraldi, P. and B. Spiegelman, *TNF-alpha and insulin resistance: summary and future prospects.* Mol Cell Biochem, 1998. **182**(1-2): p. 169-75.
258. Mohler, K.M., et al., *Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing.* Nature, 1994. **370**(6486): p. 218-20.
259. Guo, D. and D.B. Donner, *Tumor necrosis factor promotes phosphorylation and binding of insulin receptor substrate 1 to phosphatidylinositol 3-kinase in 3T3-L1 adipocytes.* J Biol Chem, 1996. **271**(2): p. 615-8.
260. Ozes, O.N., et al., *NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase.* Nature, 1999. **401**(6748): p. 82-5.
261. Gustin, J.A., et al., *The PTEN tumor suppressor protein inhibits tumor necrosis factor-induced nuclear factor kappa B activity.* J Biol Chem, 2001. **276**(29): p. 27740-4.
262. Ozes, O.N., et al., *A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling*

through insulin receptor substrate-1. Proc Natl Acad Sci U S A, 2001. **98**(8): p. 4640-5.
263. Gonzalez-Rodriguez, A., et al., *S6K1 deficiency protects against apoptosis in hepatocytes.* Hepatology, 2009. **50**(1): p. 216-29.
264. Kim, Y.L., et al., *GbetaL regulates TNFalpha-induced NF-kappaB signaling by directly inhibiting the activation of IkappaB kinase.* Cell Signal, 2008. **20**(11): p. 2127-33.
265. You, D.J., et al., *Regulation of IkappaB kinase by GbetaL through recruitment of the protein phosphatases.* Mol Cells, 2010.
266. Yuan, M., et al., *Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta.* Science, 2001. **293**(5535): p. 1673-7.
267. Lin, X., et al., *Protein kinase C-theta participates in NF-kappaB activation induced by CD3-CD28 costimulation through selective activation of IkappaB kinase beta.* Mol Cell Biol, 2000. **20**(8): p. 2933-40.
268. Arkan, M.C., et al., *IKK-beta links inflammation to obesity-induced insulin resistance.* Nat Med, 2005. **11**(2): p. 191-8.
269. Hotamisligil, G.S., et al., *Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance.* J Clin Invest, 1995. **95**(5): p. 2409-15.
270. Tsigos, C., et al., *Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral obesity.* Metabolism, 1999. **48**(10): p. 1332-5.
271. Kern, P.A., et al., *Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance.* Am J Physiol Endocrinol Metab, 2001. **280**(5): p. E745-51.
272. Koistinen, H.A., et al., *Subcutaneous adipose tissue expression of tumour necrosis factor-alpha is not associated with whole body insulin resistance in obese nondiabetic or in type-2 diabetic subjects.* Eur J Clin Invest, 2000. **30**(4): p. 302-10.
273. Hotamisligil, G.S., et al., *Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha.* J Clin Invest, 1994. **94**(4): p. 1543-9.
274. Uysal, K.T., et al., *Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function.* Nature, 1997. **389**(6651): p. 610-4.
275. Ventre, J., et al., *Targeted disruption of the tumor necrosis factor-alpha gene: metabolic consequences in obese and nonobese mice.* Diabetes, 1997. **46**(9): p. 1526-31.
276. Ofei, F., et al., *Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM.* Diabetes, 1996. **45**(7): p. 881-5.
277. Paquot, N., et al., *No increased insulin sensitivity after a single intravenous administration of a recombinant human tumor necrosis factor receptor: Fc fusion protein in obese insulin-resistant patients.* J Clin Endocrinol Metab, 2000. **85**(3): p. 1316-9.

278. Kiortsis, D.N., et al., *Effects of infliximab treatment on insulin resistance in patients with rheumatoid arthritis and ankylosing spondylitis.* Ann Rheum Dis, 2005. **64**(5): p. 765-6.
279. Yazdani-Biuki, B., et al., *Relapse of diabetes after interruption of chronic administration of anti-tumor necrosis factor-alpha antibody infliximab: a case observation.* Diabetes Care, 2006. **29**(7): p. 1712-3.
280. Borst, S.E. and G.J. Bagby, *Neutralization of tumor necrosis factor reverses age-induced impairment of insulin responsiveness in skeletal muscle of Sprague-Dawley rats.* Metabolism, 2002. **51**(8): p. 1061-4.
281. Borst, S.E., et al., *Neutralization of tumor necrosis factor-alpha reverses insulin resistance in skeletal muscle but not adipose tissue.* Am J Physiol Endocrinol Metab, 2004. **287**(5): p. E934-8.
282. Saghizadeh, M., et al., *The expression of TNF alpha by human muscle. Relationship to insulin resistance.* J Clin Invest, 1996. **97**(4): p. 1111-6.
283. Eferl, R. and E.F. Wagner, *AP-1: a double-edged sword in tumorigenesis.* Nat Rev Cancer, 2003. **3**(11): p. 859-68.
284. White, U.A. and J.M. Stephens, *Transcriptional factors that promote formation of white adipose tissue.* Mol Cell Endocrinol, 2010. **318**(1-2): p. 10-4.
285. Culbert, A.A. and J.M. Tavare, *Multiple signalling pathways mediate insulin-stimulated gene expression in 3T3-L1 adipocytes.* Biochim Biophys Acta, 2002. **1578**(1-3): p. 43-50.
286. Sabatakos, G., et al., *Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis.* Nat Med, 2000. **6**(9): p. 985-90.
287. Rowe, G.C., et al., *Increased energy expenditure and insulin sensitivity in the high bone mass DeltaFosB transgenic mice.* Endocrinology, 2009. **150**(1): p. 135-43.
288. Shi, H., et al., *TLR4 links innate immunity and fatty acid-induced insulin resistance.* J Clin Invest, 2006. **116**(11): p. 3015-25.
289. Li, G., et al., *Tumor necrosis factor-alpha induces insulin resistance in endothelial cells via a p38 mitogen-activated protein kinase-dependent pathway.* Endocrinology, 2007. **148**(7): p. 3356-63.
290. Gao, D., et al., *The effects of palmitate on hepatic insulin resistance are mediated by NADPH Oxidase 3-derived reactive oxygen species through JNK and p38MAPK pathways.* J Biol Chem, 2010. **285**(39): p. 29965-73.
291. Ruotsalainen, E., et al., *Changes in inflammatory cytokines are related to impaired glucose tolerance in offspring of type 2 diabetic subjects.* Diabetes Care, 2006. **29**(12): p. 2714-20.
292. Jager, J., et al., *Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression.* Endocrinology, 2007. **148**(1): p. 241-51.
293. Lagathu, C., et al., *Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes.* Diabetologia, 2006. **49**(9): p. 2162-73.

294. He, J., et al., *Interleukin-1alpha inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3-L1 adipocytes.* Mol Endocrinol, 2006. **20**(1): p. 114-24.
295. Garcia, M.C., et al., *Mature-onset obesity in interleukin-1 receptor I knockout mice.* Diabetes, 2006. **55**(5): p. 1205-13.
296. Somm, E., et al., *Decreased fat mass in interleukin-1 receptor antagonist-deficient mice: impact on adipogenesis, food intake, and energy expenditure.* Diabetes, 2005. **54**(12): p. 3503-9.
297. Matsuki, T., et al., *IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions.* J Exp Med, 2003. **198**(6): p. 877-88.
298. Kersten, S., B. Desvergne, and W. Wahli, *Roles of PPARs in health and disease.* Nature, 2000. **405**(6785): p. 421-4.
299. He, W., et al., *Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15712-7.
300. Hevener, A.L., et al., *Muscle-specific Pparg deletion causes insulin resistance.* Nat Med, 2003. **9**(12): p. 1491-7.
301. Ruan, H., et al., *Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory.* Diabetes, 2002. **51**(5): p. 1319-36.
302. Kudo, M., et al., *Transcription suppression of peroxisome proliferator-activated receptor gamma2 gene expression by tumor necrosis factor alpha via an inhibition of CCAAT/ enhancer-binding protein delta during the early stage of adipocyte differentiation.* Endocrinology, 2004. **145**(11): p. 4948-56.
303. Hu, E., et al., *Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma.* Science, 1996. **274**(5295): p. 2100-3.
304. Adams, M., et al., *Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site.* J Biol Chem, 1997. **272**(8): p. 5128-32.
305. Camp, H.S. and S.R. Tafuri, *Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase.* J Biol Chem, 1997. **272**(16): p. 10811-6.
306. Camp, H.S., S.R. Tafuri, and T. Leff, *c-Jun N-terminal kinase phosphorylates peroxisome proliferator-activated receptor-gamma1 and negatively regulates its transcriptional activity.* Endocrinology, 1999. **140**(1): p. 392-7.
307. Hosooka, T., et al., *Dok1 mediates high-fat diet-induced adipocyte hypertrophy and obesity through modulation of PPAR-gamma phosphorylation.* Nat Med, 2008. **14**(2): p. 188-93.
308. Guilherme, A., et al., *Tumor necrosis factor-alpha induces caspase-mediated cleavage of peroxisome proliferator-activated receptor gamma in adipocytes.* J Biol Chem, 2009. **284**(25): p. 17082-91.
309. Leff, T., *AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 224-7.

310. Dan, I., N.M. Watanabe, and A. Kusumi, *The Ste20 group kinases as regulators of MAP kinase cascades.* Trends Cell Biol, 2001. **11**(5): p. 220-30.
311. Lehmann, J.M., G. Riethmuller, and J.P. Johnson, *Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the src homology units SH2 and SH3.* Nucleic Acids Res, 1990. **18**(4): p. 1048.
312. Su, Y.C., et al., *NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain.* EMBO J, 1997. **16**(6): p. 1279-90.
313. Austin, R.L., et al., *siRNA-mediated reduction of inhibitor of nuclear factor-kappaB kinase prevents tumor necrosis factor-alpha-induced insulin resistance in human skeletal muscle.* Diabetes, 2008. **57**(8): p. 2066-73.
314. Machida, N., et al., *Mitogen-activated protein kinase kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase.* J Biol Chem, 2004. **279**(16): p. 15711-4.
315. Tesz, G.J., et al., *Tumor necrosis factor alpha (TNFalpha) stimulates Map4k4 expression through TNFalpha receptor 1 signaling to c-Jun and activating transcription factor 2.* J Biol Chem, 2007. **282**(27): p. 19302-12.
316. Wright, J.H., et al., *The STE20 kinase HGK is broadly expressed in human tumor cells and can modulate cellular transformation, invasion, and adhesion.* Mol Cell Biol, 2003. **23**(6): p. 2068-82.
317. Xue, Y., et al., *Mesodermal patterning defect in mice lacking the Ste20 NCK interacting kinase (NIK).* Development, 2001. **128**(9): p. 1559-72.
318. Kuan, C.Y., et al., *The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development.* Neuron, 1999. **22**(4): p. 667-76.
319. Collins, C.S., et al., *A small interfering RNA screen for modulators of tumor cell motility identifies MAP4K4 as a promigratory kinase.* Proc Natl Acad Sci U S A, 2006. **103**(10): p. 3775-80.
320. Zohn, I.E., et al., *p38 and a p38-interacting protein are critical for downregulation of E-cadherin during mouse gastrulation.* Cell, 2006. **125**(5): p. 957-69.
321. Bouzakri, K. and J.R. Zierath, *MAP4K4 gene silencing in human skeletal muscle prevents tumor necrosis factor-alpha-induced insulin resistance.* J Biol Chem, 2007. **282**(11): p. 7783-9.
322. Bouzakri, K., P. Ribaux, and P.A. Halban, *Silencing mitogen-activated protein 4 kinase 4 (MAP4K4) protects beta cells from tumor necrosis factor-alpha-induced decrease of IRS-2 and inhibition of glucose-stimulated insulin secretion.* J Biol Chem, 2009. **284**(41): p. 27892-8.
323. Aouadi, M., et al., *Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation.* Nature, 2009. **458**(7242): p. 1180-4.
324. Tang, X., et al., *An RNA interference-based screen identifies MAP4K4/NIK as a negative regulator of PPARgamma, adipogenesis, and insulin-responsive hexose transport.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2087-92.

325. Murphy, G.J. and J.C. Holder, *PPAR-gamma agonists: therapeutic role in diabetes, inflammation and cancer.* Trends Pharmacol Sci, 2000. **21**(12): p. 469-74.
326. Kershaw, E.E. and J.S. Flier, *Adipose tissue as an endocrine organ.* J Clin Endocrinol Metab, 2004. **89**(6): p. 2548-56.
327. Rosen, E.D. and B.M. Spiegelman, *Adipocytes as regulators of energy balance and glucose homeostasis.* Nature, 2006. **444**(7121): p. 847-53.
328. Tontonoz, P., E. Hu, and B.M. Spiegelman, *Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor.* Cell, 1994. **79**(7): p. 1147-56.
329. Vidal-Puig, A.J., et al., *Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids.* J Clin Invest, 1997. **99**(10): p. 2416-22.
330. Chawla, A., et al., *Nuclear receptors and lipid physiology: opening the X-files.* Science, 2001. **294**(5548): p. 1866-70.
331. Zhang, B., et al., *Negative regulation of peroxisome proliferator-activated receptor-gamma gene expression contributes to the antiadipogenic effects of tumor necrosis factor-alpha.* Mol Endocrinol, 1996. **10**(11): p. 1457-66.
332. Stephens, J.M., J. Lee, and P.F. Pilch, *Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction.* J Biol Chem, 1997. **272**(2): p. 971-6.
333. Hauser, S., et al., *Degradation of the peroxisome proliferator-activated receptor gamma is linked to ligand-dependent activation.* J Biol Chem, 2000. **275**(24): p. 18527-33.
334. Floyd, Z.E. and J.M. Stephens, *Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes.* J Biol Chem, 2002. **277**(6): p. 4062-8.
335. Yao, Z., et al., *A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway.* J Biol Chem, 1999. **274**(4): p. 2118-25.
336. Chinenov, Y. and T.K. Kerppola, *Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity.* Oncogene, 2001. **20**(19): p. 2438-52.
337. Pestova, T.V., et al., *Molecular mechanisms of translation initiation in eukaryotes.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7029-36.
338. Preiss, T. and W.H. M, *Starting the protein synthesis machine: eukaryotic translation initiation.* Bioessays, 2003. **25**(12): p. 1201-11.
339. Gingras, A.C., B. Raught, and N. Sonenberg, *eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation.* Annu Rev Biochem, 1999. **68**: p. 913-63.
340. Hay, N. and N. Sonenberg, *Upstream and downstream of mTOR.* Genes Dev, 2004. **18**(16): p. 1926-45.

341. Jiang, Z.Y., et al., *Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing.* Proc Natl Acad Sci U S A, 2003. **100**(13): p. 7569-74.
342. Bose, A., et al., *Glucose transporter recycling in response to insulin is facilitated by myosin Myo1c.* Nature, 2002. **420**(6917): p. 821-4.
343. Wang, X. and B. Seed, *A PCR primer bank for quantitative gene expression analysis.* Nucleic Acids Res, 2003. **31**(24): p. e154.
344. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
345. Gao, G., et al., *High throughput creation of recombinant adenovirus vectors by direct cloning, green-white selection and I-Sce I-mediated rescue of circular adenovirus plasmids in 293 cells.* Gene Ther, 2003. **10**(22): p. 1926-30.
346. Christianson, J.L., et al., *Stearoyl-CoA desaturase 2 is required for peroxisome proliferator-activated receptor gamma expression and adipogenesis in cultured 3T3-L1 cells.* J Biol Chem, 2008. **283**(5): p. 2906-16.
347. Reynolds, T.H.t., S.C. Bodine, and J.C. Lawrence, Jr., *Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load.* J Biol Chem, 2002. **277**(20): p. 17657-62.
348. Lin, T.A., et al., *Control of PHAS-I by insulin in 3T3-L1 adipocytes. Synthesis, degradation, and phosphorylation by a rapamycin-sensitive and mitogen-activated protein kinase-independent pathway.* J Biol Chem, 1995. **270**(31): p. 18531-8.
349. Lin, T.A., et al., *PHAS-I as a link between mitogen-activated protein kinase and translation initiation.* Science, 1994. **266**(5185): p. 653-6.
350. Lin, T.A. and J.C. Lawrence, Jr., *Control of the translational regulators PHAS-I and PHAS-II by insulin and cAMP in 3T3-L1 adipocytes.* J Biol Chem, 1996. **271**(47): p. 30199-204.
351. Pause, A., et al., *Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function.* Nature, 1994. **371**(6500): p. 762-7.
352. Dumont, F.J. and Q. Su, *Mechanism of action of the immunosuppressant rapamycin.* Life Sci, 1996. **58**(5): p. 373-95.
353. Mothe-Satney, I., et al., *In rat hepatocytes glucagon increases mammalian target of rapamycin phosphorylation on serine 2448 but antagonizes the phosphorylation of its downstream targets induced by insulin and amino acids.* J Biol Chem, 2004. **279**(41): p. 42628-37.
354. Scherer, P.E., *Adipose tissue: from lipid storage compartment to endocrine organ.* Diabetes, 2006. **55**(6): p. 1537-45.
355. Rasouli, N., et al., *Ectopic fat accumulation and metabolic syndrome.* Diabetes Obes Metab, 2007. **9**(1): p. 1-10.
356. Lionetti, L., et al., *From chronic overnutrition to insulin resistance: the role of fat-storing capacity and inflammation.* Nutr Metab Cardiovasc Dis, 2009. **19**(2): p. 146-52.

357. Galic, S., J.S. Oakhill, and G.R. Steinberg, *Adipose tissue as an endocrine organ.* Mol Cell Endocrinol, 2010. **316**(2): p. 129-39.
358. Michael, M.D., et al., *Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction.* Mol Cell, 2000. **6**(1): p. 87-97.
359. Pilkis, S.J. and D.K. Granner, *Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis.* Annu Rev Physiol, 1992. **54**: p. 885-909.
360. Cherrington, A.D., D. Edgerton, and D.K. Sindelar, *The direct and indirect effects of insulin on hepatic glucose production in vivo.* Diabetologia, 1998. **41**(9): p. 987-96.
361. Moore, M.C., C.C. Connolly, and A.D. Cherrington, *Autoregulation of hepatic glucose production.* Eur J Endocrinol, 1998. **138**(3): p. 240-8.
362. Postic, C., R. Dentin, and J. Girard, *Role of the liver in the control of carbohydrate and lipid homeostasis.* Diabetes Metab, 2004. **30**(5): p. 398-408.
363. Home, P.D. and G. Pacini, *Hepatic dysfunction and insulin insensitivity in type 2 diabetes mellitus: a critical target for insulin-sensitizing agents.* Diabetes Obes Metab, 2008. **10**(9): p. 699-718.
364. Boden, G., et al., *Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver.* Diabetes, 2005. **54**(12): p. 3458-65.
365. Marra, F. and C. Bertolani, *Adipokines in liver diseases.* Hepatology, 2009. **50**(3): p. 957-69.
366. Trayhurn, P. and J.H. Beattie, *Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ.* Proc Nutr Soc, 2001. **60**(3): p. 329-39.
367. Guntur, K.V., et al., *Map4k4 negatively regulates peroxisome proliferator-activated receptor (PPAR) gamma protein translation by suppressing the mammalian target of rapamycin (mTOR) signaling pathway in cultured adipocytes.* J Biol Chem, 2010. **285**(9): p. 6595-603.
368. Isakson, P., et al., *Impaired preadipocyte differentiation in human abdominal obesity: role of Wnt, tumor necrosis factor-alpha, and inflammation.* Diabetes, 2009. **58**(7): p. 1550-7.
369. Ventura, A., et al., *Cre-lox-regulated conditional RNA interference from transgenes.* Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10380-5.
370. Heikkinen, S., et al., *Evaluation of glucose homeostasis.* Curr Protoc Mol Biol, 2007. **Chapter 29**: p. Unit 29B 3.
371. Kim, J.K., *Hyperinsulinemic-euglycemic clamp to assess insulin sensitivity in vivo.* Methods Mol Biol, 2009. **560**: p. 221-38.
372. Deveaux, V., et al., *Cannabinoid CB2 receptor potentiates obesity-associated inflammation, insulin resistance and hepatic steatosis.* PLoS One, 2009. **4**(6): p. e5844.
373. McLaughlin, T., et al., *Inflammation in subcutaneous adipose tissue: relationship to adipose cell size.* Diabetologia, 2010. **53**(2): p. 369-77.

374. Khan, T., et al., *Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI.* Mol Cell Biol, 2009. **29**(6): p. 1575-91.
375. Xu, Y., S.R. Farmer, and B.D. Smith, *Peroxisome proliferator-activated receptor gamma interacts with CIITA x RFX5 complex to repress type I collagen gene expression.* J Biol Chem, 2007. **282**(36): p. 26046-56.
376. Jones, J.R., et al., *Deletion of PPARgamma in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance.* Proc Natl Acad Sci U S A, 2005. **102**(17): p. 6207-12.
377. Sugii, S., et al., *PPARgamma activation in adipocytes is sufficient for systemic insulin sensitization.* Proc Natl Acad Sci U S A, 2009. **106**(52): p. 22504-9.
378. Kim, J.Y., et al., *Obesity-associated improvements in metabolic profile through expansion of adipose tissue.* J Clin Invest, 2007. **117**(9): p. 2621-37.
379. Li, S., M.S. Brown, and J.L. Goldstein, *Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis.* Proc Natl Acad Sci U S A, 2010. **107**(8): p. 3441-6.
380. Brown, M.S. and J.L. Goldstein, *Selective versus total insulin resistance: a pathogenic paradox.* Cell Metab, 2008. **7**(2): p. 95-6.
381. Xu, H., et al., *Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance.* J Clin Invest, 2003. **112**(12): p. 1821-30.
382. Meshkani, R. and K. Adeli, *Hepatic insulin resistance, metabolic syndrome and cardiovascular disease.* Clin Biochem, 2009. **42**(13-14): p. 1331-46.
383. Klover, P.J., A.H. Clementi, and R.A. Mooney, *Interleukin-6 depletion selectively improves hepatic insulin action in obesity.* Endocrinology, 2005. **146**(8): p. 3417-27.
384. Nov, O., et al., *Interleukin-1beta may mediate insulin resistance in liver-derived cells in response to adipocyte inflammation.* Endocrinology, 2010. **151**(9): p. 4247-56.
385. Mohamed-Ali, V., et al., *Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo.* J Clin Endocrinol Metab, 1997. **82**(12): p. 4196-200.
386. Fried, S.K., D.A. Bunkin, and A.S. Greenberg, *Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid.* J Clin Endocrinol Metab, 1998. **83**(3): p. 847-50.
387. Burysek, L., P. Tvrdik, and J. Houstek, *Expression of interleukin-1 alpha and interleukin-1 receptor type I genes in murine brown adipose tissue.* FEBS Lett, 1993. **334**(2): p. 229-32.
388. Lumeng, C.N., et al., *Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes.* Diabetes, 2008. **57**(12): p. 3239-46.
389. Vaartjes, W.J., C.G. de Haas, and M. Houweling, *Acute effects of interleukin 1 alpha and 6 on intermediary metabolism in freshly isolated rat hepatocytes.* Biochem Biophys Res Commun, 1990. **169**(2): p. 623-8.
390. Gnudi, L., et al., *High level overexpression of glucose transporter-4 driven by an adipose-specific promoter is maintained in transgenic mice on a high fat diet, but*

does not prevent impaired glucose tolerance. Endocrinology, 1995. **136**(3): p. 995-1002.
391. Day, C., *Thiazolidinediones: a new class of antidiabetic drugs.* Diabet Med, 1999. **16**(3): p. 179-92.
392. Li, Y. and M.A. Lazar, *Differential gene regulation by PPARgamma agonist and constitutively active PPARgamma2.* Mol Endocrinol, 2002. **16**(5): p. 1040-8.
393. Sabio, G., et al., *A stress signaling pathway in adipose tissue regulates hepatic insulin resistance.* Science, 2008. **322**(5907): p. 1539-43.
394. Chakrabarti, P., et al., *Mammalian target of rapamycin complex 1 suppresses lipolysis, stimulates lipogenesis, and promotes fat storage.* Diabetes, 2010. **59**(4): p. 775-81.