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Defining the Importance of Fatty Acid Metabolism in Maintaining Adipocyte Function: A Dissertation

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DEFINING THE IMPORTANCE OF FATTY ACID METABOLISM IN MAINTAINING ADIPOCYTE FUNCTION

A Dissertation Presented

By

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Abstract

Although once considered a simple energy storage depot, the adipose tissue is now known to be a powerful regulator of whole body insulin sensitivity and energy metabolism. This metabolically dynamic organ functions to safely store excess fatty acid as triglyceride, thereby preventing lipotoxicity in peripheral tissues and the development of insulin resistance. In addition, the adipose tissue acts as an endocrine organ and secretes factors, called adipokines, which influence whole body insulin sensitivity and glucose homeostasis. Therefore, understanding adipose tissue development and biology is essential to understanding whole body energy metabolism.

A master regulator of adipose tissue development and whole body insulin sensitivity is the nuclear receptor, PPARγ. Due to the importance of this nuclear receptor in maintaining adipocyte function, disruptions in PPARγ activity result in severe metabolic abnormalities, such as insulin resistance and type 2 diabetes. Conversely, PPARγ activation by synthetic agonists ameliorates these conditions, demonstrating the potent control this nuclear receptor has on whole body metabolism. Therefore, understanding how PPARγ expression and activity are regulated, particularly in the adipose tissue, is paramount to understanding the pathogenesis of type 2 diabetes.

While there are several synthetic PPARγ agonists available, identifying the endogenous ligand or ligands is still an area of intense investigation. Since fatty acids can induce PPARγ activation, in the first part of this thesis, I screened several fatty acid metabolizing enzymes present in the adipocyte to identify novel modulators of PPARγ activity. These studies revealed that the fatty acid Δ9 desaturase, Stearoyl CoA Desaturase 2 (SCD2), is absolutely required for 3T3-L1 adipogenesis and to maintain adipocyte-specific

gene expression in fully differentiated cells. Although SCD2 does not appear to regulate PPARγ ligand production, it does potently regulate PPARγ activity by maintaining the synthesis of PPARγ protein. Surprisingly, this effect was found only with SCD2 and not with the highly homologous protein, SCD1. Therefore, these findings identify separate cellular functions for these SCD isoforms and reveal a novel and essential role for fatty acid desaturation in the adipocyte.

Equally important to understanding PPARγ regulation is identifying the downstream mechanisms by which PPARγ activation improves insulin sensitivity. Evidence suggests that the PPARγ target gene, Cidea, is involved in mediating insulin sensitivity by binding to lipid droplets and promoting lipid storage in the adipocyte. Therefore, the second part of thesis provides mechanistic detail into Cidea function by showing that the carboxy terminal 104 amino acids is necessary and sufficient for lipid droplet targeting and the stimulation of triglyceride storage. However, these studies also identified a novel function for Cidea, which requires both the carboxy and amino termini: to induce larger and fewer droplets from smaller dispersed droplets, indicating the possible fusion of droplets. Perhaps this striking change in lipid droplet morphology allows tighter packing and more efficient storage of triglyceride and identifies a novel role for Cidea in lipid metabolism.

The results presented in this thesis elucidate key aspects of lipid metabolism that maintain adipocyte function: SCD2 is required to maintain PPARγ protein expression in the mouse; Cidea is a downstream effector of PPARγ activity by promoting efficient triglyceride storage. Therefore, these findings enhance our understanding of adipocyte biology.

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List of Frequently Used Abbreviations

Abbreviation	Term
NIDDM	Non-Insulin Dependent Diabetes Mellitus
GLUT4	Insulin-responsive facilitated Glucose Transporter isoform 4
Myo1c	Myosin 1C
SNARE	Soluble N-ethylmaleimide-sensitive factor-attachment protein receptor
Synip	Syntaxin 4 Interacting Protein
Munc18	Syntaxin Binding Protein 1
IRS 1-4	Insulin Receptor Substrate 1-4
PDK1	3-Phosphoinositide-Dependent protein Kinase 1
AKT 1/2	Thymoma viral proto-oncogene 1
PI3K	Phosphotidylinositol 3 Kinase
AS160	TBC1D4, TBC1 domain family, member 4
GS	Glycogen Synthase
GSK	Glycogen Synthase Kinase
PKA	cAMP dependent Protein Kinase
PP1	Protein Phosphatase type 1
SHC	SRC Homology 2 domain-containing transforming protein C
GRB2	Growth Factor Receptor Bound protein 2
SOS	Son of Sevenless homolog (Drosophila)
MAPK	Mitogen Activated Protein Kinase

SHP2	SH2 domain-containing protein tyrosine Phosphatase-2
PGC1	Peroxisome Proliferative Activated Receptor gamma, Coactivator1
PEPCK	Phosphoenolpyruvate Carboxy Kinase
G6Pase	Glucose-6-Phosphatase
FoxO1	Forkhead Box O1
CBP	CREB Binding Protein
CREB	cAMP Responsive Element Binding Protein
CIDE	Cell Death Inducing DFF Effector
DFF40/45	DNA Fragmentation Factor 40/45
PAT	Perilipin/ADRP/TIP47 family
OXPAT	PAT family member associated with oxidative metabolism
ATGL	Adipose Triglyceride Lipase
ADRP	Adipocyte Differentiation Related Protein
TIP47	Mannose-6-phosphate receptor binding protein 1
HSL	Hormone Sensitive Lipase
PDE3B	Phosphodiesterase 3B
ACC	Acetyl-Coenzyme A Carboxylase
FAS	Fatty Acid Synthase
CPT1	Carnitine Palmitoyltransferase 1
SREBP1	Sterol Regulatory Element Binding Protein-1
GPAT	Glycerol-3-phosphate Acyltransferase
Fos	FBJ Osteosarcoma oncogene

Fra-1	Fos-like Antigen 1
Gab1	Growth Factor Receptor Bound Protein 2-associated protein 1
ATF4	Activating Transcription Factor 4
mTOR	Mammalian Target of Rapamycin
p70S6K 1-2	40S Ribosomal Protein S6 Kinase 1-2
4EBP1	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
eIF-4E	Eukaryotic Translation Initiation Factor 4E
IGF-1	Insulin-like Growth Factor 1
SOCS3	Suppressor of Cytokine Signaling 3
FFA	Free Fatty Acid
PKC$\tilde{\theta}$ / ζ	Protein Kinase C theta / zeta
IκKβ	Inhibitory κ B Kinase β
NFκB	Nuclear Factor kappa-B Kinase
TLR4	Toll-Like Receptor 4
DAG	Diacylglycerol
GM3	ganglioside GM3
VLDL/ HDL	Very Low/ High Density Lipoprotein
TNFα	Tumor Necrosis Factor α
IL-6	Interleukin 6
RBP4	Retinol Binding Protein 4
AMPK	AMP activated Protein Kinase
BMI	Body Mass Index

JAK	Janus Kinase 3
STAT	Signal Transducer and Activator of Transcription
CNS	Central Nervous System
WAT	White Adipose Tissue
VAT	Visceral Adipose Tissue
LPS	Lipopolysaccharide
MCP1	Monocyte Chemoattractant Protein 1
PPAR α/γ/β/δ	Peroxisome Proliferator Activated Receptor alpha/gamma/beta/delta
PPRE	Peroxisome Proliferator Activated Receptor response element
LBD	Ligand Binding Domain
N-CoR	Nuclear Receptor Co-repressor 1
SMRT	Nuclear Receptor Co-repressor 2
SRC-1	Nuclear Receptor Coactivator 1
EPA	Eicosapentaenoic acid
EET	Epoxyeicosatrienoic acid
HETE	Hydroxyeicosatetraenoic acid
HODE	Hydroxy-octadecadienoic acid
LTB4	Leukotriene B4
COX2	Cyclooxygenase 2
VCAM1	Vascular Cell Adhesion Molecule 1
TF	Tissue Factor

SCD1-5	Stearoyl CoA Desaturase 1-5
C/EBP β/δ/α	CCAAT/enhancer Binding Protein beta, delta, or alpha
PTEN	Phosphatase and Tensin homolog
eEF2	Eukaryotic translation Elongation Factor 2
eEF2K	Eukaryotic translation Elongation Factor 2 Kinase
eIF2α	Eukaryotic translation Initiation Factor 2 alpha
Scr	Scramble
siRNA	small interfering RNA

Chapter I: Introduction

The World Health Organization estimates that at least 10% of adults are obese worldwide and the percentage is as high as 25% in some western countries (8). This is of serious concern because many metabolic complications can result from obesity, including dyslipidemia, hypertension, atherosclerosis, insulin resistance and type 2 diabetes, collectively called the metabolic syndrome (4,9). The most common metabolic disease is type 2 diabetes, and the incidence of this disease is expected to continue rising. Health care costs due to this disease exceed $130 billion per year in the United States alone due to the associated complications, such as blindness, end-stage renal disease, and nontraumatic loss of limb (10). Since type 2 diabetes is rapidly becoming a global pandemic, it is crucial to unravel the complex metabolic pathways that control insulin signaling and whole body energy metabolism in order to develop more effective treatments.

Regulation of whole body energy metabolism must be flexible in order to adapt to the changing nutritional environment the body is exposed to on a daily basis. When in a fasting state, the pancreas releases the hormone, glucagon, which signals to the liver to increase glucose production through glycogenolysis and gluconeogenesis and ketone production through ketogenesis. The endogenous production of these molecules is required when not exogenously available, since the brain requires glucose or ketones as an energy source (7,11,12). Catecholamines are also produced in the adrenal glands, which stimulates lipolysis and free fatty acid release from the adipose tissue to supply the adipose tissue itself and peripheral tissues with lipids as an energy source.

However, when glucose levels rise due to food intake, the body must simultaneously increase glucose disposal, which occurs primarily in the muscle, and decrease gluconeogenesis in the liver in order to maintain a healthy serum glucose level between 4-7 mM (13). These changes in glucose metabolism initiate with an increase in insulin and a decrease in glucagon secretion from the pancreas. Insulin then signals to the liver to simultaneously inhibit gluconeogenesis and glycogenolysis and increase skeletal muscle glucose transport, glycolysis, and glycogen synthesis, thereby promoting glucose utilization and storage rather than glucose production. Insulin also increases glucose transport and utilization, decreases lipolysis, and increases lipogenesis in the adipose tissue, thereby promoting lipid storage rather than lipid utilization. Furthermore, insulin stimulates the release of cytokines from the adipose tissue called adipokines, such as adiponectin and leptin, which act to sensitize peripheral tissues to the action of insulin (see Figure 1.1).

The brain also responds to the changing nutritional environment by responding to insulin and other peripheral signals, such as leptin, to decrease food intake and hepatic glucose production. Conversely, the brain senses a decrease in these signals during nutrient deprivation and signals for the body to increase food intake and decrease energy expenditure in order to maintain an ample supply of energy (14). This complex metabolic network allows the body to use exogenous fuel when it is available and produces endogenous fuel when needed to meet the body's demand for energy. The body must therefore remain metabolically adaptable to the nutritional environment and loss in this

flexibility leads to metabolic disturbances, such as insulin resistance and type 2 diabetes mellitus.

Insulin resistance occurs when insulin loses the ability to effectively increase glucose disposal in the adipose tissue and muscle, leading to elevated serum glucose levels, which is exacerbated by reduced hepatic insulin sensitivity and increased glucose production. To compensate for this insulin resistance, the pancreas increases insulin production to maintain glucose homeostasis. However, when the pancreas fails to produce enough insulin to compensate for the decreased insulin sensitivity, then diabetes mellitus develops (15). This metabolic disease has become an epidemic and is a main cause of morbidity and mortality worldwide (4,5,16,17). While there are therapies available to treat type 2 diabetes, they have dangerous side effects, such as edema and heart failure (2,17). Therefore, dissecting the pathways of insulin action and identifying the components that dictate insulin sensitivity is of utmost importance in developing novel, more effective therapies to combat this disease.

Insulin signaling

Regulation of glucose transport

Insulin increases glucose transport by increasing the expression of the glucose transporter, GLUT4, on the cell surface. In the basal state, GLUT4 continually cycles to the cell surface, but is mainly retained in a perinuclear storage site (13,18). Upon insulin stimulation, there is rapid exocytosis of GLUT4 from this intracellular storage site as well as inhibition of GLUT4 endocytosis, resulting in a dramatic increase in GLUT4 expression on the plasma membrane (13,19-22). The translocation of GLUT4 vescicles to

the plasma membrane requires an intact microtubule and actin network, relying on kinesins and myo1C, respectively, (18,23-27). The docking and fusion of GLUT4 with the plasma membrane also appears to be regulated by insulin, possibly through the SNARE accessory proteins, Synip and Munc18 (20,28,29). Thus, insulin mediated GLUT4 translocation is regulated at several steps (See reference (30) for a more detailed review).

The signaling cascade to induce GLUT4 translocation is still under intense investigation, but many key components of the cascade have been defined. When insulin binds the insulin receptor on the cell surface, this transmembrane tyrosine kinase receptor undergoes a conformational change inducing autophosphorylation and results in multiple phosphotyrosine residues (31,32). Several of these phosphotyrosine residues function to recruit scaffolding proteins, such as IRS 1-4, which then recruit effector proteins to facilitate the insulin signaling cascade (13,33-36). The tyrosine phosphorylated IRS proteins, particularly IRS1 and IRS2, serve as docking sites for other downstream effectors, such as the type 1A phosphatidylinositol 3-kinase (PI3K) (37). The regulatory subunit of PI3K, p85, binds to the phosphorylated IRS proteins, which then activates the catalytic subunit of PI3K, p110. Although PI3K can phosphorylate many phosphoinositides, type 1A has the most activity on PI (4,5) P2 and generates PI (3,4,5) P3, which recruits PDK1 (38-42). This serine/threonine kinase then phosphorylates and activates AKT2, which is also a serine/threonine kinase. AKT2 is required for GLUT4 translocation, therefore identifying its substrates is an area of intense investigation (43-45). Although AKT2is involved in several processes and has many substrates, one that

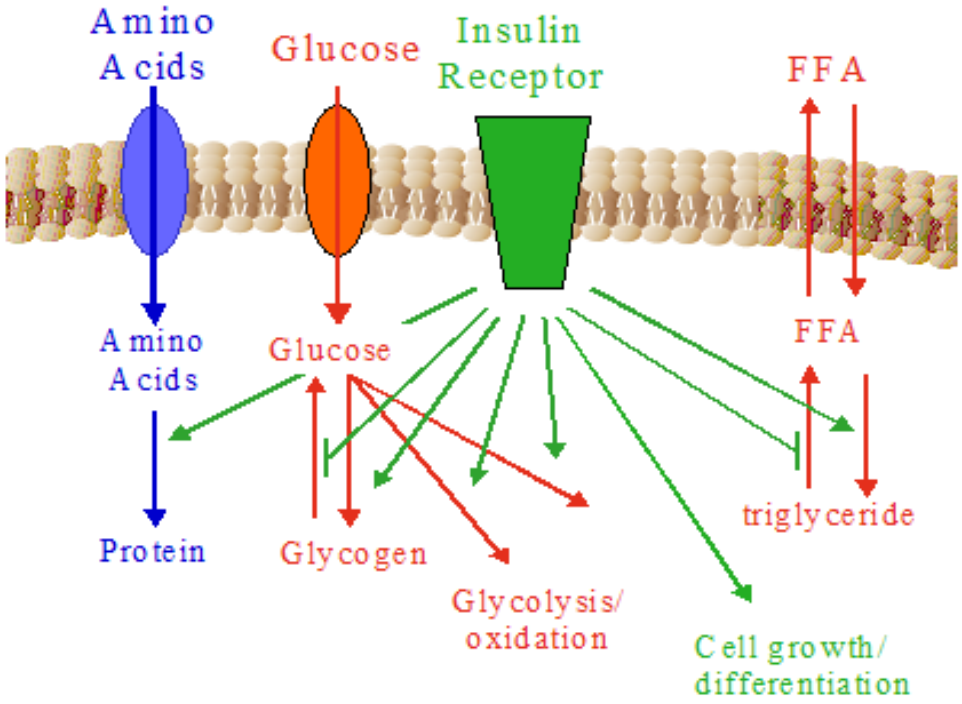
appears to negatively regulate GLUT4 translocation is AS160. This RAB-GAP protein controls the intracellular retention of GLUT4, preventing its expression on the plasma membrane in the basal state. Phosphorylation by AKT2 inactivates AS160 and allows GLUT4 translocation to the plasma membrane, leading to an increase in glucose transport (see Figure 1.2) (46,47).

In parallel to the PI3K signaling pathway, evidence suggests there is also a PI3K independent pathway involved in glucose transport, although the mechanistic details of this pathway are controversial and largely unsubstantiated (25,48).

While there are several glucose transporters present in each tissue, GLUT4 is specifically required to mediate the insulin response in glucose transport. This is evidenced by the rapid insulin resistance that occurs when GLUT4 is knocked out specifically in the adipose tissue or muscle of mice, despite the presence of other glucose transporters (15,49,50). Therefore, identifying the molecular mechanisms that dictate GLUT4 translocation to the plasma membrane is crucial in identifying the abnormalities that occur with type 2 diabetes.

Regulation of glycogen synthesis

In the muscle and liver, glycogen synthesis is mediated by glycogen synthase (GS), which incorporates uridine 5`-diphosphoglucose derived from glucose-6-phosphate (G6P), into a preexisting glycogen chain (51). GS is negatively regulated by GSK or PKA, which phosphorylates and inhibits GS activity. Upon insulin stimulation, GSK and PKA are inhibited, while protein phosphatase 1 (PP1) is activated, leading to decreased



METABOLIC PROCESS	LIVER	MUSCLE	ADIPOSE
Stimulation of glucose uptake and oxidation		X	X
Stimulation of glycogenesis	X	X	
Stimulation of fatty acid uptake			X
Stimulation of fatty acid and triglyceride synthesis	X		X
Stimulation of amino acid uptake		X	
Stimulation of protein synthesis	X	X	X
Stimulation of cell growth and differentiation		X	X
Inhibition of gluconeogenesis	X		
Inhibition of glycogenolysis	X	X	
Inhibition of lipolysis	X		X
Inhibition of protein degradation	X	X	X

Figure 1.1. The regulation of metabolism by insulin. When glucose levels rise due to food intake, the pancreas secretes insulin, which then binds to the insulin receptor expressed in the liver, muscle, and adipose tissue. Binding to the insulin receptor stimulates the uptake of glucose, amino acids and fatty acids into cells and increases the expression or activity of enzymes involved in glycogen, protein, and lipid synthesis, while inhibiting the activity or expression of those that cause degradation. Additionally, insulin inhibits the production of glucose in the liver and release of fatty acids into circulation from the adipose tissue and also promotes anabolic pathways, such as cell growth and differentiation. Together, insulin lowers serum glucose, protein, and fatty acids levels and allows the effective utilization of nutrients to support whole body metabolic processes (5).

GS phosphorylation and hence activation (5,52). Both the inhibition of GSK and the activation of PP1 are PI3K mediated (53). Although it is still unclear how PI3K regulates PP1 activity, PI3K inhibits GSK via activation of AKT2, which then phosphorylates and inhibits GSK (54,55). In the muscle, the majority of glucose is converted to glycogen and this pathway is compromised in diabetic patients. However, this defect appears to be due *to a decrease in glucose transport rather than a decrease in glycogen synthesis (See Figure 1.2) (51).*

Regulation of gluconeogenesis

Gluconeogenesis is a process unique to the liver and generates glucose from lactate, glycerol, and amino acids. This hepatic function is necessary to maintain serum glucose levels during a long term fast. However, upon feeding, insulin signals to inhibit gluconeogenesis in order to prevent an elevation of serum glucose levels and glucotoxicity. This insulin mediated signaling event requires the activation of the PI3K/AKT2 pathway via IRS1 and IRS2. AKT2 then phosphorylates and inhibits FoxO1 and PGC1α which are required to promote the transcription of PEPCK, the rate limiting enzymes in gluconeogenesis, and G6Pase (56-59). The expression of PGC1α itself also requires FoxO1 and therefore the decrease in FoxO1 activity potently inhibits gluconeogenic gene expression (59). Another transcription factor that is inhibited by insulin is CBP, a coactivator of CREB, which also promotes gluconeogenic gene expression (60). Therefore, the regulation of gluconeogenesis by insulin is through a transcriptional inhibition of gluconeogenic genes and is not a rapid process (59). Nevertheless, the maintenance of this signaling cascade is crucial to maintaining proper

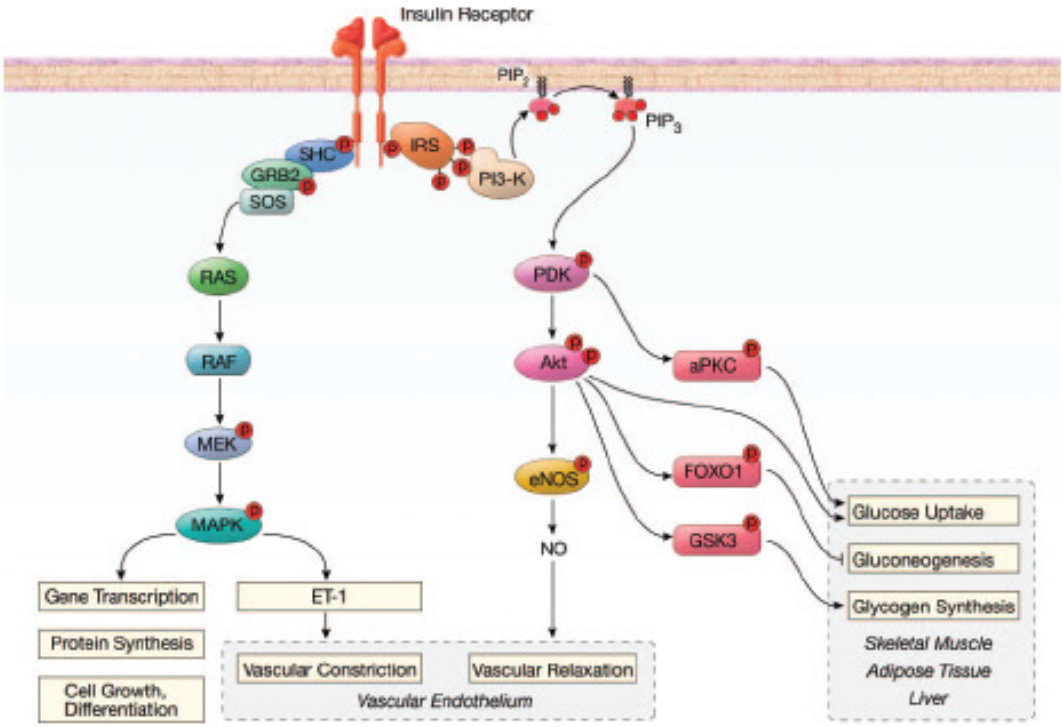


Figure 1.2 Schematic showing the general insulin signal transduction pathway. Insulin stimulation results in the tyrosine phosphorylation of the insulin receptor, which allows the docking of IRS proteins. This allows the association and activation of PI3K, which produces PI(3,4,5)P3, resulting in the recruitment and activation of PDK1, which then phosphorylates and activates both aPKCs and PKB, leading to GLUT4 translocation to the plasma membrane in the muscle and adipose tissue, decreased gluconeogenesis in the liver, and increased glycogen synthesis in the muscle and liver. In parallel, SHC binding to the insulin receptor recruits GRB2, which binds SOS. Together with SHP2 (not shown), SOS leads to activation of Ras and the MAPK cascade, leading to changes in gene expression and increased cell growth and differentiation (3).

glucose levels in the fed state and disruption of this process contributes significantly to the pathogenesis of obesity and type 2 diabetes (See Figure 1.2).

Regulation of lipid metabolism

While most tissues can store fatty acids in the form of triglyceride, this is a primary function of the adipocyte, which is capable of storing large quantities of lipid without suffering cellular toxicity. The muscle and liver can also store triglyceride, but the storage capacity is limited. When lipid levels rise in these tissues, insulin and other signaling events are disrupted and cellular toxicity ensues (2,4,6). It is therefore important that the adipocyte, in particular, maintains its ability to store or release fatty acids appropriately to prevent this lipotoxicity in peripheral tissues (See Figure 1.3). During times of fasting, the adipocyte releases free fatty acids into circulation for peripheral energy production, which occurs through lipolysis, or the lipase mediated hydrolysis of fatty acids from the glycerol backbone of triglyceride. However, in the fed state, fatty acids will not only disrupt insulin signaling, but may also compete with glucose as an energy source, thereby reducing glycolysis and glucose transport (61). In order to reduce fatty acid availability, insulin promotes the storage of fatty acids in adipocytes by preventing lipolysis, promoting fatty acid esterification and triglyceride synthesis, and inhibiting fatty acid transport into the mitochondria for α-oxidation.

Triglyceride is stored in cytoplasmic lipid droplets, which are structurally similar to lipoproteins. They contain a tightly packed neutral lipid core, consisting mostly of triglyceride and cholesterol esters, and are surrounded by a phospholipid monolayer and associated proteins (62). The prevailing theory for lipid droplet formation is that neutral

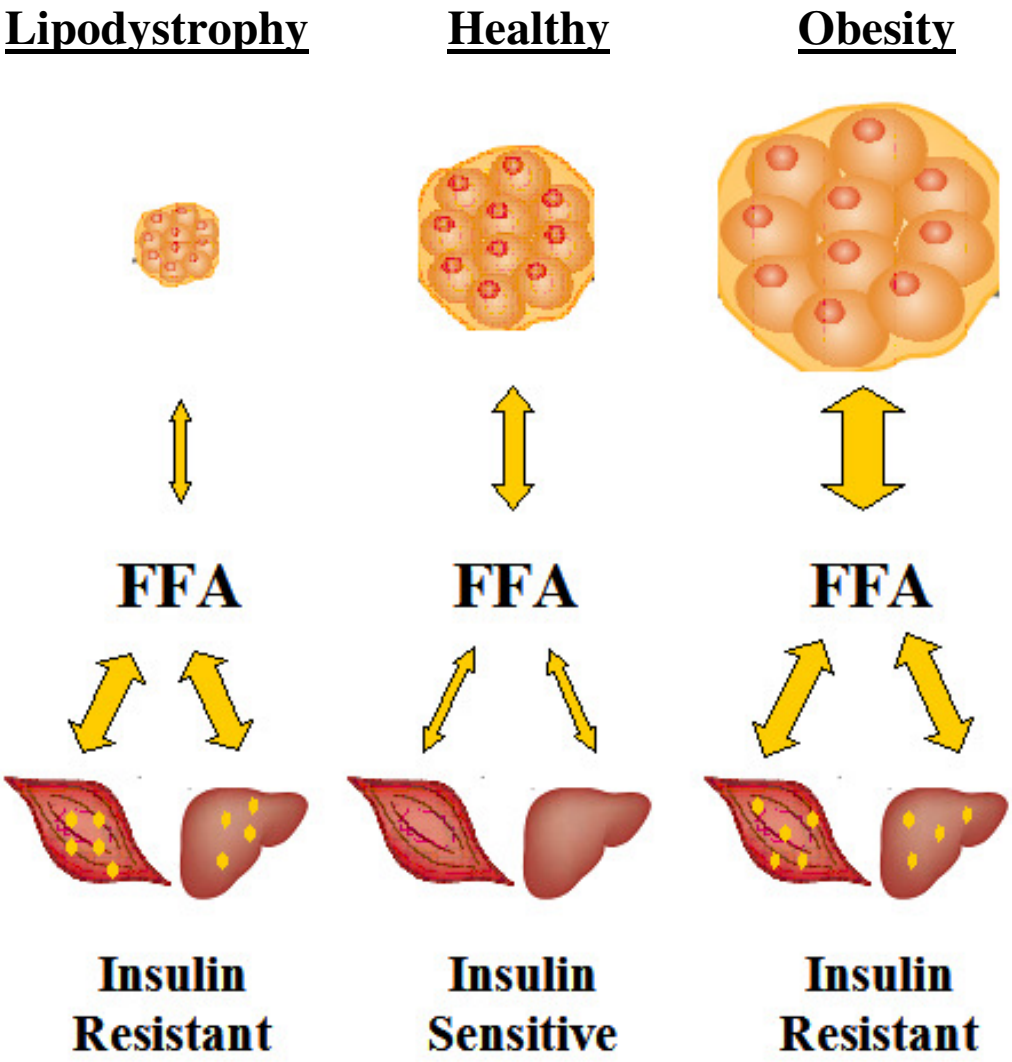


Figure 1.3. Schematic showing the importance of the adipose tissue in maintaining whole body insulin sensitivity. The middle diagram represents the insulin sensitive condition, while the left and right diagrams represent insulin resistant conditions resulting from lipoatrophy or obesity, respectively. During the lipoatrophic state, the loss in adipose tissue forces the accumulation of lipids in the liver and muscle, producing lipotoxicity in these tissues. In the obese state, the hypertrophic adipose tissue loses the ability to store free fatty acid as triglyceride, again resulting in lipotoxicity in the liver and muscle (2).

lipid accumulates between the lipid bilayer of the endoplasmic reticulum and eventually buds off from the ER into the cytoplasm, forming a lipid droplet (63,64). However, it is still not clear whether the lipid droplet remains associated with the ER or buds off completely (63-65). In adipocytes, there have been many proteins found associated with lipid droplets, but their role in lipid droplet biology is still unclear (66-69) Other proteins have a clear role in regulating triglyceride storage and turnover, such as members of the PAT family (Perilipin, Adiponectin, Tip47, S3-12 and OXPAT) and CIDE family (Cidea and Cidec, also called FSP27 in the mouse) (70-74). The PAT family of proteins contains strong sequence homology in their amino terminal regions, but interestingly, thisregion is not required for targeting the proteins to lipid droplets (70,75-77). The Cide proteins were first discovered to have homology in their amino terminal regions to the DNA fragmentation factor, DFF45, and were believed to be involved in apoptosis (78). However, more recent findings demonstrate these proteins are potent regulators of lipid storage in adipocytes (71-73,79,80).

Perilipin appears to be unique among the lipid droplet proteins, because it not only prevents lipolysis by shielding and protecting the triglyceride from lipases in a basal state, but also promotes lipolysis in response to lipolytic stimuli, such as β-adrenergic receptor agonists (81-86). OXPAT may also facilitate both lipid storage and lipolysis, but how this occurs is not yet understood (74). The two main lipases responsible for lipolysis in the white adipose tissue are ATGL and HSL (87-89). In rodents, ATGL primarily appears to regulate basal lipolysis, whereas both enzymes participate in stimulated

lipolysis; in humans, evidence suggests that both enzymes regulate basal lipolysis, but HSL seems to regulate stimulated lipolysis (89,90). During fasting, β-adrenergic signaling causes a rise in cAMP levels, which then activates PKA. PKA phosphorylates and activates HSL and also phosphorylates perilipin, allowing HSL association with perilipin and the lipid droplet, and subsequent hydrolysis of triglyceride (81,91,92). In response to insulin, AKT2 phosphorylates and activates the cyclic nucleotide phosphodiesterase, PDE3B, which then hydrolyzes cAMP, decreasing cAMP levels and PKA activation, and leading to decreased HSL activity and lipolysis (5,93).

Insulin also promotes lipid storage by simultaneously promoting endogenous fatty acid synthesis and inhibiting fatty acid oxidation in the mitochondria (5). This is accomplished by the activation of ACC1 and ACC2, producing the malonyl CoA used for either fatty acid synthesis or the inhibition of fatty acid oxidation, respectively (94). Malonyl CoA produced by ACC1, along with acetyl CoA, is utilized by FAS to produce palmitate, a sixteen carbon saturated fatty acid. Palmitate can then be esterified to glycerol to store the excess energy derived from glucose. Malonyl CoA derived from ACC2 binds to CPT1 and inhibits fatty acid transport into the mitochondria and thereby blocks fatty acid oxidation. Insulin also increases fatty acid and triglyceride synthesis via transcriptional mechanisms, by inducing SREBP-1c activity. This transcription factor increases the expression of enzymes involved in fatty acid and triglyceride synthesis, such as ACC, FAS, SCD1, and GPAT (95). These processes together increase the concentration of intracellular fatty acids, which can then be stored as triglyceride in lipid

droplets (96). This function of the adipocyte is crucial to prevent ectopic lipid accumulation and maintain whole body insulin sensitivity.

Regulation of cell growth and differentiation

In addition to regulating glucose and lipid metabolism, insulin is also a growth factor that promotes cell proliferation and/or differentiation (97,98). This occurs through increases in protein synthesis, decreases in protein degradation, and activation of various transcription factor complexes that control the transcriptional program for cell proliferation or differentiation (99-105). One such transcriptional complex is the AP-1 complex, comprised of members of the Fos [c-Fos, Fra-1 (*fos*-related antigen-1), Fra-2 and FosB] and Jun (c-Jun, JunB and JunD) families and consists of either homo or hetero-dimers. Insulin induces activation of the AP-1 complex through activation of the MAPK cascade (99,105). Upon insulin stimulation, IRS proteins and/or Shc become phosphorylated, which then recruits the adaptor protein, Grb2, which in turn binds the exchange protein, SOS (5,13,105,106). In parallel, the tyrosine phosphatase, SHP2, binds insulin receptor substrates, such as Gab1 or IRS1/2, and SHP2 together with SOS activate the serine kinase, Ras (5,13). Once activated, Ras initiates a serine kinase phosphorylation cascade beginning with MEK, which in turn activates ERK. Activated ERK translocates to the nucleus and activates various transcription factors, such as Elk1, Sap1a, and Fra1, which then induce the transcriptional changes necessary for cell proliferation or differentiation (See Figure 1.2) (13,105).

The demanding processes of proliferation and differentiation require increased protein expression, which occurs through increases in protein synthesis and decreases in

protein degradation (100-103). Insulin induces these changes both transcriptionally via activation of the transcription factor, ATF4, and through PI3K mediated activation of a serine kinase, mTOR. The activation of ATF4 leads to increased expression of amino acid biosynthetic genes and transporters required to provide the amino acids necessary for protein synthesis (107). The activation of mTOR stimulates ribosome biosynthesis by phosphorylating and activating p70 ribosomal S6 kinase (p70S6K) (107-110). Additionally, mTOR relieves the negative regulation of protein synthesis achieved by 4EBP1, which binds and inhibits the eukaryotic translation initiation factor, eIF-4E. This then allows eIF-4E to bind to the 5`cap of mRNA and promote translation initiation (111).

Taken together, insulin regulates many complex signaling cascades that coordinate glucose and lipid metabolism as well as cell growth and differentiation. While inhibition of the MAPK branch of the insulin pathway will not acutely affect glucose metabolism, it will inhibit adipocyte differentiation (112-114). Therefore, understanding these complex networks is pertinent to understanding adipocyte biology and unraveling the roots of insulin resistance.

Mechanisms of Insulin Resistance

Since metabolism is extraordinarily complex and requires the integration of many pathways and coordination of several tissues, there are many different metabolic disturbances that may lead to insulin resistance. Genetic and acquired factors can both affect insulin sensitivity, including decreases in insulin receptor concentration and kinase activity, decreases in the concentration and phosphorylation of IRS proteins, PI3K

activity, GLUT4 translocation, the activity of intracellular enzymes, or the activity of transcription factors (5,13).

Inhibition of insulin receptor signaling

The activity of the insulin receptor can be altered by either genetic mutation or changes in its phosphorylation state. While mutations in the insulin receptor itself are rare, they have been reported. The severity of insulin resistance in these individuals depends on the ability of the receptor to form hybrids with IGF-1 or other receptors and the presence of other genetic or acquired defects (5). In regards to phosphorylation, if the insulin receptor and IRS proteins are serine rather than tyrosine phosphorylated, this can also down regulate insulin signaling. Serine phosphorylation can inhibit tyrosine phosphorylation and promote association with 14-3-3 proteins, leading to decreased insulin signaling (115,116). There have been several kinases implicated in this process, including JNK, PI3K, GSK-3, AKT, mTOR, PKC, and IκB kinase (13,117,118). SOCS 1 and SOCS3 have also been shown to inhibit insulin signaling by binding IRS proteins and inducing their proteasomal degradation (119). Protein tyrosine phosphatases can also inhibit insulin action by dephosphorylating the insulin receptor and its substrates (120-124). Protein tyrosine phosphatase 1B (PTB1B) appears to be one such phosphatase, since the PTB1B knockout mice are more insulin sensitive than their wild type counterparts and are resistant to diet induced obesity (125).

Lipid mediators of insulin resistance

During insulin resistance and type 2 diabetes, there is often a chronic elevation of plasma free fatty acid and excessive lipid accumulation in non-adipose tissues (96,126).

Serum free fatty acid levels elevate during obesity due to the decreased ability of the adipose tissue to store lipids as well as decreased lipid clearance from the serum. This situation is further complicated in the insulin resistant state since insulin can no longer signal to inhibit lipolysis (127). While the adipose tissue is capable of storing excess free fatty acid as triglyceride, non-adipose tissues, such as the liver, muscle, and pancreas, have only a limited capacity for triglyceride storage. The resulting excessive lipid accumulation in the non-adipose tissues during obesity disrupts signaling pathways leading to metabolic perturbations. For example, lipid overload in the heart leads to apoptotic cell death and heart failure, in pancreatic cells causes dysregulated insulin secretion as well as apoptotic cell death, and in the liver and muscle decreases insulin sensitivity and glucose transport (96,128-131).

There have been several reasons proposed for the insulin resistance induced by free fatty acids in the liver and muscle. In 1963, Randle et al reported that elevated fatty acids increase fat oxidation and decrease glucose utilization due to a reduced flux of substrates through the glycolytic pathway. According to this mechanism, increased FFA levels lead to increased mitochondrial acetyl CoA/CoA ratios, which in turn inhibit pyruvate dehydrogenase activity and increase citrate levels, inhibiting phosphofructokinase activity. This process then leads to increased glucose-6-phosphate concentrations, which allosterically inhibits hexokinase, and thus reduces glucose transport/phosphorylation activity (132). However, in studies using ^{31}P NMR, glucose-6-phosphate concentrations are actually reduced rather than elevated in FFA-induced insulin resistant muscle. This phenomenon suggests there is a defect in glucose

transport/phosphorylation activity and not in glucose-6-phosphate inhibition of hexokinase as Randall suggested (51).

Free fatty acids have also been shown to activate PKCθ, IκKβ, as well as JNK1, leading to IRS1 serine phosphorylation, but exactly how the fatty acids lead to kinase activation is unclear. It may be through activation of the TLR4 pathway, increased reactive oxygen species generation, or endoplasmic reticulum stress (127). Conversely, lowering of FFA with the antilipolytic drug, Acipimox, enhances insulin sensitivity, supporting the idea that FFAs mediate insulin resistance (117).

In order to undergo intracellular metabolism, free fatty acids must first be activated by acyl CoA synthetase, which adds a thioester CoA to the fatty acid. The acyl CoA may then be channeled into one of three competing pathways: oxidation, glycerolipid formation, or sphingolipid formation. The majority of lipids are incorporated into glycerolipids, including phospholipids and di- and triacylglycerols. DAG (diacylglycerol) has been proposed to be a lipid intermediate of insulin resistance by inducing serine phosphorylation of IRS1 via PKCθ or IκKβ (126,131).

Sphingolipids, such as ceramide and the ganglioside GM3, are also potential lipid mediators of insulin resistance (133). In cultured muscle, adipocytes, and hepatocytes, ceramide analogs lead to decreased AKT2 activity by two mechanisms: activation of PP2A, which dephosphorylates and deactivates AKT2, and activation of PKCζ, which phosphorylates AKT on an inhibitory residue and prevents its translocation to the plasma membrane (133,134). The ganglioside GM3 has been shown to inhibit tyrosine phosphorylation of the insulin receptor and IRS1 and causes displacement of the insulin

receptor from detergent-resistant raft domains, leading to decreased insulin signaling and glucose uptake (135,136).

Some studies also suggest that cholesterol may participate in insulin resistance, since inhibitors of cholesterol synthesis have been shown to improve insulin sensitivity in rodents and humans (137,138). This may be due to decreased VLDL synthesis and delivery of triglycerides to peripheral tissues or to changes on the cellular level, since cholesterol reduction in cultured cells results in increased basal and insulin stimulated GLUT4 translocation (133). However, there is data suggesting that cholesterol depletion inhibits insulin signaling in cultured cells, possibly due to the disruption of lipid raft formation (139). Therefore, perhaps too little or too much cholesterol has a detrimental effect on insulin signaling resulting in a bell curve effect between cholesterol and insulin sensitivity.

Adipokines

The adipose tissue has long been thought of primarily as a site of energy storage, however, it is now evident that the adipose tissue also serves as an endocrine organ by secreting various factors called adipokines. These adipokines signal to peripheral tissues, such as the brain, liver, muscle, and immune system, to regulate blood pressure, glucose and lipid metabolism, inflammation, and atherosclerosis (16). Certain adipokines, such as adiponectin, leptin, omentin, chemerin, and vaspin have positive effects on insulin sensitivity, while other adipokines, such as resistin, retinol binding protein 4, IL-6, and TNFα have negative effects on insulin sensitivity (4,16,140). During insulin resistance,

there is a decrease in the production of the insulin sensitizing factors and an increase in production of the insulin resistant factors (See Figure 1.4) (141).

Adiponectin is one of the most powerful positive regulators of insulin sensitivity. This adipokine is secreted solely from adipocytes and causes suppression of hepatic gluconeogenesis, stimulation of fatty acid oxidation in the liver and skeletal muscle, stimulation of glucose uptake in the skeletal muscle, stimulation of insulin secretion from the pancreas, and modulation of food intake and energy expenditure (increased food intake and decreased energy expenditure during fasting and the opposite during refeeding) (140,141). It exerts its effects by binding to its receptors, AdipoR1 and AdipoR2, leading to the activation of AMPK, PPARα, and presumably other pathways that dictate the changes in metabolism (16).

Chemerin is another potential insulin sensitizing adipokine that is expressed in the liver and white adipose tissue and is required for normal adipocyte differentiation. It also enhances insulin stimulated glucose uptake and IRS1 tyrosine phosphorylation in 3T3-L1 adipocytes and down regulates the inflammatory response in activated macrophages (16). The correlation of chemerin expression with obesity is conflicting: a decrease in expression was found in the adipose tissue of db/db mice compared to controls, but was higher in the adipose tissue of diabetic Psammomys obesus rats compared with control rats; In humans, normal and type 2 diabetics patients had similar levels of chemerin, but when normal glucose tolerant patients were further analyzed, chemerin levels were positively correlated with BMI, triglycerides, and blood pressure (142). Therefore, it is

unclear whether chemerin has a role in increasing insulin sensitivity in vivo and requires further studies.

Omentin is a chemokine produced in the adipose tissue, but by visceral stromal vascular cells, not adipocytes. It has been shown to enhance insulin stimulated glucose transport and AKT phosphorylation in human subcutaneous and visceral adipocytes. The major isoform of omentin in the plasma is omentin 1 and its levels are negatively correlated with obesity and insulin resistance as determined by HOMA-IR and positively correlated with adiponectin and HDL levels (16). Although it is unclear what the mechanism of action is or the role omentin has in glucose metabolism, there is strong evidence that it may increase insulin sensitivity.

Leptin is the best characterized adipocyte secretory protein that positively regulates insulin sensitivity and functions by controlling food intake, energy expenditure, and neuroendocrine function (140). The regulation of glucose homeostasis appears to be mediated through separate signaling pathways from food intake and body weight. A region of the hypothalamus, called the arcuate nucleus (ARC), has been shown to be the target of leptin in regulating glucose and insulin levels and requires PI3K and AKT2. However, the regulation of food intake is dependent on the JAK-STAT pathway: Mice with a mutant leptin receptor that cannot signal through the JAK-STAT pathway, but can still activate the PI3K pathway, become obese and hyperphagic with only mild glucose intolerance. Furthermore, this glucose intolerance can be corrected with calorie restriction (16). Therefore, the regulation of glucose homeostasis requires PI3K and AKT2, while the regulation of food intake and weight gain require the JAK-STAT pathway.

Leptin also controls hepatic, muscle, and pancreatic function through direct actions on these tissues as well as through signals generated in the CNS. In liver and muscle, leptin activates AMPK, which inhibits triglyceride deposition in these tissues. In the pancreas, leptin was shown to inhibit insulin secretion in lean animals and protect the pancreas from lipid accumulation in response to a high calorie diet, thereby improving β-cell function (16,140).

Due to the potent effects of leptin on metabolism, mice deficient in leptin become severely obese and diabetic. However, during obesity, there appears to be leptin resistance not leptin deficiency. In obese individuals, a functional leptin receptor is expressed and serum leptin levels are higher than in lean individuals (143). Thus, during obesity chronic elevation of leptin may lead to leptin resistance, causing an increase in the food intake, lipid accumulation, and a decrease in insulin sensitivity.

Vaspin is another adipokine thought to be an insulin-sensitizing factor. This adipokine is a serine protease inhibitor produced in the visceral adipose tissue (VAT) and was originally identified in Otsuka Long-Evans Tokushima fatty rats, a rat model of visceral obesity and type 2 diabetes. Vaspin was abundantly and exclusively expressed in the VAT at a time when body weight and hyperinsulinemia peaked. However, vaspin mRNA decreased with disease progression and then increased again with thiazolidinedione treatment, suggesting a positive role for this protein. Consistent with this idea, recombinant vaspin improved glucose tolerance and insulin sensitivity in high fat high sucrose chow induced obese ICR mice (144). Data surrounding vaspin function

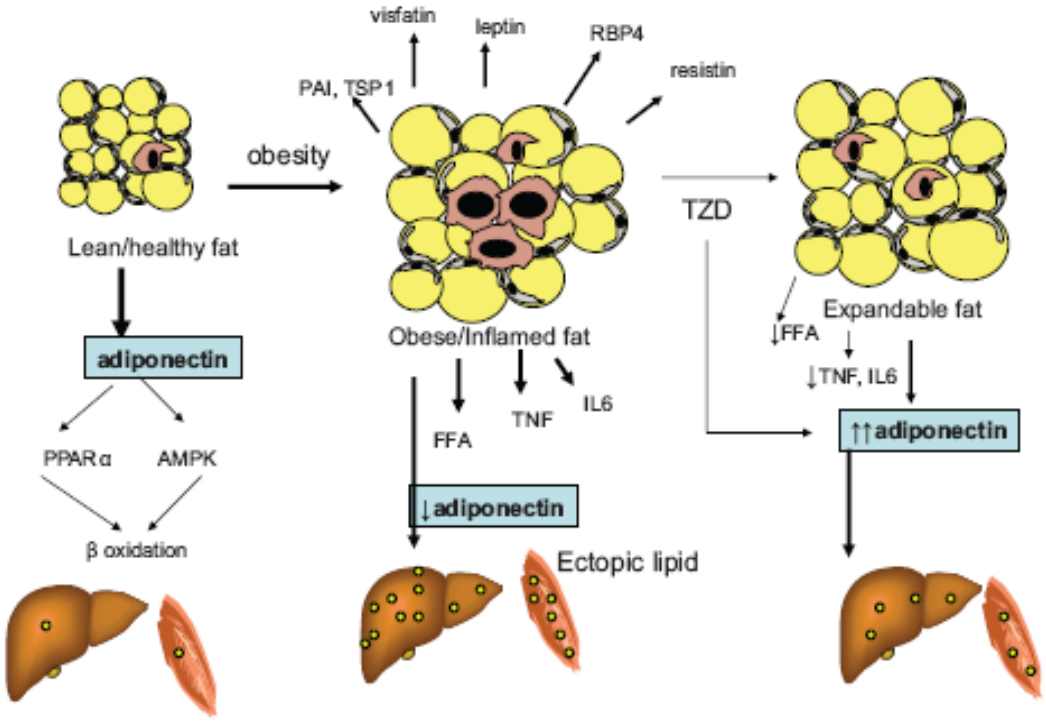


Figure 1.4. Changes in adipose tissue, liver, and muscle with obesity, insulin resistance, and thiazolidinedione (TZD) treatment. The adipose tissue of lean subjects contains few macrophages, secretes relatively high levels of adiponectin and other insulin sensitizing adipokines, and secretes low levels of inflammatory cytokines. Also, β-oxidation of lipids in the muscle is high with little ectopic fat in the muscle and liver. During obesity and insulin resistance, the adipose tissue loses the ability to store fatty acids, causing their release into circulation. Additionally, the adipose tissue secretes less insulin sensitizing adipokines and more insulin resistant adipokines. There is also increased macrophage infiltration generating inflammation within the adipose tissue. The inflammatory state along with the loss in lipid storage promotes ectopic lipid accumulation in the liver and muscle, decreasing insulin sensitivity. However, treatment with TZDs normalizes adipokine secretion, reduces inflammation, and promotes the expression of lipid synthesis and storage enzymes in the adipose tissue. Together, these changes reduce ectopic lipid accumulation and restore adipocyte function and insulin sensitivity (4).

in humans is not as clear as in rodents. In humans, vaspin mRNA was found to be expressed in both visceral and subcutaneous adipose tissue. Serum concentrations of vaspin were correlated with obesity and impaired insulin sensitivity, but no correlation was found with type 2 diabetes. Additionally, in one study the serum vaspin concentration was lower in fit versus unfit individuals, however, physical training in untrained individuals caused an increase in vaspin serum concentration (145). Therefore, it is still unclear what role vaspin has in human glucose metabolism, but it appears to be a positive regulator of insulin sensitivity in rodents.

There are also adipokines that negatively regulate insulin sensitivity. One such adipokine is resistin. In rodents, resistin is produced by adipocytes, while in humans it is produced by monocytes and macrophages, and was named for its ability to induce insulin resistance (140,146). Studies in rodents reveal that in several models of obesity, serum resistin levels rise, while the adipose tissue mRNA declines. Loss in resistin function leads to increased insulin sensitivity and hepatic AMPK phosphorylation as well as increased adipose tissue and body weight. Conversely, resistin treatment inhibits adipogenesis and systemic treatment or transgenic overexpression of resistin inhibits the ability of insulin to suppress hepatic glucose production, which may be explained by a decrease in AMPK phosphorylation and an increase in SOCS expression (147). The receptor for resistin is still unknown, but it may target the liver directly or the hypothalamus, since the effects on hepatic insulin sensitivity and glucose production can be repeated with resistin administration or blockade in the hypothalamus alone. In humans, the correlation of resistin with glucose abnormalities is still controversial.

However, resistin levels do positively correlate with TNFα and IL-6 levels and are likewise induced with low levels of LPS injection, suggesting resistin is related to inflammation and may therefore have a role in human glucose homeostasis (140).

Another adipokine that impairs insulin sensitivity is retinol binding protein 4 (RBP4). Serum levels of this adipokine were found elevated in several mouse models of obesity and insulin resistance and also in humans with these conditions (148). Moreover, studies found that RBP4 mRNA expression is increased in visceral compared with subcutaneous adipose tissue, correlating with the idea that abnormalities in the visceral rather than subcutaneous adipose tissue are linked with insulin resistance (149). Additionally, serum RBP4 concentrations correlate with RBP4 mRNA expression, intra-abdominal fat mass, total body fat mass, and insulin resistance (150). In addition, transgenic overexpression of human RBP4 in mice or treatment with recombinant RBP4 induced insulin resistance by increasing hepatic PEPCK expression and gluconeogenesis as well as inhibiting muscle insulin signaling (16). On the contrary, genetic deletion of RBP4 in mice resulted in increased insulin sensitivity and the synthetic retinoid, fenretinide, which decreases serum levels of RBP4 by promoting urinary excretion, corrected the insulin resistance found in mice fed a high fat diet (148). Taken together, the studies indicate that RBP4 is a strong potential target for the treatment of insulin resistance and type 2 diabetes in humans. Nevertheless, studies also exist that fail to correlate RBP4 levels with obesity, insulin resistance, type 2 diabetes, or components of the metabolic syndrome. These discrepancies may be explained by differences in the patient populations or the methodology used to measure RBP4 levels.

IL-6 is a proinflammatory cytokine that is produced by many cell types, but production by the adipose tissue is enhanced during obesity. The secretion is higher from visceral than subcutaneous adipose tissue, consistent with the idea that visceral adipose tissue is more inflammatory than subcutaneous adipose tissue. However, the majority of the IL-6 produced during obesity is thought to come from the cells of the stromal vascular fraction (preadipocytes, endothelial cells, and macrophages) rather than the adipocytes themselves. Despite the correlation of IL-6 levels and obesity, there is conflicting data regarding the role of this cytokine in insulin resistance: It was found to decrease insulin mediated glycogen synthesis in the liver and glucose uptake in adipocytes, but increase both parameters in myotubes (16). Studies examining the effect of IL-6 on hepatic glucose production also produce conflicting results; therefore, much work is needed to clarify these discrepancies (4). One idea is that during obesity and type 2 diabetes, IL-6 levels are chronically elevated, causing insulin resistance, whereas transient increases contribute to normal glucose homeostasis (16).

TNFα is another proinflammatory cytokine that is implicated as a cause of insulin resistance. This cytokine is mainly produced by macrophages and lymphocytes, but can also be produced by adipocytes. Deletion of TNFα or the TNFα receptors significantly improved insulin sensitivity in diet induced obese mice and in leptin deficient ob/ob mice. Furthermore, neutralization of TNFα in obese rats improved insulin resistance, suggesting a strong role for TNFα in inducing insulin resistance in rodents (16). Both TNFα and IL-6 are thought to mediate insulin resistance through several distinct mechanisms, including JNK1 mediated serine phosphorylation of IRS-1, IκB kinase

mediated NFκB activation, and induction of SOCS3 expression (151-153). Additionally, TNFα increases the level of circulating free fatty acids by increasing lipolysis and decreasing triglyceride synthesis in the adipose tissue (117). TNFα also decreases PPARγ expession, a crucial adipocyte transcription factor required to maintain adipocyte function, leading to decreased insulin sensitivity in this tissue (154). In humans, adipose tissue TNFα expression has been shown to correlate with BMI, percent body fat, and serum insulin levels, whereas weight loss decreased TNFα levels. Other studies, however, failed to show an increase in TNFα in obese versus lean patients, challenging the idea that TNFα is important in the pathophysiology of insulin resistance in humans. Additionally, in contrast to rodents, infusion of TNFα neutralizing antibody did not improve insulin sensitivity in obese, insulin resistant or type 2 diabetic patients (16). Therefore, the role of this cytokine in human insulin resistance still needs clarification.

Inflammation

It appears that during obesity there exists a chronic low-grade inflammation with infiltration of macrophages in the white adipose tissue, which may lead to altered adipokine expression, insulin resistance, and diabetes (155). The mechanisms that initiate and drive this inflammation are poorly understood, but it seems to involve cross talk between the adipocytes and other cell types found within the white adipose tissue, such as macrophages and endothelial cells. Increased levels of inflammatory markers, such as TNFα and IL-6, characterize this inflammation, but whether the production of these molecules originates from adipocytes or macrophages is still under investigation (146,156,157).

It is also unclear what recruits the macrophages to the adipose tissue during obesity. It appears to be related to body weight, since a small reduction in body weight is enough to decrease macrophage infiltration and inflammatory gene expression (146). One theory suggests that adipocytes undergo apoptosis, possibly due to reduced angiogenesis in the expanding adipose tissue and subsequent hypoxia, which then recruits macrophages to the adipose tissue (157-160). Another theory suggests that altered adipokine secretion recruits inflammatory monocytes/macrophages to the adipose tissue, possibly through the chemokine, MCP1 (monocyte chemoattractant protein 1) (146,161). However, its role in promoting macrophage infiltration is controversial, despite its increased expression during obesity (117,146). Additionally, leptin, which is primarily thought to be an insulin sensitizing factor by controlling food intake and energy expenditure, is also an immune modulator that is increased during obesity and could cause macrophage recruitment and activation (162). Furthermore, it has been proposed that other factors secreted from hypertrophied human adipocytes can activate endothelial cells present in the adipose tissue, causing the expression of adhesion molecules and promoting monocyte adhesion and migration into the tissue (146).

Nevertheless, macrophage recruitment alone does not decrease insulin sensitivity; it's the inflammatory response that is generated by the macrophages that negatively impacts adipocyte function and whole body insulin sensitivity. It is unclear what initiates the inflammatory process, but one possibility is through activation of the LPS receptor, TLR4 (163,164). During obesity, lipolysis increases in the adipocyte, releasing more fatty acids into circulation (89). The fatty acids may activate TLR4, which is expressed in

adipocytes, monocytes, and macrophages, leading to NFκB activation and the transcription of many proinflammatory genes in either cell type. The data regarding fatty acid activation of TLR4 is controversial, but TLR4 deficient mice have a decrease in the expression of inflammatory markers in response to lipid infusion and high fat diet and remain insulin sensitive compared to wild type mice (163,164). Additionally, isolated adipocytes from TLR4 deficient mice do not release TNFα or IL-6 in response to free fatty acid treatment (164). Clearly, these results support a role for TLR4 in inflammation and insulin sensitivity, but LPS contamination of the fatty acid treatments may be responsible for the activation of TLR4 rather than the fatty acids themselves.

Role of PPARs in maintaining insulin sensitivity

Peroxisome proliferator-activated receptors (PPARs) are members of the steroid hormone nuclear receptor superfamily that regulate the transcription of genes involved in lipid metabolism, energy balance, inflammation, and atherosclerosis (165,166). The importance of these nuclear receptors is evidenced by the use of synthetic agonists that target PPARs and are effective therapies in treating dyslipidemia and diabetes (17). The three known isotypes of PPARs, PPARα, PPARγ, and PPARβ/δ, become activated through either ligand dependent or independent mechanisms. When a cognate ligand binds to the ligand binding domain (LBD), it induces a conformational change that facilitates complex formation with another ligand-activated receptor, retinoid X receptor (RXR). This change in conformation also induces the release of co-repressor proteins, like N-CoR and SMRT, and the binding of co-activator proteins, like SRC-1 and PGC-1, and subsequent expression of genes containing a PPRE response element in their

promoter region (167). Ligand binding can also repress gene expression by inducing protein-protein interactions with other transcription factors, such as NFκB, STAT, and AP-1, and inhibiting their activity (168-171). These interactions may explain the anti-inflammatory effects of PPAR activation.

Ligand independent activation has also been reported for PPARα and PPARγ via phosphorylation of the receptor, possibly involving MAP kinases (167,172). Other evidence of ligand independent activation comes from a mutant PPARγ receptor that does not bind several of the known ligands, but is still functional both during adipogenesis and in fully differentiated adipocytes (173). Additionally, blockade of the LBD with an antagonist does not inhibit transcriptional activity in fully differentiated adipocytes, suggesting a ligand is not necessary for activity (174).

Although all three PPAR isoforms can regulate the same genes and contain common elements in their expression and regulation, each isoform has a distinct biological role in metabolism (see Figure 1.5).

PPARα

Issemann and Green first identified PPARα in mouse liver in 1990, but has since also been found expressed in the heart, skeletal muscle, intestine, pancreas, vasculature, immune cells, and in many other tissues at low levels (165,175). This PPAR isoform is activated by long chain fatty acids, such as palmitate, stearate, linoleic acid, linolenic acid, eicosapentaenoic acid (EPA), and epoxyeicosatrienoic acids (EETs), as well as eicosanoids, such as hydroxyl-eicosatetraenoic acid (HETE), 9 and 13 hydroxydecanoic acids (9, 13-HODE), and leukotriene B4 (176-180). Given that these ligands are fatty

acids and immune modulators, it is not surprising that PPARα regulates the expression of genes involved in lipid metabolism, fatty acid oxidation, and inflammation (171,181-184).

Much of the information regarding PPARα function has been elucidated by studies in PPARα deficient mice, which have impaired hepatic fatty acid uptake and oxidation, hypoglycemia, elevated levels of LTB4, and increased inflammation (165,176,181). These findings illustrate a central role for PPARα in regulating whole body metabolism; a decrease in fatty acid oxidation causes an over-compensatory shift towards glucose for energy, resulting in hypoglycemia. Additionally, in the absence of PPARα, the increased inflammatory state may be explained by an increase in NFκB activity and inflammatory gene expression, as well as increased levels of the proinflammatory lipid mediator, LTB4 (171,176).

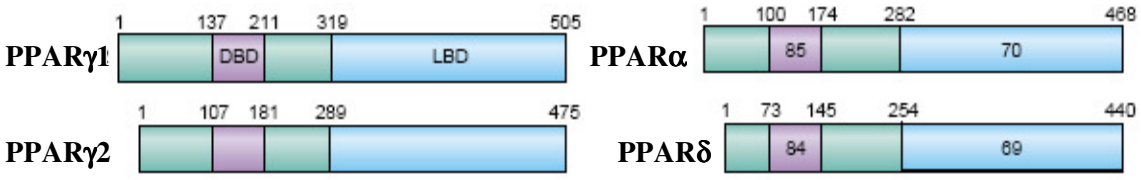
Activation of PPARα by synthetic agonists, called fibrates, also improves serum lipid levels by decreasing plasma triglyceride and VLDL concentrations. This occurs partly by enhanced hepatic fatty acid uptake and catabolism and increased HDL concentration (185,186). Consistent with the mouse knock out model, synthetic agonists also decrease the expression of inflammatory genes, such as IL-6, COX2, VCAM1, and tissue factor (TF) (165,182,184,186). These hypolipidemic and anti-inflammatory effects of PPARα activation may explain the beneficial effects of fibrates on atherosclerotic lesion progression found in clinical studies (186). However, there is also evidence suggesting PPARα is involved in hypertension and diabetic cardiomyopathy, indicating potential pathological consequences of PPARα activation (165,187). Perhaps the

metabolic outcome of its activation depends on the chemical structure of the ligand; different ligands may induce different conformational changes and result in the recruitment of distinct co-activator complexes (17).

PPARγ

The PPARγ gene gives rise to at least three mRNAs, PPARγ1, PPARγ2, and PPARγ3, that differ in their 5' end due to alternate promoter usage and splicing. This results in identical proteins from PPARγ1 and PPARγ3, but an additional 30 amino acids on the amino terminal end of PPARγ2 (167). This latter PPARγ isoform is expressed primarily in the adipose tissue, whereas PPARγ1 is expressed in the adipose tissue, skeletal muscle, liver, heart, intestine, vaculature, and immune cells (165,188-190). Similar to PPARα, extensive data support PPARγ as an integral component of lipid metabolism, glucose homeostasis, inflammation, and atherosclerosis. The beneficial effects of the highly specific synthetic PPARγ agonists, thiazolidinediones, illustrate the important role of PPARγ in the metabolic syndrome. These agonists lower serum glucose, insulin, FFA, cholesterol, and triglyceride levels as well as reduce inflammation and atheroslerosis (6,17,191-194). The mechanism of action of thiazolidinediones is mainly through PPARγ activation in the adipose tissue, promoting lipid storage and metabolism in the adipose tissue and reducing lipid accumulation in peripheral tissues, as well as normalizing adipokine secretion (see Figure 1.4) (6,195,196).

A.



B.

	PPARα	PPARγ	PPARδ
			
Tissue Expression	Liver Heart Kidney Adrenal	Adipose tissue Spleen Adrenal Colon	Many tissues
Cell-specific Expression	Endothelial cells Macrophages Smooth muscle cells	Macrophages T cells	Many cell types
Biological Functions	Triglyceride-rich lipoprotein synthesis and metabolism β-oxidation Anti-inflammation	Fat cell development Glucose homeostasis Anti-inflammation	Endothelial biology Energy utilization Lipid metabolism
Ligands	PUFAs 8(S)-HETE	PUFAs 15d-PGJ2 13-HETE 9-HODE	PUFAs
Disease targets	Hypertriglycerdemia	Type 2 diabetes	Metabolic syndrome?
Drugs	Fibrates	TZDs	

Figure 1.5. Summary of the PPAR subfamily of nuclear receptors. A. Diagram showing the domain structure of PPARγ2, PPARγ1, PPARα, and PPARδ. With the exception of the N terminal 30 amino acids of PPARγ2, both PPARγ isoforms are identical. The percentage similarity of PPARγ with PPARα and PPARδ in the LBD and DBD are shown. B. Listed are the major sites of expression in the body, major biological roles, naturally occurring ligands, and classes of drugs that are in clinical use for each PPAR (6,7).

Due to the efficacy of synthetic PPARγ agonists in improving metabolic parameters, intense investigation has surrounded the identification of the endogenous ligands for PPARγ. There have been a number of proposed endogenous PPARγ ligands, including long chain polyunsaturated fatty acids, lysophosphatidic acid, nitrated oleic and linoleic acid, HODEs, and 15d-prostaglandin J2 (178,197-201). However, the physiological significance of these ligands remains unclear; while PPARγ activity can be induced by these ligands *in vitro*, it is not known if they are functional ligands *in vivo*. Taken together, evidence suggests that either PPARγ is a promiscuous receptor that can be activated by several different mechanisms, or there are one or more highly specific ligands that have not yet been identified.

PPARγ has the highest expression in the adipose tissue, where it is the master regulator of adipogenesis and also of adipocyte gene expression in fully differentiated adipocytes (98,202,203). Consistent with this, mice deficient in PPARγ only in mature adipocytes had a significant reduction in brown and white adipose tissue mass. These mice were hyperlipidemic, with elevated serum FFA and triglyceride levels, and decreased serum levels of the adipokines, leptin and adiponectin. Clamp studies on these mice revealed adipose and hepatic insulin resistance along with hepatic lipid accumulation. Surprisingly, however, whole body insulin sensitivity was maintained on a chow diet. This effect was most likely due to the maintenance of muscle insulin sensitivity, which is responsible for the majority of glucose disposal. Nevertheless, these mice did become insulin resistant on a high fat diet (196).

Another mouse model of fat specific PPARγ deletion resulted in one-week-old animals that had no adipose tissue, severe hepatic steatosis, and lipid accumulation in the heart, skeletal muscle, and kidneys, as well as insulin resistance and glucose intolerance. When these mice reached adulthood, serum FFA and triglyceride levels decreased, presumably due to increased FFA oxidation in the muscle, but they were still glucose intolerant due to elevated hepatic gluconeogenic enzymes (196). Together, these studies highlight the importance of adipose PPARγ in maintaining whole body insulin sensitivity, particularly in the presence of high fat diets. This is due the crucial role of PPARγ in regulating adipose tissue development and protecting peripheral tissues from lipotoxicity as well as regulating the expression of insulin sensitizing adipokines.

While the primary role of PPARγ is in the adipose tissue, this nuclear receptor is also responsible for maintaining whole body insulin sensitivity in other tissues. For example, deletion of PPARγ in the muscle and liver also resulted in whole body insulin resistance, despite its low expression in these tissues. In the muscle, PPARγ deficiency resulted in secondary insulin resistance in the adipose tissue and liver; in the liver, PPARγ deficiency resulted in decreased hepatic steatosis, but increased adiposity, hyperlipidemia, and insulin resistance (196,204,205). Additionally, PPARγ deletion in macrophages resulted in disruption of alternative macrophage activation and subsequent diet induced obesity, insulin resistance, and glucose intolerance (190). Therefore, PPARγ appears to regulate insulin sensitivity by various mechanisms in distinct tissues and may promote the appropriate cross talk between these tissues necessary for proper metabolism.

PPARβ/δ

Similar to PPARα and PPARγ, PPARδ (also known as PPARβ) is activated by long chain fatty acids, including polyunsaturated fatty acids and eicosanoids, and its target genes are likewise involved in lipid metabolism (165,177). PPARδ is ubiquitously expressed, suggesting a role in basic cellular functions, such as lipid synthesis and turnover, but it may also have a role in cell proliferation and differentiation (165,175). However, the expression of PPARδ is the highest in skeletal muscle, where it regulates genes involved in fatty acid metabolism, mitochondrial respiration, thermogenesis, and the programming of muscle fiber type (206,207)

Since there is still not a synthetic agonist used clinically in humans, most of the information regarding PPARδ function has been generated in rodent models. Mouse knockout studies support its function in fundamental cell processes, since mice die at an early age and have placental and myelination defects, altered wound healing and responses to skin inflammation, and decreased adipose tissue mass (175). However, adipocyte-specific PPARδ deletion does not reduce fat mass, suggesting PPARδ is not specifically required for fat storage in the adipose tissue (165). Conversely, activation of PPARδ with a high affinity ligand, GW501516, has been shown to increase HDL levels, decrease inflammation, cause weight loss, and improve whole body insulin sensitivity in mice (175,207). The improved metabolic phenotype appeared to be mediated by increased lipid catabolism and oxidative phosphorylation in the adipose and muscle and decreased hepatic glucose output. The effects in the liver were attributed to increased glycolysis and flux through the pentose phosphate shunt and enhanced fatty acid

synthesis (207). Additionally, PPARδ may regulate some aspects of alternative macrophage activation, which has been shown to positively regulate whole body insulin sensitivity (175,190). Taken together, PPARδ appears to be a potent regulator of glucose homeostasis by controlling metabolic gene expression in the major insulin responsive tissues, but future studies need to elucidate the role of this receptor in humans.

Specific Aims

Maintaining adipocyte function is critical to maintaining insulin sensitivity and glucose homeostasis due to the ability of adipocytes to safely store excess free fatty acid as triglyceride and secrete factors, called adipokines, which enhance insulin sensitivity in peripheral tissues. The development of adipose tissue occurs through adipogenesis, a process whereby preadipocyte precursors differentiate into mature adipocytes. When exposed to a high calorie diet, the adipose tissue will expand due to increased triglyceride storage within the adipocytes and increased cell number due to adipogenesis. Both of these processes are crucial to maintaining insulin sensitivity and glucose homeostasis when exposed to a high calorie diet. A critical regulator of both triglyceride storage and adipogenesis is the nuclear receptor, PPARγ. Not only is the activation of PPARγ necessary and sufficient to drive these processes, but also the depletion of PPARγ results in decreased adipocyte specific gene expression, lipid storage, and insulin sensitivity. Therefore, PPARγ is a master regulator of adipogenesis, adipocyte function, and whole body insulin sensitivity.

Understanding the endogenous regulation of PPARγ has been an intense area of research since the discovery that this receptor mediates the therapeutic effects of

thiazolidinediones (TZDs) in glucose homeostasis. PPARγ activity can be regulated by its expression level and through ligand dependent and independent activation (167,168,173,178). Manipulating these endogenous regulatory mechanisms may provide the therapeutic benefits found with TZDs, while not inducing the same negative side effects, such as heart failure and edema (17). Therefore, identifying these endogenous regulatory mechanisms is critical to developing safer, more effective diabetic therapies.

Equally important to understanding PPARγ regulation is identifying the downstream mechanisms by which PPARγ activation improves insulin sensitivity. Although increased lipid storage in the adipose tissue is thought to mediate the insulin sensitizing effects of PPARγ agonists, the mechanisms by which PPARγ target genes control this adipocyte function are still unclear (4). Defining these parameters will provide insight into understanding adipocyte biology and further elucidate how this tissue regulates whole body insulin sensitivity.

Therefore, to enhance our understanding of PPARγ regulation and function, the specific aims of this study were:

1. **To identify novel mechanisms by which PPARγ is regulated by fatty acid metabolism in adipocytes.** siRNA mediated depletion of 24 enzymes in preadipocyte fibroblasts was performed to identify fatty acid metabolizing enzymes that are required for adipogenesis, a PPARγ dependent process. This siRNA-based screen identified Stearoyl CoA Desaturase 2 (SCD2) as a required factor for 3T3-L1 adipogenesis and was therefore further tested in fully differentiated adipocytes to determine if these effects were due to

specific regulation of PPARγ. These studies found that SCD2 is required for general protein synthesis and hence PPARγ protein expression and function in fully differentiated adipocytes and may explain the requirement for this fatty acid metabolizing protein during adipogenesis.

2. **To determine the mechanism by which a PPARγ target gene, Cidea, functions to increase lipid storage in adipocytes.** Immunofluorescent confocal microscopy was used to determine which domain of Cidea is responsible for its targeting to lipid droplets. Analysis of cells expressing either the full length or various domains of Cidea fused to HA or GFP determined that the carboxy terminus is necessary and sufficient for lipid droplet localization. Additionally, overexpression of Cidea leads to enhanced triglyceride storage in COS cells, which is also specifically dependent on the carboxy terminus. Furthermore, while the carboxy terminus is necessary for lipid droplet localization, the amino terminus is required to induce the formation of large and few lipid droplets from small, dispersed lipid droplets. Thus, the carboxy terminus is required to increase triglcyeride storage, but the amino terminus is required for this yet unrecognized function of Cidea to change the lipid droplet morphology.

Chapter II: Stearoyl CoA Desaturase 2 is required for PPARγ expression and adipogenesis in cultured 3T3-L1 cells

Disclaimer: The author performed all experiments, except Sarah Nicoloro generated affymetrix gene chip data shown in Figure 2.1 (gene changes during 3T3-L1 adipogenesis and in mice fed a normal versus high fat diet) and Juerg Straubhaar generated the affymetrix database from the gene chip data for analysis and the heat map shown in Figure 2.6.

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Abstract

Based on recent evidence that fatty acid synthase and endogenously produced fatty acid derivatives are required for adipogenesis in 3T3-L1 adipocytes, we conducted a siRNA-based screen to identify other fatty acid metabolizing enzymes that may mediate this effect. Of 24 enzymes screened, Stearoyl CoA Desaturase 2 (SCD2) was found to be uniquely and absolutely required for adipogenesis. Remarkably, SCD2 also controls the maintenance of adipocyte-specific gene expression in fully differentiated 3T3-L1 adipocytes, including the expression of SCD1. Despite the high sequence similarity between SCD2 and SCD1, silencing of SCD1 did not downregulate 3T3-L1 cell differentiation or gene expression. SCD2 mRNA expression was also uniquely elevated 44 fold in adipose tissue upon feeding mice a high fat diet, while SCD1 showed little response. The inhibition of adipogenesis caused by SCD2 depletion was associated with a decrease in PPARγ mRNA and protein, while in mature adipocytes loss of SCD2 diminished PPARγ protein levels, with little change in mRNA levels. In the latter case, SCD2 depletion did not change the degradation rate of PPARγ protein, but decreased the

metabolic labeling of PPARγ protein using [35S] methionine/cysteine, indicating protein translation was decreased. This requirement of SCD2 for optimal protein synthesis in fully differentiated adipocytes was verified by polysome profile analysis, where a shift in the mRNA to monosomes was apparent in response to SCD2 silencing. These results reveal that SCD2 is required for the induction and maintenance of PPARγ protein levels and adipogenesis in 3T3-L1 cells.

Introduction

The ability of adipocytes to sense and respond to circulating fatty acid levels is important in maintaining the proper balance between fatty acid storage and fatty acid release for energy utilization. In the case of energy excess, fatty acids are stored in the form of triglyceride, and new adipocytes are generated to efficiently metabolize amino acids, glucose, and fatty acids to triglyceride (196). The key regulator of adipogenesis, the process whereby preadipocytes differentiate into fully mature adipocytes, is the ligand-activated nuclear receptor, PPARγ (208). Cultured mouse 3T3-L1 preadipocytes are an excellent model system for the study of adipogenesis. These cells differentiate into adipocytes with multilocular lipid droplets through a transcriptional cascade beginning with the rapid and transient expression of C/EBPβ and C/EBPδ (98,209). The upregulation of these transcription factors precedes the expression of PPARγ and C/EBPα, which are critical for the completion of adipogenesis as well as the maintenance of adipocyte-specific gene expression in fully differentiated cells (98,209). Other transcription factors have also been shown to play significant roles in adipogenesis and adipocyte biology (for reviews, see (98,210,211). However, since PPARγ controls the

expression of large sets of genes required to maintain the adipocyte phenotype, including C/EBPα itself, a loss in the activity or expression of PPARγ leads to a loss in adipocyte function (203).

While it is unclear whether ligands are actively modulating PPARγ activity in fully differentiated adipocytes, ligand-mediated activation of PPARγ appears to be required for transcriptional activity during adipogenesis (174). The requirement for a PPARγ ligand during differentiation is supported by the fact that preadipocytes will not differentiate in the presence of a PPARγ antagonist that blocks ligand binding. In addition, it appears that differentiating adipocytes can fully synthesize a PPARγ ligand, since preadipocytes will differentiate and produce a PPARγ ligand in the absence of exogenous fatty acids (201,212). Although the ligand has not been identified, ligand production is dependent on sterol regulatory element binding protein-1 (SREBP1), since the expression of a dominant negative SREBP1 blocks adipogenesis and further addition of a PPARγ synthetic ligand restored differentiation. All together, this suggests that a PPARγ ligand is necessary for adipogenesis and that ligand production relies on SREBP1 activity. Furthermore, the overexpression of SREBP1 in adipocytes apparently increases ligand production (213), while inhibition of acetyl CoA carboxylase (ACC) (214) or fatty acid synthase (FAS) (215) inhibits adipogenesis. SREBP1 is a transcription factor that controls the expression of many fatty acid metabolizing enzymes, including ACC and FAS. Since ACC and FAS work sequentially to produce palmitate, it is possible that SREBP1 promotes PPARγ ligand production through a pathway involving ACC and FAS. While there may be several explanations for the requirement of SREBP1, ACC, or

FAS for adipogenesis apart from PPARγ ligand production, these studies do support the notion that endogenously synthesized fatty acids are required for adipogenesis.

Since PPARγ has a large hydrophobic ligand binding domain (216) and activation occurs in response to fatty acids (217), endogenous long chain fatty acids or their derivatives have been proposed as natural ligands. These include oleate, linoleate, nitrolinoleate (LNO_2), nitro-oleate ($OA\text{-}NO_2$), 9-hydroxydecaenoic acid (9-HODE), arachadonic acid, and 15-deoxy-prostaglandin J2 (198,200,201,218,219). Despite the many proposed ligands, LNO_2 and $OA\text{-}NO_2$ are the only fatty acids with a high binding affinity, but it has not yet been verified that these fatty acids are truly endogenous PPARγ ligands in adipocytes (200,219). Since several low affinity fatty acid ligands activate PPARγ (198-200,218,220), this nuclear receptor may instead serve as a general fatty acid sensor, allowing proper expression of fatty acid metabolizing enzymes and the generation of new adipocytes.

Since adipocytes express multiple fatty acid metabolizing enzymes, these cells apparently produce highly diverse lipid species that may affect cellular signaling events, including PPARγ activation. Thus, the aim of the present study was to identify enzymes involved in fatty acid synthesis or metabolism that may mediate such signaling pathways through their fatty acid products. To achieve this goal, we set up a screen in which 24 fatty acid metabolizing enzymes were individually depleted using siRNA oligonucleotides in order to identify enzymes that are required for adipocyte-specific gene expression. Through this siRNA screen, we identified the fatty acid Δ9 desaturase, Stearoyl CoA Desaturase 2 (SCD2), as a required enzyme for 3T3-L1 cell adipogenesis

and for the maintenance of adipocyte-specific gene expression in fully differentiated cells. Importantly, SCD2 was found to be required for PPARγ induction during differentiation of 3T3-L1 cells and for PPARγ expression in fully differentiated adipocytes. Related to this latter effect, SCD2 expression was found to promote protein translation, secondarily affecting PPARγ protein levels. Surprisingly, although SCD1 and SCD2 exhibit high sequence similarity, are both expressed in the endoplasmic reticulum of the adipocyte, and are predicted to produce the same products, SCD1 depletion failed to attenuate PPARγ expression or adipogenesis. Therefore, these results identify SCD2 as a key regulator of adipocyte function by promoting PPARγ protein synthesis, and reveal a novel and specific role for SCD2 versus SCD1 in the adipocyte.

Experimental Procedures

Animals- All procedures were carried out following the University of Massachusetts Medical School Institution Animal Care and Use Committee (UMMA-IACUC) guidelines. Four-week-old male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and mantained in a 12hr light/dark cycle. Half of the mice were fed a standard mouse chow (10%kcal fat) and the other half was fed a high fat diet (55%kcal fat) ad libitum for eighteen weeks. The animals were fasted for 18 hours prior to harvesting the tissues. Animals were sacrificed and epididymal fat pads were harvested from the mice and placed in KRH buffer pH 7.4 supplemented with 2.5% BSA and RNA was collected using TRIzol (Invitrogen) for subsequent Affymetrix GeneChip analysis.

Materials- Rosiglitazone was purchased from Biomol (Plymouth Meeting, PA). The proteasome inhibitor, MG132, was purchased from Calbiochem Biotechnology (San Diego, CA). Mouse monoclonal anti-PPARγ, mouse monoclonal anti-AKT1, mouse monoclonal anti-β catenin, rabbit polyclonal anti-PPARγ, and rabbit polyclonal anti-C/EBPα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-AMPK, eEF2, RS6K, and eIF2α were purchased from Cell Signaling (Danvers, MA). Protein A-Sepharose beads were purchased from Sigma. Rabbit PTEN antiserum was purchased from Upstate Biotechnology (Charlottesville, VA). The MTS cell proliferation assay kit and TUNEL kit were purchased from Promega (Madison, WI). The iScript cDNA synthesis kit and the iQ SYBR green supermix kit were purchased from Bio-Rad (Hercules, CA). [^{35}S] methionine/cysteine was purchased from Perkin Elmer (Waltham, MA).

SiRNA Duplexes- The siRNA purchased from Dharmacon Inc.(Lafayette, CO) were designed to target the following cDNA sequences: scrambled, 5'-CAGTCGCGTTTGCG ACTGG-3'; SCD2, 5'-GAGCAGATGTTCGCCCTGATT-3'; PPARγ, 5'-GACATGAA TTCCTTAATGA-3'; SCD1,5'GCCTAGAACTGATAACT AATT -3'; and proprietary SMART-pool siRNA duplexes were used to target all other transcripts.

Cell culture and electroporation-3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium DMEM supplemented with 10% fetal bovine serum, 50 μg/mL streptomycin, and 50 units/mL penicillin (45). For experiments performed during differentiation, fibroblasts were cultured for 7 days and $5x10^6$ cells were electroporated with 20 nmol siRNA. The electroporation was performed using a Bio-Rad gene pulser II

at the setting of 0.18 kV and 960 microfarads. Immediately after electroporation, the cells were reseeded into 2 wells of a 6 well plate. After 24 hours, differentiation media consisting of 2.5 µg/mL insulin, 0.25 µM dexamethasone, and 0.5 mM IBMX (3-Isobutyl-1-methyl-xanthine) in the culture media described above was added for 72 hours in the absence or presence of 1µM rosiglitazone. After 72 hours, differentiation media was replaced with culture media for an additional 24 hours, and then RNA or protein was collected. For experiments in mature adipocytes, fibroblasts were cultured for 8 days, differentiated into mature adipocytes as described above, and cultured for an additional 7 days. Adipocytes were then electroporated (20 nmol siRNA/ $5x10^6$ cells) as described above. After electroporation, cells were reseeded into multiple-well plates and RNA or protein was collected 4-72 hours post-electroporation.

Affymetrix Gene Chip Analysis- Total RNA was collected from day 10 adipocytes after 72 hours of siRNA treatment or from preadipocyte fibroblasts, adipocytes, and primary fat tissue as described (221). Subsequent reactions were carried out as already described (222).Only signals considered present were used for further analysis. If more than one probe is present, only one representative probe is shown.

RNA isolation and Real Time-PCR- Total RNA was collected using TRIzol (Invitrogen, Carlsbad, CA) and reverse transcription and real time-PCR analysis were carried out as already described (1,222). Primers were chosen from the PrimerBank online database (http://pga.mgh.harvard.edu/primerbank) (223). AKT1 was used as the internal control.

Immunoblotting- Cells were solubilized with lysis buffer containing 25 mM Hepes (pH 7.5), 0.5% Nonidet P-40, 1mM EGTA, 1 mM EDTA, 1% SDS, 12.5 mM NaF, 5mM sodium pyrophosphate, 5 mM β-glycerophosphate, 5 mM sodium vanadate, 1mM PMSF, 5 μg/mL aprotinin, and 10 μg/mL leupeptin. Protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL) and then resolved on a 8% SDS-PAGE gel, electrotransferred to nitrocellulose, blocked with 5% BSA and 5% nonfat milk in TBST (0.05% Tween 20 in Tris-buffered Saline), washed with TBST, and incubated with specific antibody at 4°C, overnight. The blots were then washed with TBST and a horseradish peroxidase anti-mouse or anti-rabbit secondary antibody was applied. Proteins were visualized using an enhanced chemiluminescent substrate kit (Amersham Pharmacia Biotech, Piscataway, NJ) and immunoblot band intensities were quantified by scanning densitometry using Photoshop.

Oil Red O staining- Cells were fixed with 4% formaldehyde for 1 hour at room temperature, washed three times with PBS, permealized with P-buffer (0.5% Triton X-100, 1% fetal bovine serum, and 0.05% sodium azide) for 20 minutes, incubated with Oil Red O solution (5 mg/mL Oil Red O solid dissolved in isopropanol, then diluted to a 60% working solution with ddH_20) for 30 minutes, washed three times with distilled water, and analyzed by light microscopy or visual inspection.

^{35}S-Methionine/cysteine labeling and immunopreciptiation of PPARγ- Seventy two hours after electroporation of cells with siRNA, one 100 mm plate of cells was starved of methionine and cysteine for 2 hours and then labeled with 500 uCi of [^{35}S] methionine/cysteine for 4 hours. Cells were then lysed in ice-cold buffer containing 25

mM Hepes (pH 7.5), 0.5% Nonidet P-40, 1mM EGTA, 1 mM EDTA, 1% SDS, 12.5 mM NaF, 5mM sodium pyrophosphate, 5 mM β-glycerophosphate, 5 mM sodium vanadate, 1mM PMSF, 5 μg/mL aprotinin, and 10 μg/mL leupeptin. Total cell lysates of 1 mg of protein were immunoprecipitated overnight with 20 μg of mouse monoclonal antibody against PPARγ followed by incubation with 50 μl of protein A-sepharose beads for 2 h at 4°C. The beads were then washed 5 times with lysis buffer before boiling for 5 minutes in Laemmli buffer. Protein was then separated on an 8% SDS gel, transferred to nitrocellulose, and exposed to a phosphor screen for 60 hours. The screen was then visualized with a Phosphorimager (Molecular Dynamics). The nitrocellulose was then immunoblotted as described above using goat polyclonal antibody against PPARγ to detect the efficiency of the immunoprecipitation.

Polysome profile and RT-PCR- Polysome profiles were generated as described previously (224-226). Briefly, after siRNA transfection, cells were reseeded into one 10-cm dish. After 24 or 72 hours, cycloheximide (Sigma) was added at a final concentration of 100 mg/ ml for 10 min. Cells were then washed with PBS, trypsinized, pelleted and resuspended in polysome buffer (20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl2) containing 150 μg/mL cycloheximide and 100 units/mL Rnasin (Promega). After determining the cell number in each sample, Triton-X 100 was added to the cell suspension at a final concentration of 0.3% (v/v) and cells were passed through a 27-gauge needle five times to ensure lysis. The nuclei were then pelleted by centrifugation at 4°C and 12,000 x *g* for 5 min. The supernatant was then layered on a linear 10-50% sucrose gradient in polysome buffer containing 10μg/mL cycloheximide and 3.3

units/mL Rnasin and the gradients were centrifuged in a Beckmann SW41Ti Rotor at 141,000 x *g* at 4°C for 4 h. The gradients were fractionated into 1-ml fractions, and the UV absorption at *A*254 was recorded. Twelve fractions were collected and RNA was then extracted from each fraction using TRIzol (Invitrogen). Equal volumes of each fraction were then reverse transcribed and real time PCR was performed as already described (222).

Results

Expression of fatty acid metabolizing enzymes in cultured adipocytes and primary adipose tissue. In order to establish a siRNA-based screen of broad scope, we first identified key enzymes in the major pathways of fatty acid metabolism that are clearly expressed in both mouse 3T3-L1 adipocytes and primary mouse adipose tissue. Figure 2.1 illustrates 8 pathways of fatty acid metabolism that were considered for our studies, which include ω-oxidation, β-oxidation, α-oxidation, elongation, desaturation, nitration, epoxygenation/hydroxylation, and isomerization. Identification of the enzymes shown in Figure 2.1 was accomplished by Affymetrix GeneChip microarray analysis of samples obtained from 3T3-L1 preadipocytes versus 3T3-L1 adipocytes (6 days after initiation of differentiation) and from the adipose tissue of mice fed a normal diet versus a high fat diet for 16 weeks. Table 2.1 presents the list of specific genes we selected by this analysis, all of which were found to be significantly expressed in both model systems.

The fatty acid metabolizing enzymes shown in Table 2.1 and Figure 2.1 allow the generation of many different fatty acid products and derivatives from the same initial fatty acid substrate. A saturated fatty acid, such as palmitate, may be: 1.) ω- oxidized by

the cytochrome P450 enzyme CYP4f16, forming a dicarboxylic acid; 2.) β- oxidized in peroxisomes by the acyl CoA oxidases, ACOX1 and ACOX2, or in the mitochondria by the acyl CoA dehydrogenases, ACADl and ACADvl, cleaving two carbons per cycle from the fatty acid; 3.) α- oxidized in peroxisomes by phytanoyl CoA hydroxylase, cleaving one carbon per cycle from the fatty acid; 4.) elongated by ELOVL1, ELOVL3, ELOVL5, and ELOVL6, which are present in the endoplasmic reticulum, adding two carbons per cycle to the fatty acid; or 5.) desaturated by various enzymes found in the endoplasmic reticulum, including stearoyl CoA desaturase 1 or stearoyl CoA desaturase 2, forming a cis-double bond between the 9 and 10 carbons, or fatty acid desaturase 1, fatty acid desaturase 2, or fatty acid desaturase 3, forming a cis-double bond between 5 and 6, 6 and 7, and possibly the 4 and 5 carbons, respectively. In addition, the double bond in an unsaturated fatty acid may change position through the isomerase, ALOXe3, found in the cytoplasm, be nitrated by nitric oxide species produced by nitric oxide synthase, found in the cytoplasm, or be oxidized by the cytochrome P450 enzymes, CYP2f2, CYP2c55, CYP20a1, CYP26b1, CYP1b1, found in the endoplasmic reticulum, adding an epoxide, hydroxyl, or peroxyl group to the fatty acid. An epoxide may then be further metabolized by the epoxide hydrolase, EPHX1, present in the endoplasmic reticulum, or EPHX2, present in the cytoplasm, producing dihydrodiols. Additionally, these pathways can operate in tandem, changing the carbon length or position of a side

Gene Symbol	Day 0 Signal	Day 6 Signal	3T3-L1 Adipogenesis (fold change)	*P* value	NC Signal	HFD Signal	NC vs HFD (fold change)	*P* value
Pparg	1397.00	7378.12	5.28	0.047	4522.00	2695.19	-1.68	0.011
Fasn	1853.79	10802.39	5.83	0.003	2679.69	7938.53	2.96	0.021
Scd1	313.88	11057.21	35.23	0.009	7304.06	9111.11	1.25	0.044
Scd2	4895.31	14465.96	2.96	0.009	65.83	2900.53	44.06	0.000
Scd3	103.88	448.77	4.32	0.010	11.60	13.67	1.18	0.071
Fads1	1648.81	3117.44	1.89	0.110	370.41	431.98	1.17	0.276
Fads2	1317.32	3045.47	2.31	0.170	65.57	139.48	2.13	0.002
Fads3	329.77	651.62	1.98	0.182	1020.65	1994.35	1.95	0.001
Elovl1	2111.08	2188.70	1.04	0.847	244.99	358.40	1.46	0.005
Elovl2	23.51	24.17	1.03	0.622	6.88	6.72	-1.02	0.690
Elovl3	232.19	1475.64	6.36	0.004	12.22	25.29	2.07	0.009
Elovl4	27.54	28.87	1.05	0.471	8.27	8.14	-1.02	0.603
Elovl5	2088.32	2522.17	1.21	0.354	1302.75	2108.06	1.62	0.006
Elovl6	240.29	136.43	-1.76	0.075	47.17	239.63	5.08	0.010
Acadvl	2210.50	9122.99	4.13	0.001	2373.59	2792.72	1.18	0.241
Acadl	1435.63	3993.85	2.78	0.001	4052.60	5396.23	1.33	0.016
Acox1	1198.38	5517.91	4.60	0.000	4693.07	4998.84	1.07	0.520
Acox2	189.52	1126.28	5.94	0.011	39.90	45.21	1.13	0.540
Acox3	204.71	430.64	2.10	0.002	107.82	122.26	1.13	0.200
Ephx1	2089.01	2380.93	1.14	0.856	590.19	1733.25	2.94	0.006
Ephx2	577.50	1980.53	3.43	0.003	5111.05	6246.07	1.22	0.025
Nos3	125.62	257.42	2.05	0.010	448.31	661.26	1.48	0.029
Cyp2f2	218.51	1251.98	5.73	0.011	483.70	166.21	-2.91	0.024
Cyp2c55	612.21	364.27	-1.68	0.049	9.09	9.13	1.00	0.960
Cyp4f16	356.39	252.67	-1.41	0.078	67.92	72.98	1.07	0.539
Cyp20a1	312.63	323.74	1.04	0.795	152.89	146.35	-1.04	0.781
Cyp1b1	969.70	408.26	-2.38	0.093	156.72	54.98	-2.85	0.012
Cyp51	673.89	3924.58	5.82	0.007	116.02	472.96	4.08	0.000
Aloxe3	411.81	443.56	1.08	0.356	59.06	63.00	1.07	0.489

Table 2.1. Affymetrix Gene Chip analysis of fatty acid metabolizing enzymes in differentiating 3T3-L1 adipocytes and primary adipocytes from mice fed a normal chow or high fat diet. RNA was collected from 3T3-L1 cells prior to differentiation or 2, 4, and 6 days post differentiation and subjected to Affymetrix Gene Chip analysis. RNA from three different samples was collected and pooled and then analyzed on one array; each experiment was done in triplicate resulting in a total of 9 RNA samples and three arrays for each timepoint. From the primary adipocytes, RNA was collected from 23 mice fed a normal chow diet and 14 mice fed a high fat diet for 18 weeks. The mice were divided into three groups within each diet condition, and the RNA from each group was pooled and then analyzed on one array, resulting in three arrays for each diet condition. Shown are representative values for the fold changes in gene expression during adipogenesis or due to high fat diet. Boldface shows values that are significantly up-regulated or down-regulated in response to 3T3-L1 differentiation. The values obtained for SCD1 and SCD2 are in the rectangle. The asterisk denotes a p value < 0.05.

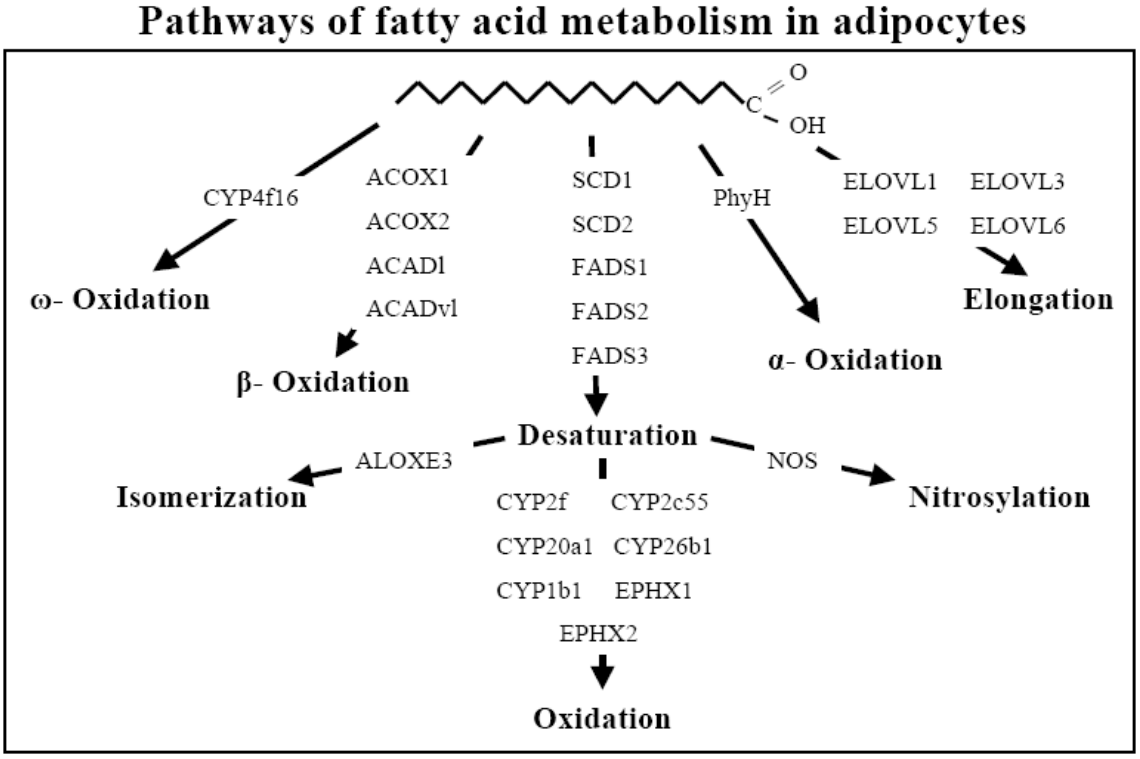
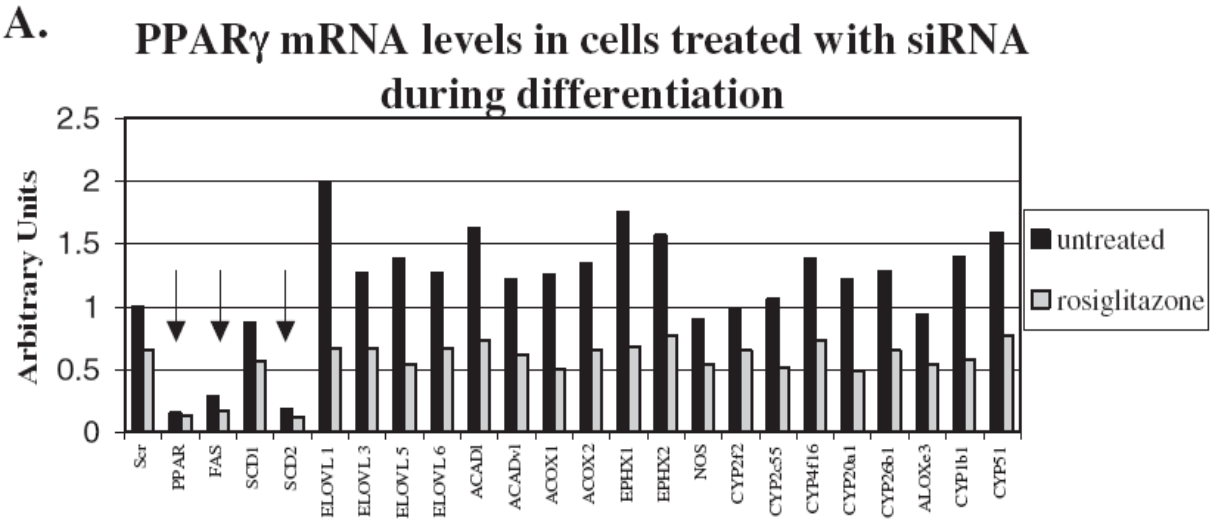


Figure 2.1. Diagram showing the multiple pathways of fatty acid metabolism in adipocytes. A saturated fatty acid may be ω- oxidized, forming a dicarboxylic acid; β- oxidized, cleaving two carbons per cycle from the fatty acid; α- oxidized, cleaving one carbon per cycle from the fatty acid; elongated, adding two carbons per cycle to the fatty acid; or desaturated, forming a cis-double bond between the 9 and 10, 5 and 6, 6 and 7, or possibly the 4 and 5 carbons. The double bond in a desaturated fatty acid may then change position through isomerases or be nitrated or oxidized, producing various side groups on the fatty acid (see text for further details).

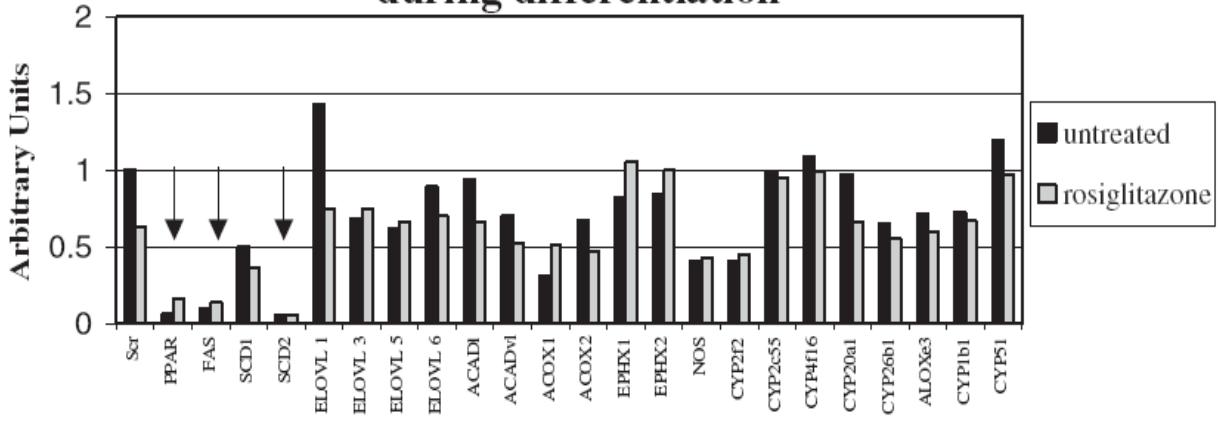
group or double bond within the fatty acid. Since a fatty acid produced from any one of these pathways may affect cell signaling events or other processes, these enzymes listed in Table 1 were targeted in a siRNA-based screen to determine if they affect adipocyte gene expression in 3T3-L1 cells.

SCD2, but not SCD1, is required for 3T3-L1 adipogenesis. To identify fatty acid metabolizing enzymes that are required for 3T3-L1 adipogenesis, siRNA oligonucleotides directed against each of the enzymes identified by the microarray analysis in Table 2.1 were electroporated into 3T3-L1 preadipocytes prior to differentiation. Since PPARγ appears to be activated by an endogenous ligand during adipogenesis (174,198-200,218), we reasoned that if a depleted enzyme is required specifically for the production of a PPARγ ligand, the addition of an exogenous ligand may reverse the effect of such enzyme depletion. Thus, in our screen the enzymes were also depleted in the presence of the PPARγ specific ligand, rosiglitazone, as a control. The initial screen monitored the mRNA transcript levels by real time PCR of the differentiation-induced proteins, PPARγ and GLUT4 (Figure 2.2). As expected, the well-established required factors for adipocyte differentiation, PPARγ and FAS (215), did indeed attenuate PPARγ and GLUT4 expression in this screen when depleted by siRNA, and acted as positive controls. In addition, rosiglitazone treatment did not restore PPARγ or GLUT4 levels upon siRNA-based depletion of PPARγ (Figure 2.2 and Supplementary Figure 2.1). Importantly, of the remaining 24 enzymes screened, only SCD2 depletion potently inhibited gene expression during adipogenesis (Figure 2.2A and 2.2B).

A.


PPARγ mRNA levels in cells treated with siRNA during differentiation
Arbitrary Units
2.5
2
1.5
1
0.5
0
Scr
PPAR
FAS
SCD1
SCD2
ELOVL1
ELOVL3
ELOVL5
ELOVL6
ACADl
ACADvl
ACOX1
ACOX2
EPHX1
EPHX2
NOS
CYP2f2
CYP2c55
CYP4f16
CYP20a1
CYP26b1
ALOXe3
CYP1b1
CYP51
untreated
rosiglitazone

B.


Glut4 mRNA levels in cells treated with siRNA during differentiation
Arbitrary Units
2
1.5
1
0.5
0
Scr
PPAR
FAS
SCD1
SCD2
ELOVL 1
ELOVL 3
ELOVL 5
ELOVL 6
ACADl
ACADvl
ACOX1
ACOX2
EPHX1
EPHX2
NOS
CYP2f2
CYP2c55
CYP4f16
CYP20a1
CYP26b1
ALOXe3
CYP1b1
CYP51
untreated
rosiglitazone

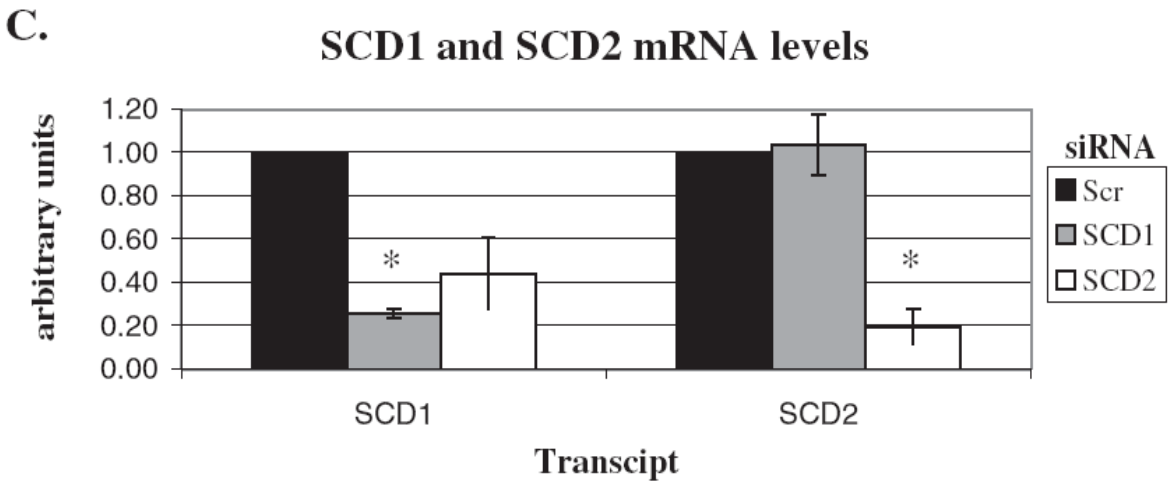


Figure 2.2. Depletion of SCD2 inhibits the mRNA expression of adipogenic markers in differentiating 3T3-L1 adipocytes. Confluent fibroblasts were electroporated with scrambled nucleotide as a control or SMART-pool siRNA directed against various fatty acid metabolizing enzymes and reseeded in duplicate wells as described in materials and methods. After 24 hours, the cells were differentiated in the presence or absence of 1 μM rosiglitazone. On the fourth day of differentiation, RNA was collected to determine the expression of (A) PPARγ, (B) GLUT4, or (C) SCD1 and SCD2 by real time PCR using AKT1 as an internal control. The results shown in (A) and (B) were performed once as part of the initial screen; the results shown in (C) are an average of three independent experiments and the asterisk denotes a p value < 0.01.

Interestingly, despite the predicted similarity in substrate selectivity between SCD1 and SCD2 (227), depletion of SCD1 in 3T3-L1 cells did not inhibit PPARγ or GLUT4 expression (Figures 2.2 and Supplementary Figure 2.1). Furthermore, the addition of rosiglitazone did not restore the transcript levels of PPARγ or GLUT4 upon loss of SCD2 or FAS (Figure 2.2 and Supplementary Figure 2.1). This suggests that if SCD2 and FAS are involved in PPARγ ligand production during adipogenesis, the enzymes are also required for an independent function.

In an attempt to confirm and extend these findings, expression of PPARγ protein was measured in a second screen of 10 enzymes, again revealing that SCD2, but not SCD1, is absolutely required for expression of this transcription factor (Figure 2.3A). In addition, when preadipocytes differentiate into adipocytes, the cells become smaller and rounder, losing their fibroblastic morphology. The cells also acquire the ability to accumulate lipid in the form of triglyceride, appearing as lipid droplets in the cytoplasm (98,201). Oil Red O staining of accumulated neutral lipids in cells four days after the initiation of differentiation confirms that PPARγ and SCD2 are required for the lipid accumulation (Figure 2.3B) and morphological changes (data not shown) that occur during adipogenesis, while SCD1 is not. Therefore, SCD2, but not SCD1, is required for several aspects of adipogenesis, including the induction of adipocyte specific genes, the increase in lipid accumulation, and the gain in the adipocyte morphology.

To verify that the inhibition of adipogenesis by depletion of PPARγ, FAS, or SCD2 is not due to general toxicity, metabolic activity was measured in the cells using the tetrazolium compound, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-

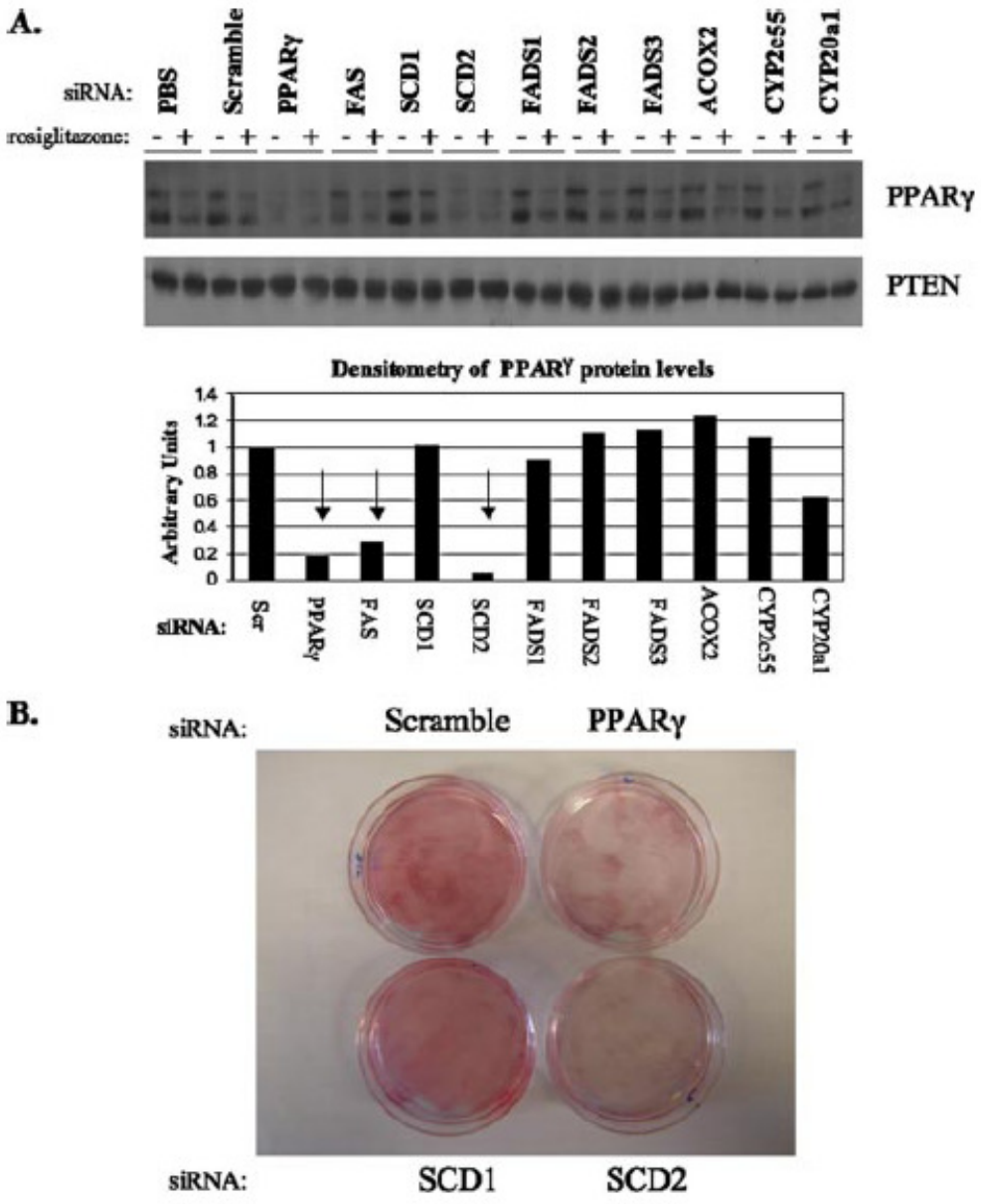


Figure 2.3. Depletion of SCD2 inhibits adipogenic protein expression and morphological changes in differentiating 3T3-L1 adipocytes. Confluent fibroblasts were electroporated with scrambled nucleotide as a control or SMART-pool siRNA directed against various fatty acid metabolizing enzymes and reseeded in duplicate wells as described in materials and methods. After 24 hours, the cells were differentiated in the presence or absence of 1 μM rosiglitazone. On the fourth day of differentiation, (A) protein was collected to determine the expression of PPARγ by western blot using PTEN as a loading control; densitometry values reflect the ratio of PPARγ to PTEN. (B) Cells were electroporated and differentiated as described in (A), then fixed and stained with Oil Red O four days post differentiation.

2-(4-sulfophenyl)-2H-tetrazolium]. MTS is reduced by the cells to a colored formazan product, presumably by NADPH or NADH produced by dehydrogenase enzymes, and therefore is an indirect measure of dehydrogenase activity. As seen in Figure 2.4A, depletion of the various enzymes using siRNA did not cause a reduction in dehydrogenase activity, and therefore the inhibition of adipogenesis does not appear to be due to general toxicity.

We also found an increase in the expression of several caspases with SCD2 depletion (Supplementary Table 2.1). Since caspases are involved in apoptosis, a TUNEL (TdT-mediated dUTP Nick-End Labeling) assay was performed to ensure that the siRNA treatment does not induce apoptosis. This assay utilizes flourescein-12-dUTP and terminal deoxynucleotidyl transferase to fluorescently label the fragmented DNA of apoptotic cells on the free 3'OH DNA ends. The fluorescence of the cell population is then quantitated by flow cytometry to determine the extent of apoptosis occurring within the cell population. As can be seen in Figure 2.4B, depletion of PPARγ, FAS, SCD1, or SCD2 does not induce apoptosis, and therefore the ffects on gene expression are not due to this toxic event.

SCD2, but not SCD1, is required for adipocyte-specific gene expression in fully differentiated adipocytes. Real time PCR analysis reveals that SCD2 expression is higher in preadipocyte fibroblasts than SCD1 expression (Supplementary Figure 2.2A and 2.2C), but six days after the induction of differentiation, SCD1 expression increases by 23 fold (Supplementary Figure 2.2B), while SCD2 expression only increases by approximately 8 fold (Supplementary Figure 2.2B and 2.2C). This dramatic induction of

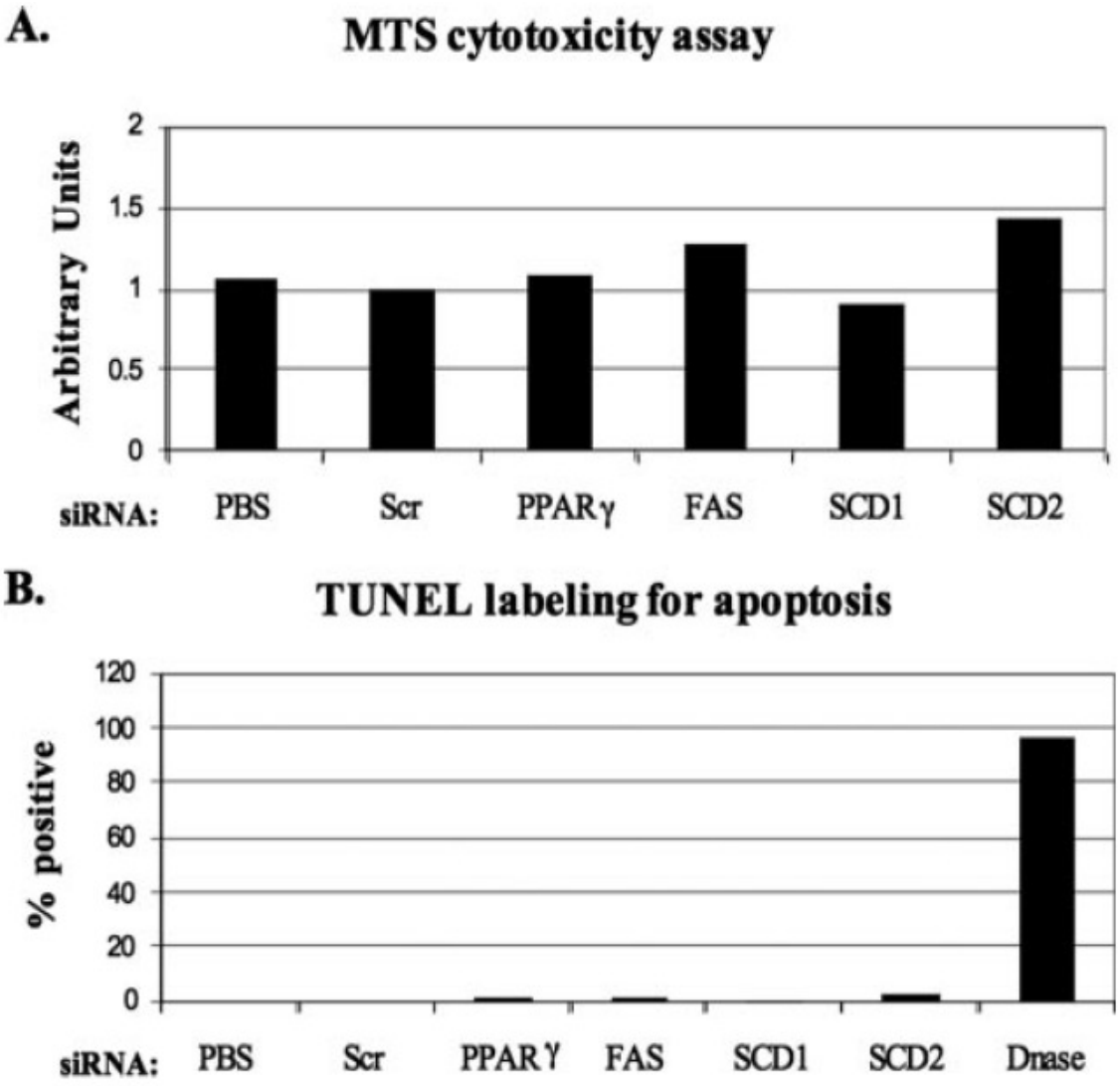


Figure 2.4. The treatment of cultured 3T3-L1 cells with siRNA does not induce toxicity. (A) Confluent 3T3-L1 fibroblasts were transfected with PBS or scrambled nucleotide as controls or SMART-pool siRNA against PPARγ, FAS, SCD1, or SCD2 transcript and differentiated as described. Toxicity was then determined using the colorimetric MTS cell proliferation assay (Promega). (B) Seven days post differentiation, adipocytes were electroporated with PBS or scrambled nucleotide as controls or siRNA against PPARγ, FAS, SCD1, or SCD2 transcript. After 72 hours, the cells were labeled for apoptosis using the TUNEL assay kit (Promega), and positively labeled cells were determined by FACS analysis. Dnase treated cells acted as the positive control.

SCD1 expression results in higher SCD1 than SCD2 expression in fully differentiated cells (Supplementary Figure 2.2A and 2.2C). Since SCD2 depletion inhibits the increase in SCD1 expression during adipogenesis (Figure 2.2C), perhaps the inhibition of adipogenesis is not due solely to SCD2 depletion, but is dependent on a decrease in total desaturase activity. Therefore, perhaps the more profound effect of SCD2 depletion on adipogenesis is simply due to its higher expression in the preadipocyte. We therefore tested whether SCD1 or SCD2 are required to sustain adipocyte-specific gene expression in fully differentiated adipocytes (seven days after initiation of differentiation), when SCD1 expression is dramatically higher than SCD2 expression (Supplementary Figure 2.2C). Remarkably, real time-PCR analysis of the products of several adipocyte genes revealed that SCD2, but not SCD1, is necessary for optimal expression of the PPARγ regulated genes, PEPCK and ACCβ, in fully differentiated cells (Figure 2.5A). However, SCD2 knockdown in these fully differentiated adipocytes only caused a minor decrease in PPARγ mRNA expression (Figure 2.5A), in contrast to SCD2 depletion in cells prior to differentiation (Figure 2.2A). Therefore, the expression of PPARγ1 and PPARγ2 protein was determined by western blot (Figure 2.5B). Surprisingly, the protein levels of both PPARγ isoforms were markedly decreased in fully differentiated adipocytes upon siRNA-mediated depletion of SCD2 and not affected by depletion of SCD1. FAS is also required for PEPCK and ACCβ expression in fully differentiated cells, but this effect is not due to a decrease in PPARγ expression, since FAS depletion did not cause a significant decrease in PPARγ mRNA or protein expression (Figures 2.5A and 2.5B). Thus, the maintenance of PPARγ protein in fully differentiated cultured adipocytes is

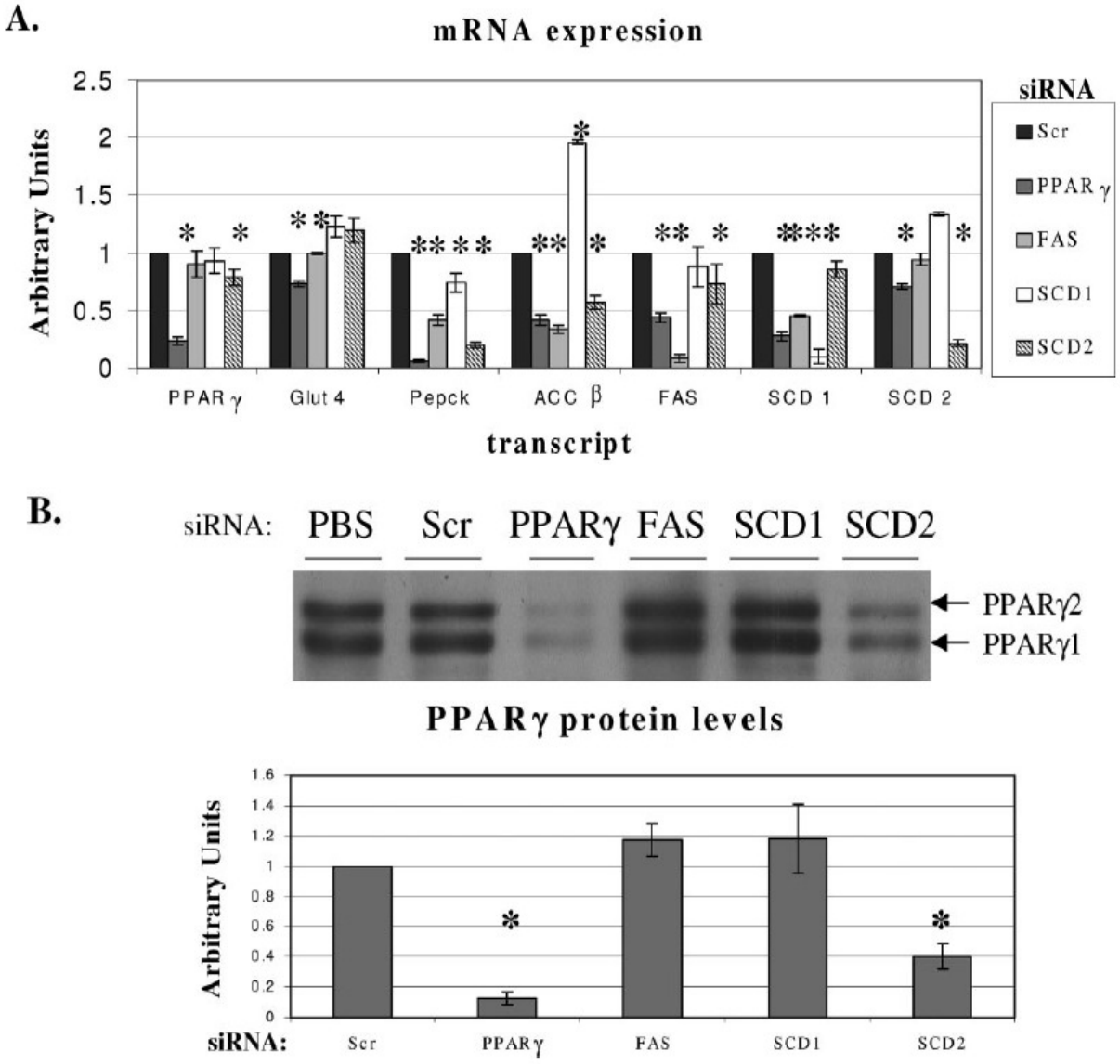


Figure 2.5. SCD2 is required for PPARγ protein, but not mRNA expression, as well as the expression of the PPARγ regulated genes, Pepck and ACCβ, in fully differentiated 3T3-L1 adipocytes. Seven days post differentiation, adipocytes were electroporated with PBS or scrambled nucleotide as controls or siRNA against PPARγ, FAS, SCD1, or SCD2 transcript. After 72 hours, (A) RNA was collected to determine the expression of adipogenic markers by real time PCR using AKT1 as an internal control; or (B) protein was collected to determine the expression of PPARγ by western blot. Changes in protein expression were quantified by densitometry; the values for PPARγ represent both PPARγ1 and PPARγ2 isoforms, since both isoforms show a similar decrease. The values represent the average of 3 independent experiments and the asterisk denotes a p value < 0.05.

specifically dependent on SCD2 activity, explaining the requirement of SCD2 for PEPCK and ACCβ gene expression.

In order to compare the sets of adipocyte genes regulated by SCD2 depletion versus PPARγ depletion, Affymetrix Gene Chip analysis was performed in fully differentiated adipocytes electroporated with siRNA directed against PPARγ, SCD1, or SCD2. Figure 2.6 illustrates the results of this analysis as a heat map showing the comparison of genesthat change in expression with the different siRNA treatments. The green bars represent genes that are significantly upregulated and the red bars represent genes that are significantly down regulated in the cells treated with siRNA versus scrambled nucleotide control. Not surprisingly, SCD2 depletion has a profound effect on gene expression that strongly parallels the effects of PPARγ depletion, while loss of SCD1 shows no similarity to PPARγ depletion in its effect on gene expression (Figure 2.6). Likewise, a closer analysis of genes highly expressed in the adipocyte reveals similar changes in gene expression due to PPARγ and SCD2 depletion, but not SCD1 depletion (Supplementary Table 2.1). In these experiments, PPARγ depletion by siRNA was only about 50% (data not shown). Therefore, these results demonstrate the powerful requirement of PPARγ for optimal adipocyte-specific gene expression, as previously published (203). Furthermore, these results illustrate the distinct roles that the highly similar desaturases, SCD1 and SCD2, fulfill in the fully differentiated adipocyte.

SCD2 is required for optimal protein synthesis in 3T3-L1 adipocytes. The reduction in PPARγ protein but not mRNA expression in response to SCD2 depletion in fully differentiated adipocytes may be due to a decrease in its synthesis or an increase in its

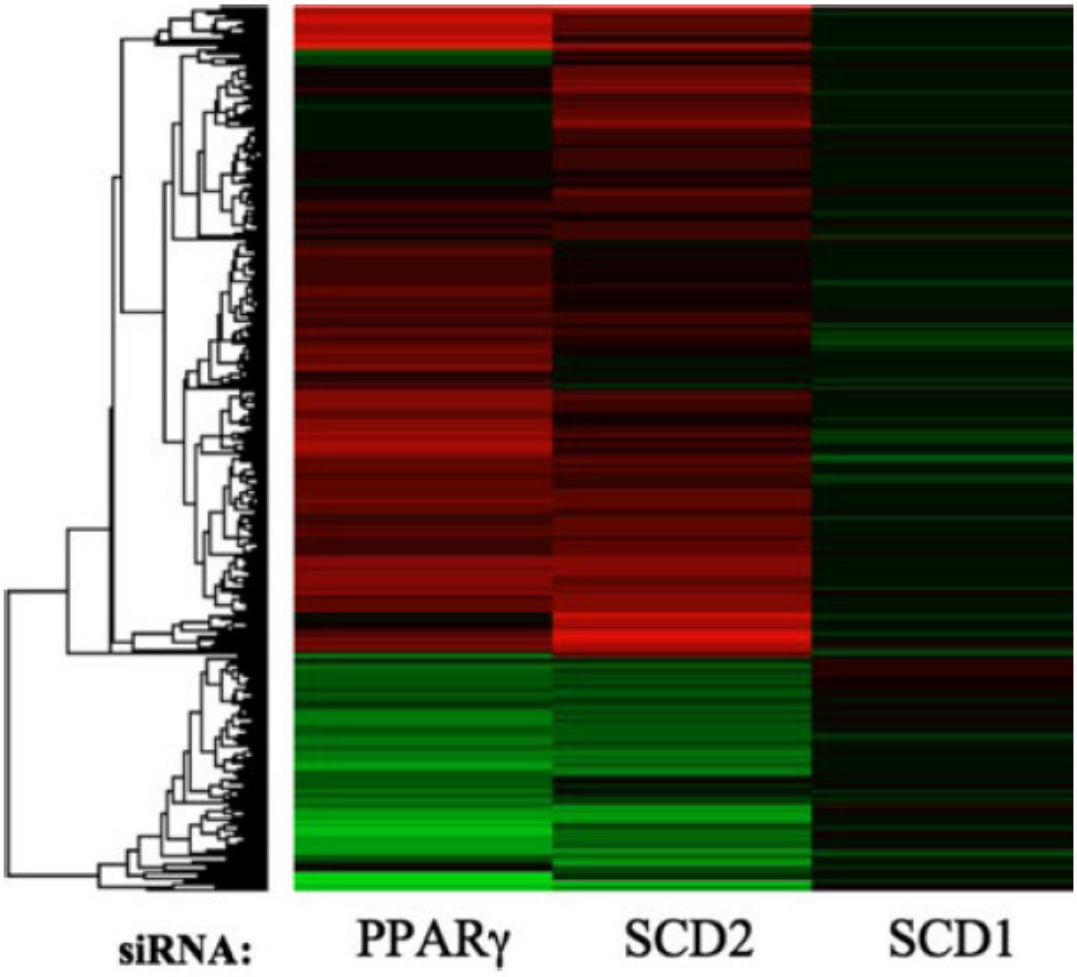


Figure 2.6. Depletion of SCD2, but not SCD1, attenuates the expression of PPARγ-regulated genes. Heat map showing the comparison of genes that change in expression with PPARγ, SCD2, or SCD1 depletion. Seven days post differentiation, adipocytes were transfected with siRNA against PPARγ, SCD2, SCD1, or scramble nucleotide control and RNA was collected after 72 hours to perform Affymetrix GeneChip analysis. The first column is a comparison of scrambled nucleotide versus PPARγ depletion; the second column is a comparison of scrambled nucleotide versus SCD2 depletion; the third column is a comparison of scrambled nucleotide versus SCD1 depletion. Green bars represent genes that are significantly up regulated and red bars represent genes that are down regulated ($p<0.05$).

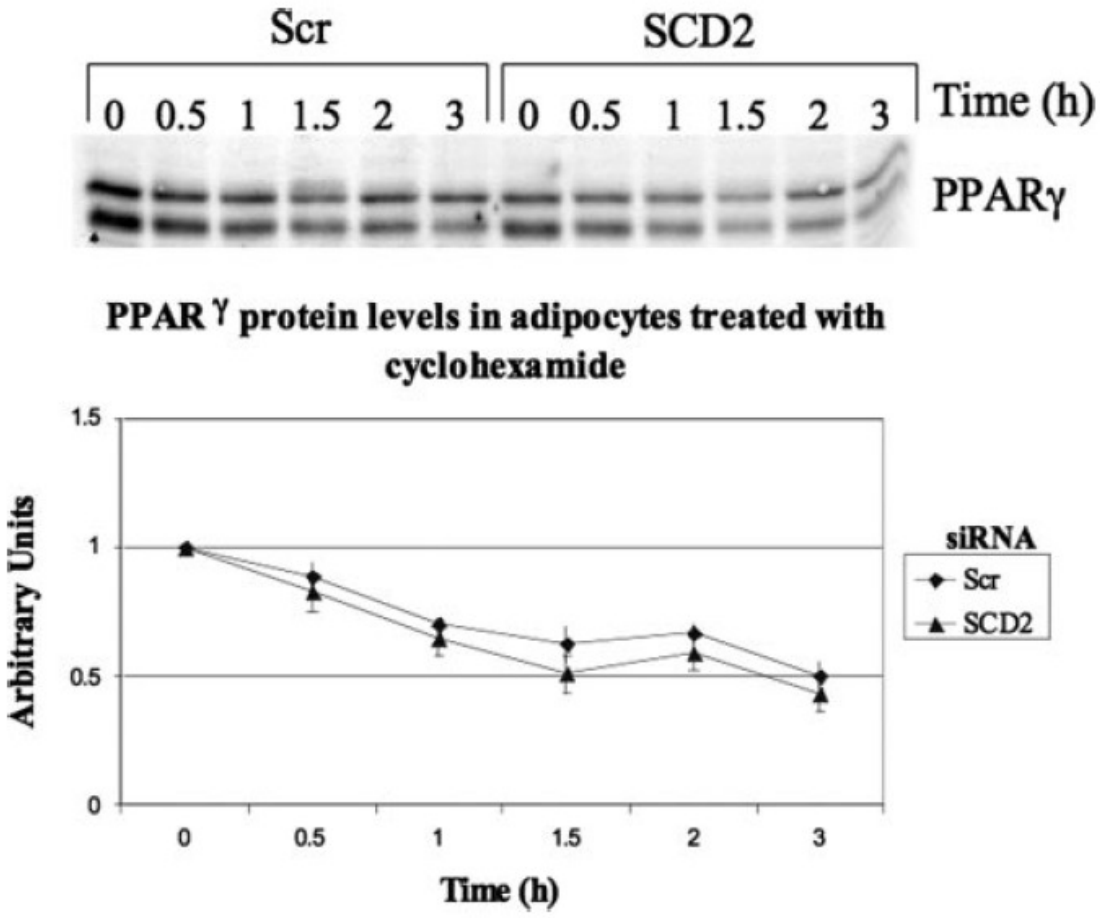


Figure 2.7. SCD2 depletion does not enhance degradation of PPARγ. Seven days post differentiation, adipocytes were electroporated as described and after 24 hours of siRNA transfection, cells were treated with 5 μM cycloheximide. Protein was collected at the indicated timepoints and analyzed by western blot. Shown is a representative blot. Proteins were quantified by densitometry and the 0.5-3h timepoints were normalized to the time 0 timepoint for each condition to calculate the fold change in time for protein turnover. The graph illustrates the average of 6 independent experiments.

degradation. Cultured adipocytes were therefore treated with cycloheximide to inhibit protein synthesis and determine if PPARγ degradation is increased upon loss of SCD2. Using this standard method to determine the protein degradation rate in the presence of cycloheximide, PPARγ protein levels were assessed in adipocytes that were electroporated with scrambled siRNA or siRNA directed against SCD2. As seen in Figure 2.7, the rate of loss of PPARγ protein is rapid upon this treatment, exhibiting ashort half life of approximately 1.5 hours similar to what has been previously reported (228). However, the rate of PPARγ degradation is similar between control and SCD2-depleted cells, indicating no change in response to loss of SCD2. Therefore, these results confirm rapid turnover of PPARγ protein in adipocytes and indicate that SCD2 is not promoting PPARγ degradation.

The results in Figure 2.7 indicate that the decrease in PPARγ protein levels in response to the loss of SCD2 in fully differentiated adipocytes is due to decreased synthesis of PPARγ protein. In order to determine if SCD2 is required for PPARγ protein synthesis, newly synthesized protein was labeled with [^{35}S] methionine/cysteine and PPARγ protein was immunoprecipitated from control and SCD2 depleted cells. The radioactive signal generated from the immunoprecipitated protein indicates protein that has been newly synthesized, whereas the western blot of the immunoprecipitated protein shows the total amount of protein present. As seen in Figure 2.8, newly synthesized PPARγ1 and PPARγ2 are reduced by approximately 50% in the SCD2 depleted cells, which is similar to the decrease in total protein levels (Figures 2.8 and 2.5B). Therefore,

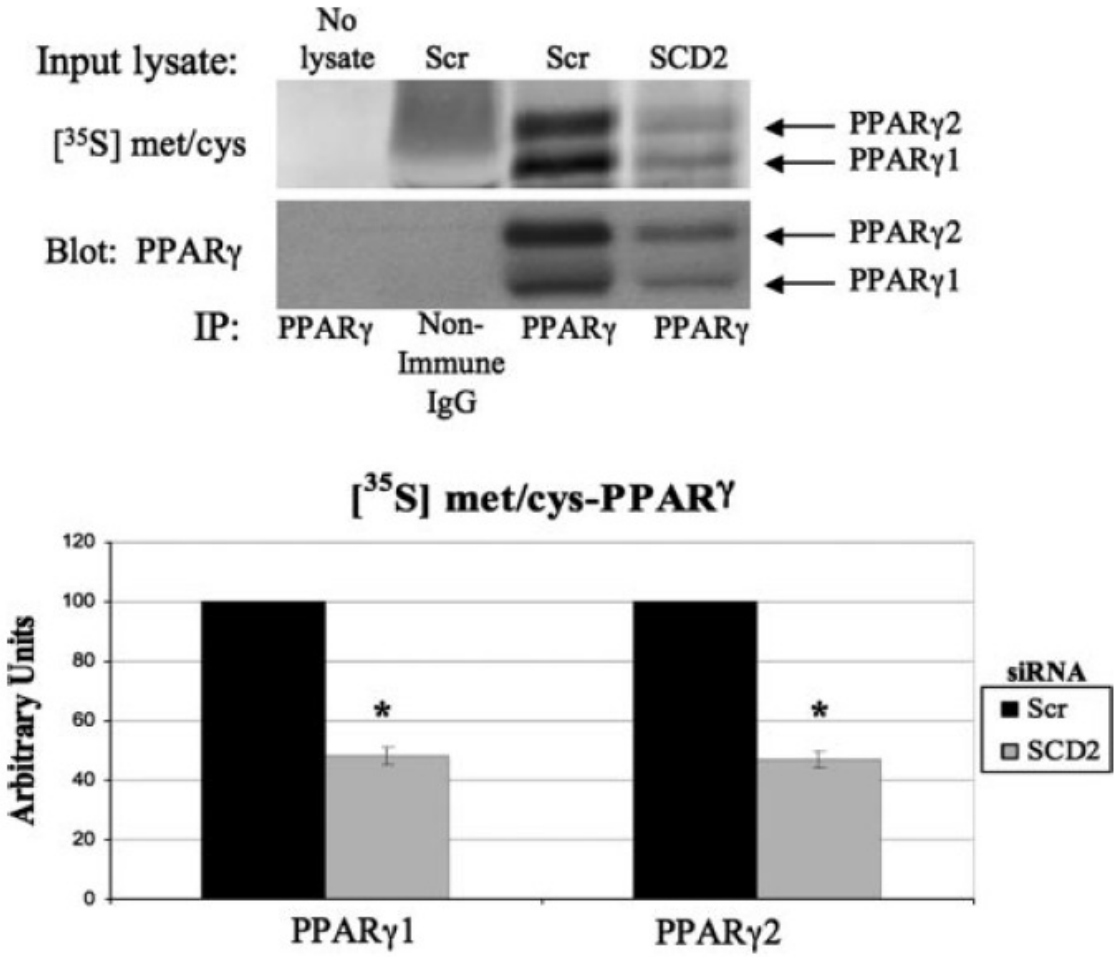


Figure 2.8. SCD2 depletion inhibits the synthesis of PPARγ protein. Seven days post differentiation, adipocytes were electroporated as described and 72 hours after siRNA transfection, cells were metabolically labeled with [^{35}S] Met/Cys, washed several times, and protein was collected for PPARγ immunoprecipitation and western blot. The radioactive signal was visualized using a phosphorimager (Molecular Dynamics) and quantified by densitometry. Shown is a representative immunoprecipitation of PPARγ labeled with [^{35}S] Met/Cys or anti-PPARγ antibody. The graph illustrates the average of three experiments and the asterisk denotes a p value < 0.01.

since PPARγ degradation is not altered (Figure 2.7), the decrease in newly synthesized protein appears to be due to a decrease in protein synthesis.

A common method to monitor the translational efficiency of a particular mRNA is by polysome profile analysis. This methodology separates monosomes from polysomes using a sucrose density gradient, which is then fractionated to generate an absorbance profile indicating which fractions contain monosomes and polysomes. Subsequently, mRNA is isolated from each fraction to determine the degree to which a particularmRNA associates with monosomes or polysomes. To verify that translation of PPARγ is indeed decreased in response to SCD2 depletion, polysome profile analysis was performed and the distribution of PPARγ mRNA with monosomes and polysomes was determined. The UV absorbance at A_{254} reveals a decrease in the absorbance in the heavy polysome fractions and an increase in absorbance in the light polysome and 80S monosome fractions in cells depleted of SCD2, suggesting that less ribosomes are associated with mRNA and there is a global reduction in translation. Real time PCR analysis also reveals that the PPARγ mRNA shifts toward the lighter polysome and monosome fractions, confirming that PPARγ is less efficiently translated in the absence of SCD2 (Figure 2.9). Therefore, the decrease in PPARγ protein expression is due to a decrease in general protein synthesis and is not specifically affecting PPARγ translation.

Discussion

The major finding reported here is the unexpected requirement of the fatty acid desaturase isoform SCD2 for both adipogenesis and the maintenance of the adipocyte phenotype in cultured 3T3-L1 cells (Figures 2.2, 2.3, 2.5, 2.6, and Supplementary Figure

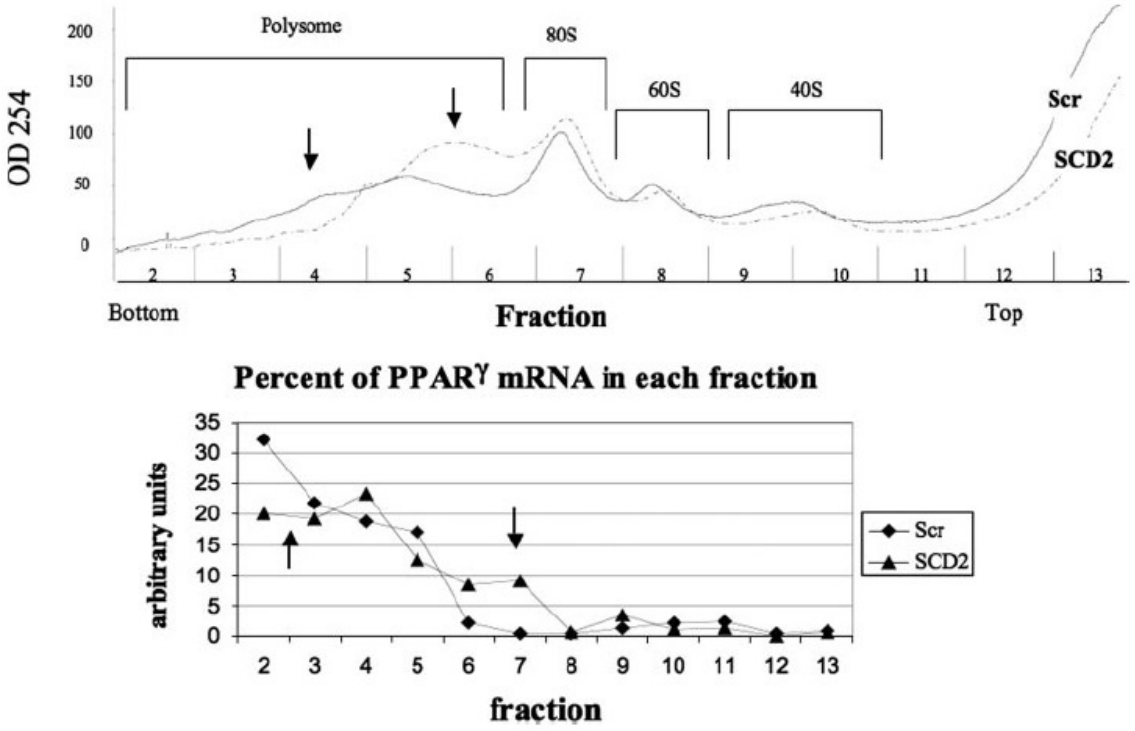
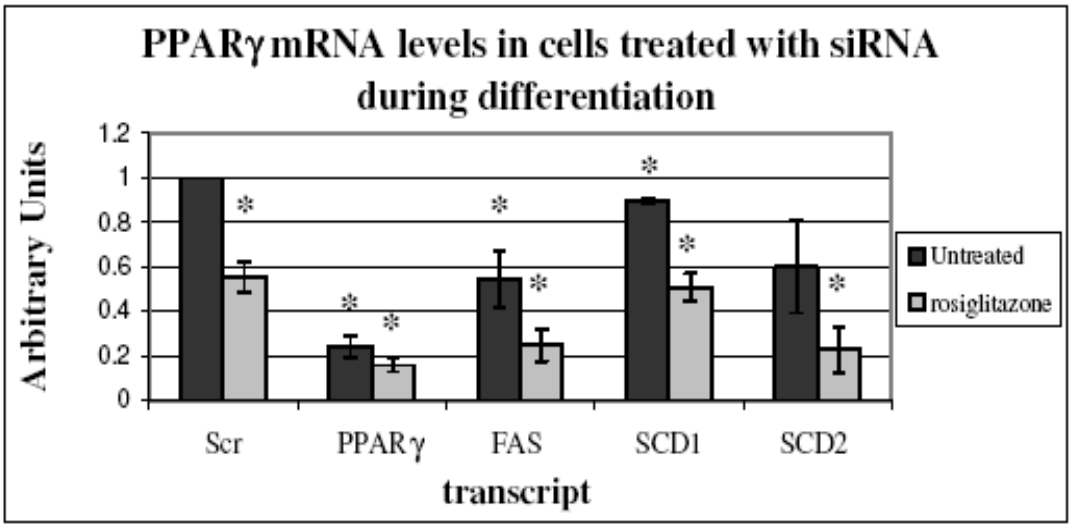
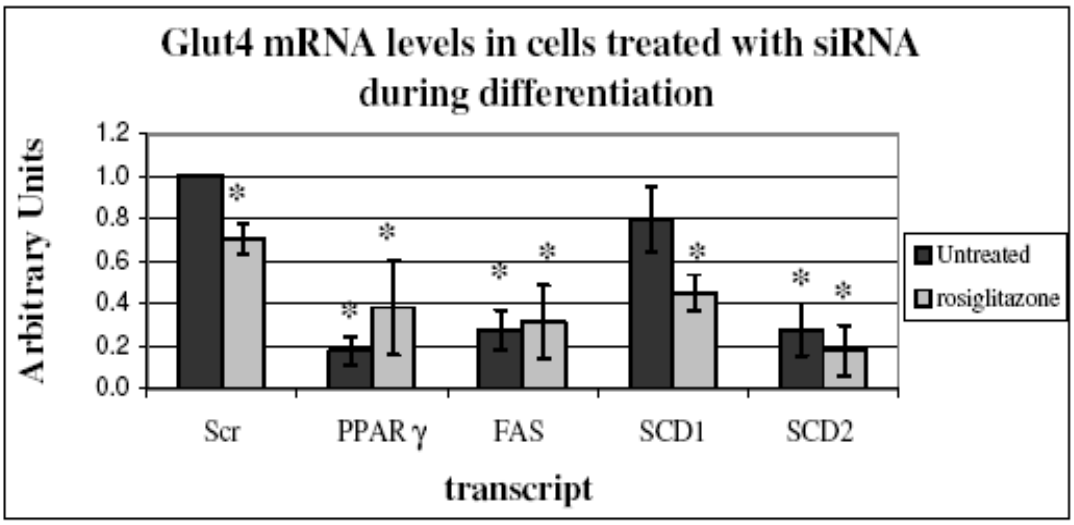


Figure 2.9. SCD2 depletion decreases polysome association with mRNA in cultured adiocytes. Seven days post differentiation, adipocytes were electroporated as described and after 24 hours of siRNA transfection, cytoplasmic extracts were prepared and fractionated on a 10-50% sucrose gradient. The absorbance of each fraction was determined at A254 and total RNA was extracted from fractions 2-13. PPARγ mRNA was quantified from equal volumes of the fractions using real time PCR and expressed as a percentage of the maximum PPARγ mRNA in each sample. The data shown represents one of four experiments with similar results.

2.1). SCD2 regulates adipogenesis at least in part by controlling the transcription of the nuclear receptor PPARγ (Figure 2.2A and Supplementary Figure 2.1), while in fully differentiated adipocytes SCD2 is required for optimal protein synthesis, including PPARγ translation (Figures 2.7, 2.8, and 2.9). Thus, in 3T3-L1 preadipocytes and adipocytes PPARγ protein levels are remarkably dependent on the expression levels of SCD2. Interestingly, the inhibition of adipogenesis by SCD2 depletion was not restored by the addition of the PPARγ-specific ligand, rosiglitazone (Figures 2.2, 2.3, and Supplementary Figure 2.1). Therefore, SCD2 does not appear to be regulating the production of a PPARγ ligand. Rather, these data indicate that in preadipocytes one or more unsaturated fatty acids generated by the SCD2 enzyme or a protein-protein interaction dependent on SCD2 is necessary for the normal functioning of the transcriptional machinery that drives PPARγ expression and also to maintain protein synthesis rates in mature adipocytes.

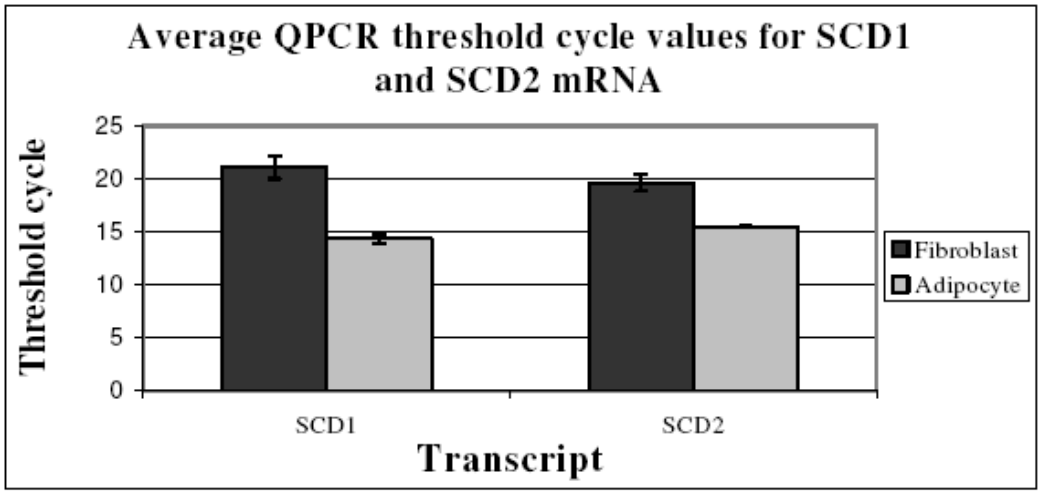
The surprisingly powerful effects of depleting SCD2 in cultured adipocytes suggest a special role for this enzyme in adipocyte function. We tested the effects of depleting 24 enzymes that catalyze reactions in fatty acid metabolism in our siRNA-based screen, but only FAS and SCD2 were found to be necessary for adipogenesis (Figure 2.2, 2.3, and Supplementary Figure 2.1). Mice express 4 isoforms of SCD (SCD1-4), which exhibit approximately 80% sequence similarity, while humans have two isoforms (SCD1 and SCD5), which are approximately 60% similar in sequence (229-231). However, all four mouse SCD isoforms are nearly 80% similar to human SCD1



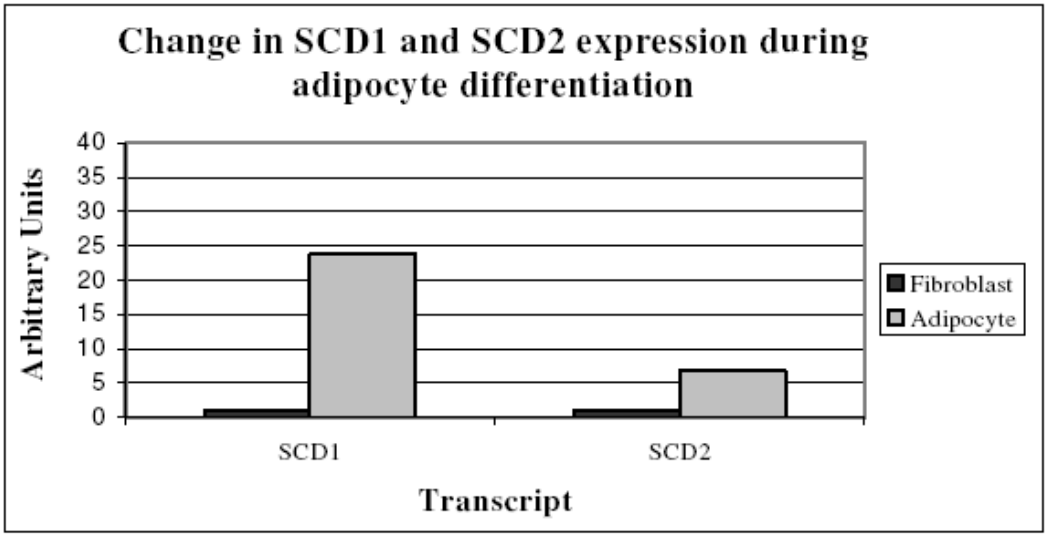


Supplementary Figure 2.1. Depletion of SCD2 inhibits PPARγ and GLUT4 mRNA expression in differentiating 3T3-L1 adipocytes. The experiments were performed as described in Figure 2 and the results show the average of three independent experiments. The asterisk denotes a p value < 0.01.

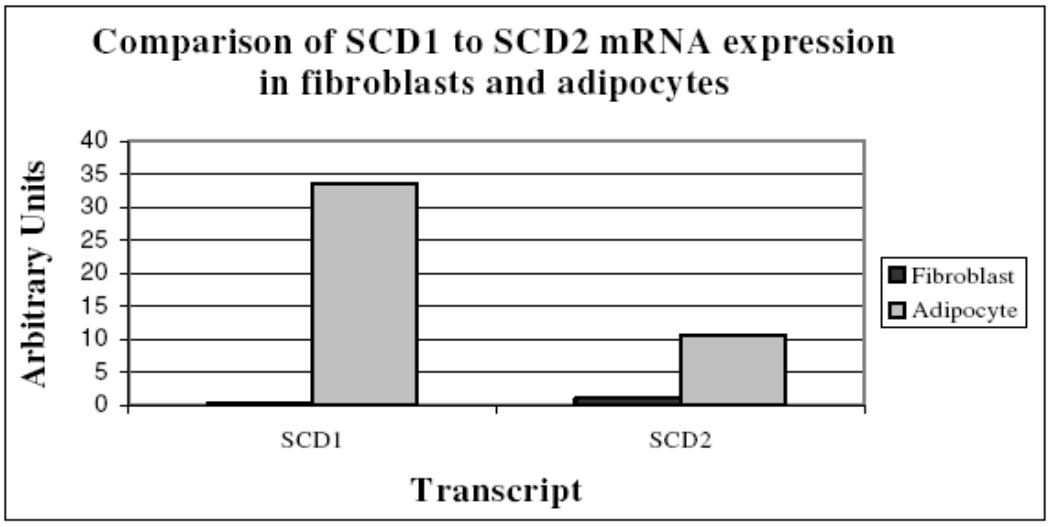
A.


Average QPCR threshold cycle values for SCD1 and SCD2 mRNA
Threshold cycle
25
20
15
10
5
0
SCD1
SCD2
Transcript
Fibroblast
Adipocyte

B.


Change in SCD1 and SCD2 expression during adipocyte differentiation
Arbitrary Units
40
35
30
25
20
15
10
5
0
SCD1
SCD2
Transcript
Fibroblast
Adipocyte

C.



Supplementary Figure 2.2. SCD2 is more highly expressed than SCD1 in preadipocyte fibroblasts, but SCD1 is more highly expressed than SCD2 in fully differentatiated adipocytes. RNA was collected from confluent 3T3-L1 fibroblasts prior to differentiation or from cultured adipocytes four days post differentiation and analyzed by real time PCR using AKT1 as an internal control. (A) The threshold cycle values for SCD1 and SCD2 mRNA in fibroblasts and adipocytes; threshold cycle values represent the number of PCR cycles required for the amplified target to reach a fixed threshold and hence a higher threshold cycle value indicates less RNA (1). (B) The changes in SCD1 and SCD2 mRNA expression during differentiation; the values for SCD1 and SCD2 in adipocytes are normalized to the respective value for SCD1 and SCD2 in fibroblasts. (C) A comparison of SCD1 and SCD2 expression in fibroblasts and adipocytes; the values for SCD1 and SCD2 in both fibroblasts and adipocytes are normalized to the value for SCD2 expression in fibroblasts to allow a direct comparison of mRNA levels in both cell types.

Gene Symbol/ Name	Scramble vs PPARγ siRNA (fold change)	*P* value	Scramble vs SCD2 siRNA (fold change)	*P* value	Scramble vs SCD1 siRNA (fold change)	*P* value
PPARγ	-3.6	0.000	-1.44	0.070	1.05	0.063
C/EBPα	-2.53	0.000	-2.02	0.050	-1.02	0.710
GLUT4	-2.07	0.000	-1.74	0.039	-1.02	0.507
FASN	-2.01	0.000	-1.9	0.010	-1.09	0.077
Adipsin	-2.07	0.000	-1.22	0.031	1	0.671
Adiponutrin	-3.65	0.000	-2.57	0.041	1.04	0.635
FSP27	-4.45	0.000	-2.33	0.015	-1.17	0.001
GPD1	-3.18	0.001	-2.450	0.023	1.02	0.774
DGAT2	-3.01	0.000	-3.45	0.004	-1.07	0.209
ACSL1	-3.37	0.000	-2.64	0.036	1.05	0.316
KLF15	-2.02	0.000	-2.07	0.043	-1.17	0.003
Lipin 1	-3.99	0.000	-2.74	0.012	-1.06	0.267
GPR120	-19.14	0.000	-8.76	0.008	-1.57	0.002
FABP4	-4.62	0.000	-2.57	0.007	-1.02	0.445
AKT2	-1.8	0.000	-2.15	0.004	1	0.912
PEPCK	-16.63	0.000	-7.19	0.043	-1.01	0.774
Caspase 2	1.95	0.000	1.69	0.001	-1.42	0.000
Caspase 3	1.26	0.001	1.73	0.000	-1.04	0.343
Caspase 4	1.35	0.015	5.76	0.000	1.01	0.860
Caspase 6	1.09	0.201	1.19	0.038	-1.13	0.076
Caspase 7	1.42	0.002	2.12	0.000	1.18	0.089
Caspase 12	2.8	0.001	3.24	0.002	-1.01	0.918

Supplementary Table 2.1. SCD2 and PPARγ depletions cause parallel changes in adipocyte gene expression, while SCD1 depletion does not. The experiments were performed as described in Figure 6 and the Table shows values for changes in expression of specific selected genes due to siRNA treatment.

(229,230,232,233). Mouse SCD1 is the best characterized SCD isoform and is expressed in adipose tissue, liver,muscle, and sebaceous glands; SCD2 is expressed ubiquitously; SCD3 is expressed in the harderian gland and in sebocytes in the skin; and SCD4 is expressed in the heart (229). The reason for multiple highly homologous isoforms in the mouse has remained unclear, especially since SCD1 and SCD2 apparently utilize the same substrates with the same efficiency (227). One possible explanation for the redundancy in SCD isoforms is the need for differential expression in various tissues during specific stages of development (229). However, depletion of SCD1 in fully differentiated cells did not have a major impact on adipocyte-specific gene expression, despite the higher expression of SCD1 versus SCD2 (Supplementary Figure 2.2C). Therefore, in spite of the predicted similarity in substrate usage, and common cellular localizations of the enzymes, SCD1 and SCD2 appear to have disparate cellular functions in 3T3-L1 adipocytes (227,229,232,233). Interestingly, Affymetrix Gene Chip analysis reveals that when mice are put on a high fat diet, SCD2 expression increases 44 fold, while SCD1 expression shows little change (Table 2.1). These data suggest that SCD2 may also have a specific role in promoting adipogenesis *in vivo*, since its expression increases during a time of increased adipogenesis (234), despite the already high expression of SCD1 (232,235).

It should be noted that the requirement for a Δ9 desaturase during adipogenesis is somewhat surprising since Gomez et al showed that adipogenesis of 3T3-L1 cells is not affected when induced in the presence of the SCD chemical inhibitor, sterculic acid (236). Perhaps this discrepancy can be explained by a selectivity of the inhibitor for the

highly homologous protein, SCD1, thereby preserving SCD2 activity and adipogenesis. This would be consistent with our results showing that the depletion of SCD1 did not attenuate adipogenesis. Another possibility is that SCD2 activity is not required, only its expression. Nevertheless, our studies presented here are not the first evidence suggesting separate cellular functions of the enzymes, since SCD1 deficiency leads to skin abnormalities despite SCD2 expression in the skin (230).

PPARγ protein expression was dramatically reduced upon SCD2 depletion in mature adipocytes (Figures 2.3A and 2.5B), which explains why there is a decrease in the expression of many PPARγ-regulated genes (Figure 2.5A). Furthermore, the reduction in PPARγ protein expression is due to a decrease in general protein synthesis and not degradation, since the turnover of PPARγ protein when protein synthesis is inhibited by cycloheximide is unaffected by the depletion of SCD2 (Figure 2.7). Consistent with this interpretation, there is a decrease in newly synthesized PPARγ protein as determined by [^{35}S] methionine/cysteine labeling (Figure 2.8) and in the association of actively translating ribosomes with mRNA, including PPARγ mRNA (Figure 2.9).

Since SCD2 is required for general protein synthesis, PPARγ is not the only protein that is reduced in expression upon SCD2 depletion. In fact, examination of the total lysate from cells labeled with [^{35}S] methionine/cysteine shows a significant 15% decrease in newly synthesized protein from SCD2 depleted cells (data not shown). Unlike PPARγ however, many proteins decrease in expression on the transcript level; conversely, many transcripts also increase in expression with SCD2 depletion (Figure 2.6), which taken together makes it difficult to determine the effect of SCD2 on total protein synthesis.

SCD2 depletion does result in the posttranscriptional decrease in expression of proteins other than PPARγ, such as AKT1 and β catenin. The decreased expression of these proteins also appears to be due to a decrease in translational efficiency since the association of AKT1 and β catenin mRNA shifts from polysomes to monosomes (data not shown). However, we have not verified that the synthesis of these proteins is decreased using [^{35}S] methionine/cysteine metabolic labeling or determined if the degradation rate of these proteins increases with SCD2 depletion; therefore, we can not conclude that the decrease in their expression is due to a decrease in translation.

Altogether, our data indicate that unsaturated fatty acids may regulate a pathway to enhance the machinery of protein translation in adipocytes. Since oleate is a major unsaturated fatty acid product of SCD2, we tested whether exogenous addition of oleate would restore the decrease in PPARγ protein levels with SCD2 depletion (227,229). However, even addition of oleate at a concentration as high as 1mM, did not restore PPARγ levels (data not shown). Therefore, perhaps SCD2 is required to produce an unsaturated fatty acid other than oleate, or is required for the proper shuttling of an unsaturated fatty acid, as seen with linoleate in the SCD2 knockout mouse (229), or is necessary for a protein-protein interaction that regulates translation.

To our knowledge, the only previously published evidence of regulation by unsaturated fatty acids of protein synthesis is by arachadonic acid or eicosapentaenoic acid (EPA). Arachadonic acid has been shown to both activate and inhibit protein translation in diverse cell systems, while EPA has been shown to inhibit translation initiation by inducing eIF2α phosphorylation (237-239). Therefore, we examined eIF2α

phosphorylation in response to depletion of SCD2, but did not find a difference between SCD2-depleted and control adipocytes (data not shown). Protein synthesis can also be controlled through the protein kinases AMPK and mTOR (240). An increase in AMPK activity could lead to decreased peptide elongation through activation of eEF2 kinase, which then phosphorylates and inhibits eEF2, a factor that promotes protein chain elongation. Interestingly, this pathway may be regulated by unsaturated fatty acids, since SCD1 deficiency in mice leads to increased AMPK activity in the liver (241). In SCD2 depleted adipocytes, we did find an approximate 80% increase in AMPK phosphorylation and a small 20% increase in eEF2 phosphorylation compared to control cells (data not shown). However, these increases in AMPK and eEF2 phosphorylation associated with SCD2 depletion do not appear to mediate the decrease we observe in protein synthesis, since eliminating the increase in phosphorylation of eEF2 by the dual depletion of eEF2 kinase and SCD2 did not restore PPARγ protein levels (data not shown). It is reported that mTOR positively regulates protein synthesis by phosphorylating and activating RS6K and 4EBP1 (242,243). While SCD2 depletion causes a reduction in RS6K and 4EBP1 protein levels, it does not reduce the phosphorylation of these proteins, suggesting the mTOR pathway is not affected (data not shown). Consistent with these results, inhibition of mTOR with rapamycin also decreases RS6K1 and RS6K2 activity, but does not affect PPARγ levels (242,244,245). Therefore, it remains unclear how SCD2 regulates mRNA association with polysomes, and this is an important question for future studies to address.

It will also be interesting in future studies to test whether SCD2 plays a unique role in modulating glucose homeostasis in mice. White adipose tissue is a key regulator of whole body metabolism through its ability to control glucose disposal and insulin sensitivity in peripheral tissues (196,220). This regulation appears to be mediated by two main mechanisms (195,196,220): 1.) storing excess fatty acids in the form of triglyceride to prevent lipotoxicity in peripheral tissues and 2.) secreting insulin-sensitizing factors, such as adiponectin. PPARγ plays a central role in both of these processes by promoting expression of genes involved in fatty acid esterification to triglyceride (195) and the expression of adiponectin (195,246). SCD2 may have profound influence on these processes through its regulation of PPARγ and adipogenesis. Unfortunately, SCD2 -/- mice do not survive and can't be studied in this regard. Thus, these important questions regarding the physiological role of SCD2 in whole body metabolism must await the generation of mouse models with tissue-specific depletion of this enzyme.

Limitations and Future Perspectives

While the findings in this study clearly support the role of SCD2 in maintaining PPARγ expression in fully differentiated adipocytes, there are still many questions that remain regarding SCD2 function both during adipogenesis and in fully differentiated cells. In mature adipocytes, PPARγ expression is dramatically dependent on SCD2 expression, but whether it is dependent on SCD2 activity remains to be determined. It could be that SCD2 controls the assembly of protein synthesis machinery by acting as a scaffolding protein rather than as a desaturase. To clarify this, a desaturase dead mutant of SCD2 could be overexpressed in SCD2 depleted cells to determine if PPARγ protein

and adipocyte gene expression are restored. If SCD2 desaturase activity is required to regulate protein synthesis, then identification of its fatty acid products would be beneficial to understanding how fatty acids regulate adipocyte biology. This could be accomplished by global lipid analysis using mass spectrometry and identifying the desaturated lipids that are reduced in cells depleted of SCD2, but show no change with depletion of SCD1. While this methodology would be intensive, given the multitude of desaturated fatty species present in adipocytes, it could be accomplished. Then, SCD2 depleted cells could be cultured in the presence of the identified fatty acids to determine which fatty acids restore protein synthesis.

It is also unclear if the effects of SCD2 depletion on gene expression are due solely to a loss in PPARγ expression. To address this question, adenoviral overexpression of PPARγ could be used in cells depleted of SCD2 to determine if adipocyte gene expression is rescued. If rescue occurs, then the effects of SCD2 depletion are due to a loss specifically in PPARγ protein and not other proteins. However, since protein synthesis is attenuated, a high titer of adenovirus may be needed to restore PPARγ protein levels. In addition to regulating PPARγ expression, SCD2 may also regulate PPARγ ligand production. If adenoviral overexpression of PPARγ does not rescue gene expression, but then the addition of a synthetic agonist does, then this suggests that SCD2 also regulates PPARγ ligand production.

In this study, SCD2 was required for adipogenesis as well as the maintenance of gene expression in fully differentiated adipocytes. However, it is not known if the function of SCD2 during adipogenesis is also to maintain PPARγ protein expression. To

determine if SCD2 also regulates protein synthesis in preadipocytes, polysome analysis could be performed in preadipocytes depleted of SCD2 to determine if ribosome association with mRNA is attenuated. Also, to determine if the inhibition of adipogenesis is due to a loss in PPARγ specifically, adenoviral overexpression of PPARγ could be used to determine if adipogenesis is restored in the absence of SCD2. Additionally, it is unclear if SCD1 has any role during adipogenesis, since SCD2 depletion inhibits the induction of SCD1. Therefore, adenoviral overexpression of SCD1 in the absence of SCD2 would determine if this isoform also has a role during adipogenesis.

Although these findings increase the understanding of adipocyte biology *in vitro*, they are only important if also found *in vivo*. This would require analysis of the preadipocytes and adipocytes from the SCD2 knockout mouse to determine if PPARγ expression, adipogenesis, and protein synthesis are decreased. Additionally, in the small number of SCD2 knockout mice that survive past weaning, SCD1 expression increases and they return to a wild type phenotype, suggesting the SCD1 can compensate for the loss in SCD2 function *in vivo* (229). However, these mice were never put on a high fat diet, which is when SCD2 expression increases in the adipose tissue. Perhaps under these conditions, SCD1 functions in triglyceride synthesis and SCD2 is required for other synthetic pathways, such as protein synthesis. Therefore, in mice fed a high fat diet, SCD2 may be required to maintain adipocyte function and perhaps the SCD2 knockout mice would be more insulin resistant and glucose intolerant than their wild type counterparts.

While rodent models provide exceptional insight into protein function, the true importance is how the findings relate to human biology. Humans only produce one SCD isoform that is highly homologous to both mouse SCD1 and SCD2 (247). Therefore, it would be interesting to see if depletion of SCD1 in human preadipocytes attenuates protein synthesis and adipogenesis. In summary, this study identifies a novel function for SCD2 in mouse adipocyte biology, but the findings need to be substantiated in primary cells and extended to human adipocytes.

Chapter III: The carboxy terminus of Cidea facilitates lipid droplet binding and triglyceride accumulation

Disclaimer: The author performed all experiments, except Anil Chawla helped design and generate the GFP and HA tagged constructs used for all experiments.

Abstract

It is now known that the CIDE-domain containing protein, Cidea, inhibits lipolysis and promotes lipid storage in both human and mouse adipocytes. In mice, data suggests that Cidea may prevent lipolysis by binding and shielding the lipid droplet from lipase association. However, it was not known if the human isoform of Cidea also binds to lipid droplets and the mechanism by which binding occurs. Here, we show that human Cidea binds to lipid droplets in both adipocytes and non-adipocyte cell lines and stimulates triglyceride accumulation. Additionally, both lipid droplet localization and triglyceride accumulation are solely dependent on the carboxy terminal 104 amino acids, indicating the presence of both the lipid droplet targeting and trigylceride shielding domains. Furthermore, Cidea overexpression causes a striking change in lipid droplet morphology in which lipid droplets become large in size and few in number, suggesting that Cidea may promote lipid droplet fusion. This change in lipid droplet morphology requires both the amino and carboxy terminal domains and may be important to regulate the efficient packing and storage of triglyceride in adipocytes, revealing a novel role for Cidea.

Introduction

An important mechanism to survive famine is the ability to store energy when nutrients are available to use during times when nutrients are not available. One efficient

method of storing excess energy is in the form of triglyceride, which provides 9300 kcalories of energy per kilogram of fatty acid (89). The major triglyceride storage site in the body is the adipose tissue, since it expresses many lipid-metabolizing enzymes and is able to accumulate large amounts of lipid without suffering cellular toxicity. However, if the adipose tissue loses the ability to store triglyceride, then fatty acids accumulate in peripheral tissues, such as the muscle and liver, causing lipotoxicity and metabolic perturbations (4,96,126). This phenomenon commonly occurs during obesity, which is now a global health devastation due to its associated medical disorders, such as type 2 diabetes, hypertension, dyslipidemia, and atherosclerosis (8,248). Therefore, understanding how adipocytes regulate fatty acid storage and release is detrimental in identifying how obesity causes these serious metabolic disorders.

The adipose tissue stores triglyceride and other neutral lipids in lipid droplets, which are composed of a neutral lipid core surrounded by a phospholipid monolayer and associated proteins (62,64). There have been many proteins found associated with lipid droplets and include members of the PAT family (Perilipin, ADRP, Tip47, S3-12, and OXPAT) , the CIDE family (Cidea, Cideb and Cidec, or FSP27 in mice), caveolin 1, SNARE proteins, various lipid synthesizing enzymes, lipases (HSL and ATGL), the RAB family of GTPases, and the small GTPase, ARF1 (63,65,66,69-74,249-252). These proteins are involved in lipid droplet formation and fusion and in regulating lipid storage and turnover. However, the list of associated proteins continues to grow, as does our understanding of lipid droplet biology.

Although once considered a simple storage site, the lipid droplet is now considered a dynamic organelle that greatly impacts many cellular processes. It provides fatty acids for energy production, phospholipid, and membrane biosynthesis, cholesterol for membrane rigidity, and acts as a depot for proteins involved in transcription and apoptosis (64,67). It is also possible that different lipid droplets are involved in different cellular functions, with the function being dictated by which proteins are associated. This idea is supported by the fact that some proteins are found on mutually exclusive subsets of lipid droplets, as seen with RAB18 and ADRP (64,65). Additionally, in response to lipolytic stimuli, not all droplets are associated with the same proteins or lipases, suggesting only a subset is involved in fatty acid release under these conditions (64,65,82).

In addition to their important role in maintaining cellular function, lipid droplet proteins also regulate whole body energy homeostasis. For example, Perilipin, Cidea, and FSP27 knockout mice remain lean and insulin sensitive when put on a high fat diet. Consistent with the idea that these proteins regulate lipid storage, these mice have lower intracellular triglyceride levels and adipose tissue weight (80,253,254). These findings suggest that these proteins may promote obesity and insulin resistance by increasing fat storage. However, mice have large amounts of brown adipose tissue, which is dense with mitochondria and efficiently oxidizes fatty acids. Therefore, a loss in the lipid storage capability in the adipose tissue of mice may not lead to peripheral lipotoxicity, if the BAT effectively oxidizes and lowers serum fatty acid levels. In fact, the cidea knockout mice

have increased lipolysis in the BAT, but not increased fatty acid release, suggesting the BAT is indeed capable of oxidizing the released fatty acids (80).

Humans, on the other hand, only have a very small amount of brown adipose tissue. Thus, if the white adipose tissue loses the ability to efficiently store lipid, it is likely to lead to peripheral lipotoxicity and insulin resistance. Consistent with this idea, Puri et al found that in BMI matched patients, there was a positive correlation with Cidea, Cidec, and perilipin expression and insulin sensitivity in the adipose tissue (72). This suggests that these proteins protect humans from developing insulin resistance when high levels of fatty acids are present. Furthermore, Nordstrom et al found that siRNA mediated depletion of Cidea in human preadipocytes resulted in increased lipolysis, supporting the idea that, similar to mouse cidea, human cidea regulates adipocyte lipid storage (79). However, in contrast to mice, these two studies suggest that a loss in Cidea function in the human results in a negative rather than a positive effect on insulin sensitivity.

Due to the impact that Cidea has on whole body metabolism in mice and possibly humans, it is crucial to understand how Cidea regulates adipocyte lipid storage. In this study, we show that human Cidea binds to lipid droplets and stimulates triglyceride storage. Since previous studies suggest that Cidea inhibits lipolysis, the increase in triglyceride is most likely due to a decrease in lipolysis rather than an increase in triglyceride synthesis (79,80). We also determined that the binding to the lipid droplet and the stimulation of triglyceride accumulation is solely dependent on the carboxy terminus. Additionally, Cidea induces large lipid droplet formation while simultaneously reducing the number of lipid droplets, suggesting that Cidea may regulate lipid droplet

fusion. This change in lipid droplet morphology is not required for triglyceride accumulation, but is dependent on both the amino and carboxy terminal domains. While this function of Cidea does not appear to have a significant impact on triglyceride storage, it may allow more compact lipid storage within the adipocyte and reduce the intracellular space occupied by triglyceride. Altering lipid storage in this manner would be beneficial to adipocytes, since they accumulate excessive amounts of triglyceride and need to maximize their efficiency in storing lipid.

Experimental Procedures

Materials- Rabbit anti-human calreticulin IgG was purchased from Calbiochem. Mouse anti-HA IgG (monoclonal HA.11) was purchased from Covance. Alexa 568 donkey anti-rabbit IgG, Alexa 488 chicken anti-mouse IgG, and Prolong Gold anti-fade reagent were purchased from Invitrogen. The free glycerol and triglyceride determination kit and oil-red-O were purchased from Sigma.

*Plasmids***-** Cidea cDNA was purchased from Open Biosystems. PCR was performed to generate cDNA encoding a full length or truncated product by using a 5'-linker containing a Bgl2 restriction site and a 3'-linker containing a Sal1 site. After cutting with the restriction enzymes, the purified PCR fragments were cloned into pEGFPC1 vector (Clontech, USA). For the generation of HA tagged proteins, PCR was performed using a 5'-linker containing a MLU1 restriction site and a 3'-linker containing a Sal1 site. After cutting with the restriction enzymes, the purified PCR fragments were cloned into 3XHA-pCMV5 (F3) vector. This vector was generated from the pCMV5 vector (gift from Dr. David Russel, Southwestern Medical Center, Dallas, TX) by

inserting a linker containing the DNA sequences for a 3 HA tag at the EcoR1 and MLU1 sites.

Cell culture and electroporation-Cells were cultured in Dulbecco's modified Eagle's medium DMEM supplemented with 10% fetal bovine serum, 50 μg/mL streptomycin, and 50 units/mL penicillin (45). For experiments performed in preadipocytes, $2.5x10^6$ cells were electroporated with 8 ug of plasmid DNA. The electroporation was performed using a Bio-Rad gene pulser II at the setting of 0.18 kV and 960 microfarads. Immediately after electroporation, the cells were reseeded into 1 well of a 6 well plate. For experiments in mature adipocytes, fibroblasts were cultured for 8 days, differentiated into mature adipocytes by adding differentiation media (2.5 μg/mL insulin, 0.25 μM dexamethasone, and 0.5 mM IBMX in the culture media described above) for 72 hours, then cultured for an additional 24-48 hours. Adipocytes were then electroporated as described above using $5x10^6$ cells and 15ug of plasmid DNA. After electroporation, cells were reseeded into 1 well of a 6 well plate. For experiments in COS-7 cells, transfection was performed using TransIT-LT1 tranfection reagent according to the manufacturers instructions.

Oil-red-O staining for intracellular triglycerides- To stain neutral lipids, cells were first washed with PBS and then fixed in 4% formaldehyde solution in PBS (1 hour). After three PBS washes, the cells were stained with oil-red-O solution (5 mg/mL oil red O solid dissolved in isopropanol, then diluted to a 60% working solution with ddH20 and filtered through a 0.45μm filter) for 30 min at room temperature, followed by six washes with water.

Immunofluorescence- Cells were washed with PBS and fixed in 4% paraformaldehyde in PBS (1 hour), then washed three times with PBS, permeabilized with 0.05% Triton X-100 in PBS containing 1% FBS (15 min), and incubated overnight at 4°C with primary antibody against HA or calreticulin in permeabilization buffer. Cells were then washed, labeled with secondary antibody, and washed again with permeabilization buffer before mounting on slides using Prolong Gold.

Confocal microscopy- Images were taken on a Zeiss Axiophot microscope equipped with a Hamamatsu digital camera and processed using Metamorph imaging software, version 6.1 (Universal Imaging, Downingtown, PA).

FACS analysis- GFP positive cells were sorted on a BD Biosciences [San Jose, CA] FACS Vantage DiVa Cell Sorter. The acquisition software used was FACS DiVa 6.0.

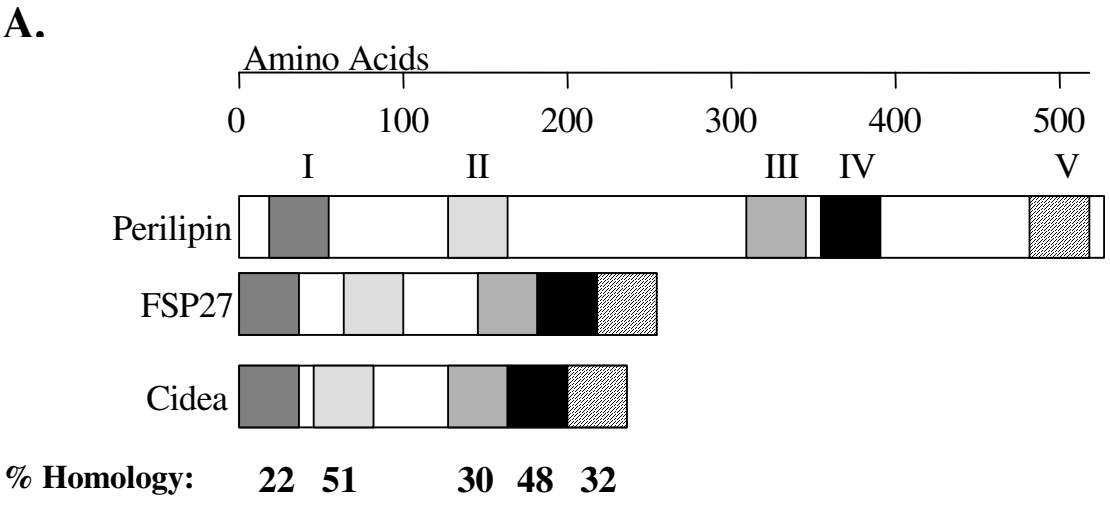
Triglyceride Determination-Cells were transfected as described above and GFP positive cells were sorted by FACS. The cells were then lysed with water, sonicated, and quantitated for triglyceride using the Triglyceride Determination Kit (Sigma) via the manufacturer's instructions.

Statistical analysis- Quantitative data is represented as mean ± SEM. For statistical analysis the differences between groups were examined with student's paired t-test and p value of < 0.05 was considered statistically significant.

Results

The carboxy terminus of human Cidea is necessary and sufficient for lipid droplet targeting

Although the functional domains of many lipid droplet associated proteins have been identified (75,76,86,255), analysis of the functional domains of Cidea has not yet been performed. In order to answer this question, various domains of human Cidea were fused to GFP or HA to identify which segments are required for lipid droplet targeting and triglyceride shielding (Figure 3.1). Since human and mouse Cidea are approximately 90% homologous, the domains were chosen based on the sequence similarity previously described for mouse Cidea to the PAT family protein, perilipin, which is a well-characterized lipid droplet associated protein (72). These regions also share homology with another CIDE family protein that binds lipid droplets, FSP27 (72). The first region of Cidea (I) shares approximately 22% homology with perilipin within a short amino terminal sequence, which also shares homology with the other PAT family proteins, Adipophilin and TIP47. No function has yet been identified for this region, but the shared homology among the lipid droplet proteins may indicate an unidentified function for this motif. The second region of Cidea (II) shares 51% homology with the amino terminal triglyceride-shielding domain of Perilipin. This segment of Perilipin is responsible for blocking lipase association with the lipid droplet and preventing lipolysis. Since Cidea also inhibits lipolysis, this region is potentially important for Cidea function. The third and fourth regions of Cidea (III and IV) share approximately 30% and 48% homology with the two regions of Perilipin responsible for lipid droplet targeting and anchoring and



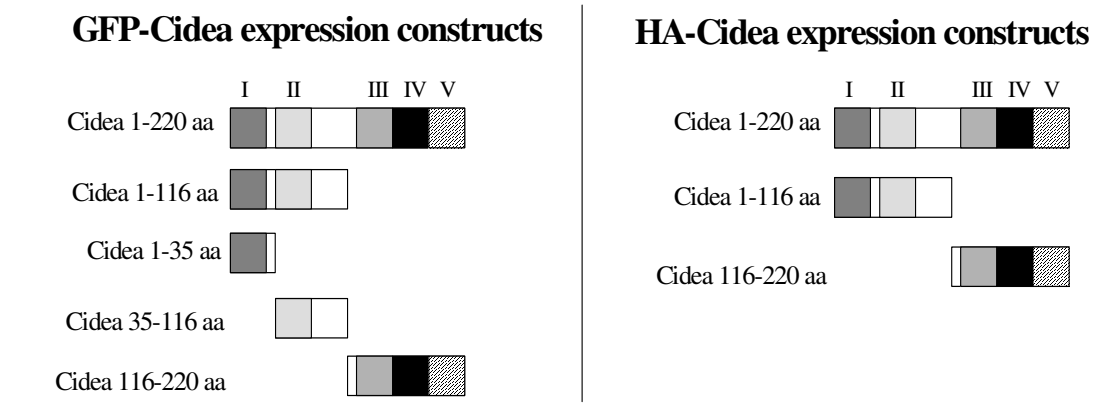


Figure 3.1. Schematic diagram showing the predicted amino acid similarity of Cidea and FSP27 to Perilipin and the Cidea constructs generated based on these motifs. A. The adipophilin-like sequence of perilipin (aa 11-38; Region I) shows a sequence similarity of 32% with FSP27 (aa 2-29) and 22% with Cidea (aa 2-28). Region II of perilipin (aa 120-152) is responsible for shielding triglyceride from cytosolic lipases and has a sequence similarity of 40% with FSP27 (aa 46-77) and 51% with Cidea (aa 38-69). Regions III (aa 313-352) and IV (aa 365-391) of perilipin are responsible for lipid droplet targeting and anchoring and have 40% and 30 % similarity with the respective sequences of FSP27 (aa 137-173 and aa 174-200) and 38% and 48% similarity with Cidea (aa 122-158 and 159-185). The homology between Cidea and Perilipin within each domain is shown. B. Cidea constructs of GFP or HA fused to the full length or various fragments of Cidea were generated based on the homology to the known perilipin domains.

therefore may be required to target Cidea to the lipid droplet (72). Finally, the fifth region of Cidea (V) shares 32% homology with the second triglyceride shielding domain found in the carboxy terminal portion of Perilipin. The constructs generated include the full length protein, the amino terminal 116 amino acids, encompassing regions I and II, or the carboxy terminal 104 amino acids, encompassing regions III and IV, fused to either GFP or HA. Additionally, in order to further analyze the amino terminal region, GFP constructs were also generated to either region I or region II (Figure 3.1).

Not surprisingly, ectopic expression of the full-length Cidea protein fused to GFP in 3T3-L1 adipocytes confirmed that human Cidea targets to lipid droplets similarly to mouse Cidea (72). As seen in Figures 3.2 and Supplementary Figure 3.1, Cidea is found concentrated around the periphery of lipid droplets stained with oil red, a lipophylic dye which binds to neutral lipids, such as triglyceride and cholesterol esters. Conversely, the GFP control and three amino terminal constructs show diffuse cytoplasmic and nuclear staining, indicating no distinct intracellular localization. However, the carboxy terminal construct shows a localization pattern similar to the full-length protein, with ring-like labeling surrounding lipid droplets (Figures 3.2 and Supplementary Figure 3.1).

To ensure that these findings were not an artifact of the GFP tag, the experiments were repeated with an HA tag fused to the full length, amino terminus, or carboxy terminus of Cidea. These experiments showed a similar intracellular localization pattern for the GFP and HA tagged proteins, indicating that Cidea lipid droplet localization is not influenced by the presence of the GFP tag (Supplementary Figure 3.2). Furthermore,

these experiments reveal that Cidea targeting to the lipid droplet is solely dependent on the carboxy terminus in adipocytes.

In order to determine if the targeting of human Cidea is an adipocyte specific phenomenon, the GFP tagged constructs were expressed in 3T3-L1 fibroblasts and COS-7 monkey kidney cells. Similar to 3T3-L1 adipocytes, in 3T3-L1 fibroblasts, both the full-length protein and carboxy terminus target to lipid droplets, while the GFP control and amino terminus show diffuse cytoplasmic and nuclear staining (Figure 3.3). These findings suggest that Cidea targeting to lipid droplets is not dependent on a protein specifically expressed in adipocytes.

However, 3T3-L1 fibroblasts are preadipocytes and may express low levels of an adipocyte protein that targets Cidea to the lipid droplets. Therefore, Cidea expression was also analyzed in the more distinct cell line, COS-7 kidney cells. These experiments again show that the GFP control and amino terminus of Cidea do not have a distinct localization pattern, but the full-length Cidea and the carboxy terminus of Cidea localize to the lipid droplets. However, neither Cidea nor the carboxy terminus shows as tight an association with the droplets in COS cells as in adipocytes or preadipocytes. While both Cidea and the carboxy terminus show punctate staining in the vicinity of lipid droplets, with some colocalization, they do not form the same ring-like structures surrounding the lipid droplets, as seen in adipocytes and preadipocytes (Figures 3.4 and Supplementary Figure 3.3). This data suggests there may be a protein expressed in adipocytes and preadipocytes that enhances Cidea association or prevents Cidea dissociation from the lipid droplets.

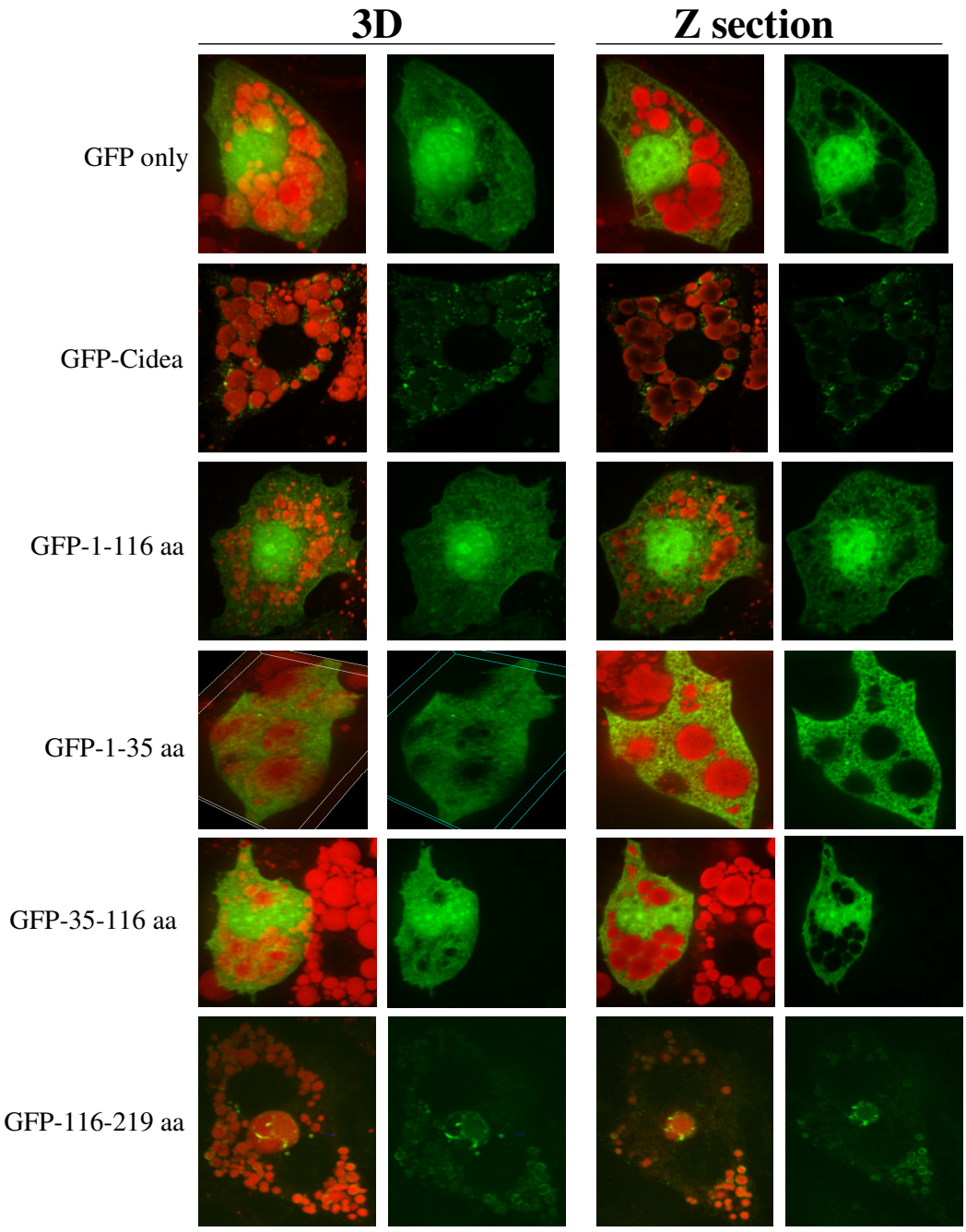


Figure 3.2. The C terminus of Cidea (116-219 amino acids) is necessary and sufficient for lipid droplet localization in adipocytes. GFP only or the GFP-Cidea constructs were expressed in day 5 adipocytes for 24 hours, the cells were then fixed, and the neutral lipid was labeled with oil red O. The left two panels show 3D images in either the green and red channels together to visualize the Cidea localization to lipid droplets or the green channel only to visualize the cellular distribution of Cidea. The right two panels show confocal Z sections in either the green and red channels together or the green channel only. All images are 100X.

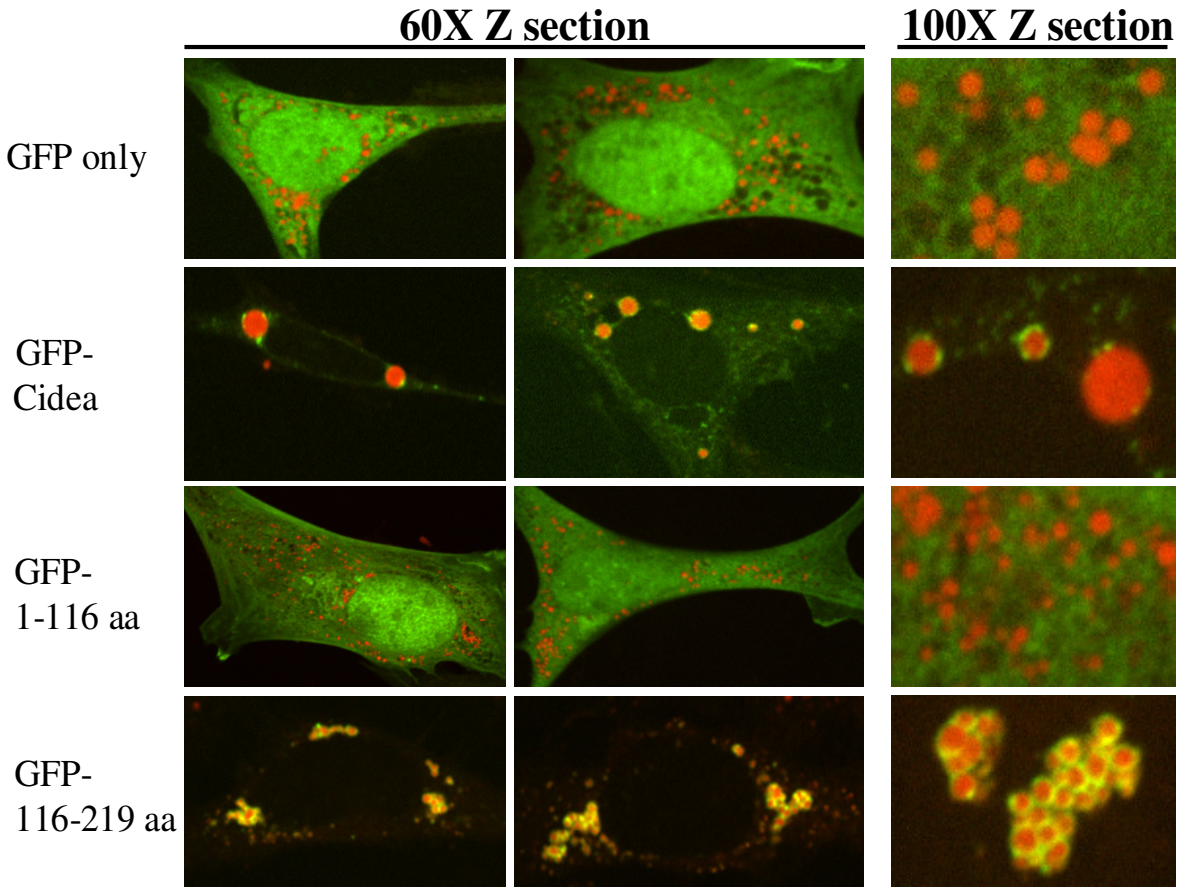


Figure 3.3. The C terminus of Cidea (116-219 amino acids) is necessary and sufficient for lipid droplet localization in preadipocytes. GFP only or the GFP-Cidea constructs were expressed in 3T3-L1 preadipocytes for 24 hours, the cells were then fixed, and the neutral lipid was labeled with oil red O. The left two panels show a 60X confocal Z section of two different cells. The right panel shows a 100X confocal Z section for closer inspection of cidea localization to the lipid droplet. The images for each construct are from three different cells.

Cidea and the carboxy terminus of Cidea induce distinct changes in lipid droplet morphology

Interestingly, these experiments in preadipocytes and COS-7 cells also revealed a striking change in lipid droplet morphology with overexpression of the full length or carboxy terminus of Cidea compared to the GFP control or amino terminus. In cells expressing the GFP control or amino terminus, lipid droplets remain small and dispersed throughout the cytoplasm. However, in cells expressing the full-length protein, the lipid droplets tend to be fewer in number and larger in size, suggesting that Cidea may facilitate the fusion of lipid droplets. In cells expressing the carboxy terminus of Cidea, the lipid droplets cluster together, but do not form large droplets during the timecourse analyzed (Figure 3.4). This clustered morphology has been reported for other lipid droplet shielding proteins, such as Perilipin and MLDP, suggesting that the carboxy terminus may contain the shielding domain required to inhibit lipase association with the droplet (85,256). To determine if the carboxy terminus could induce large lipid droplet formation in the presence of the amino terminus, both constructs were expressed together. In cells expressing both constructs, lipid droplets were clustered, but not large and few. Therefore, dual expression of the amino and carboxy termini together did not result in the full length phenotype, suggesting the carboxy terminus is required for the proper targeting of the amino terminus for large lipid droplet formation and the amino terminus cannot function without the presence of the carboxy terminus (data not shown).

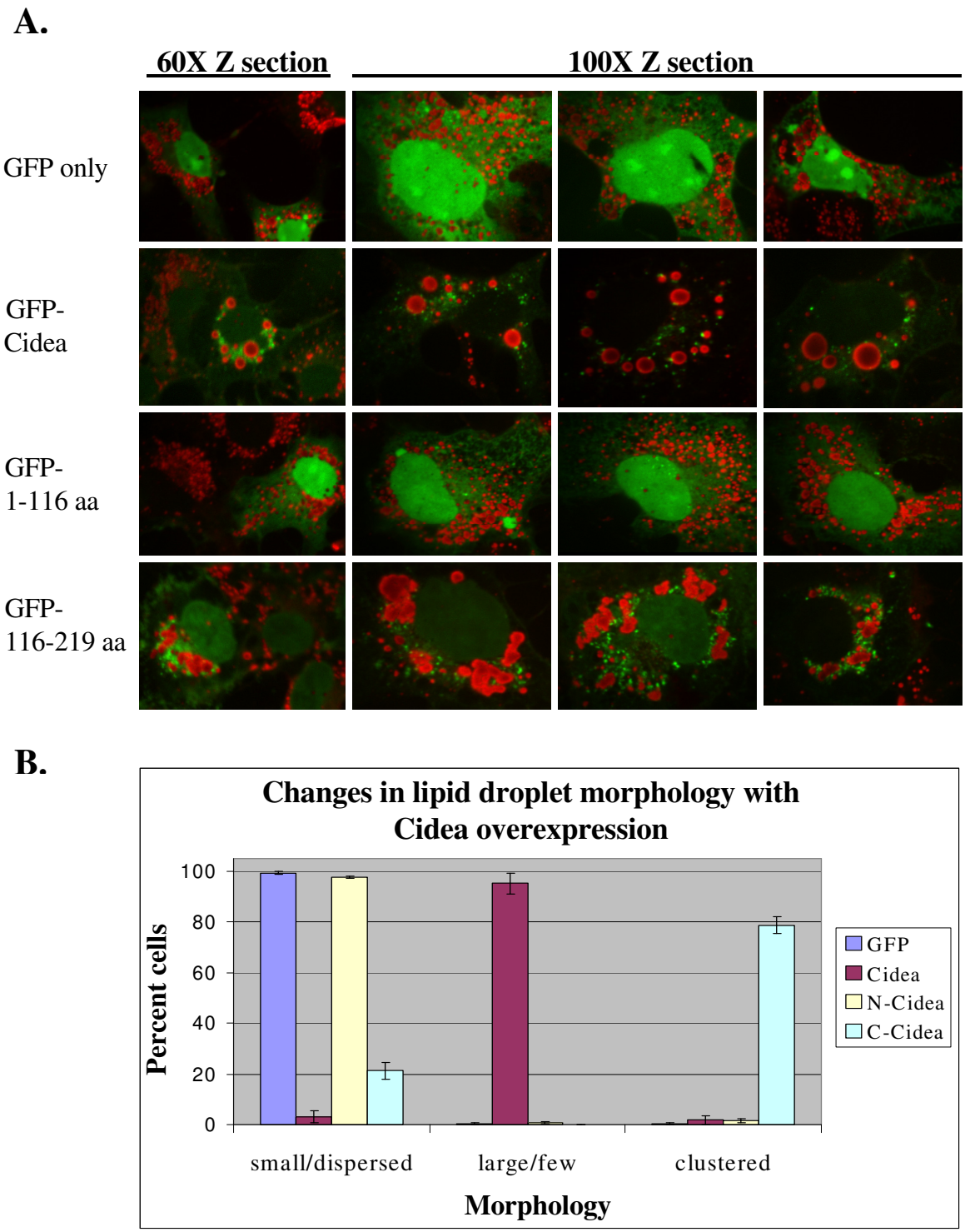


Figure 3.4. Cidea changes lipid droplet morphology in COS-7 cells, but does not tightly associate with lipid droplets. A. GFP only or the GFP-Cidea constructs were expressed in COS-7 for 24 hours in the presence of 400µM oleate, the cells were then fixed, and the neutral lipid was labeled with oil red O. The left panel shows a 60X confocal Z section of a cell expressing GFP. The right panel shows a 100X confocal Z section for closer inspection of cidea localization to the lipid droplet. The images for each construct are from four different cells. B. Lipid droplet morphology was determined in 200 cells from three independent experiments as being small and dispersed, large and few, or clustered.

To ensure that the morphological changes are not due to the GFP tag, the experiments were repeated expressing the proteins fused to an HA tag. These experiments resulted in the same phenotype, indicating that the morphological changes are not due to the GFP tag (Supplementary Figure 3.4). Furthermore, even after 72 hours of expression, the carboxy terminus did not induce the large and few lipid droplets seen with the full-length protein. This data again suggests that the carboxy terminus is required to localize Cidea to the lipid droplets, but the amino terminus is required to stimulate large lipid droplet formation.

The C terminus of Cidea is necessary and sufficient to stimulate triglyceride storage

Multiple lines of evidence indicate that Cidea promotes lipid storage by inhibiting lipolysis. To date, all support for this Cidea function has been through loss of function studies (79,80). To determine if Cidea overexpression can also stimulate lipid storage, the GFP-Cidea constructs were overexpressed in COS-7 cells. The GFP-positive cells were then isolated through FACS to determine the triglyceride levels in cells expressing each of the constructs. Not surprisingly, overexpression of the amino terminus of Cidea caused no significant change in triglyceride level compared to the GFP control. However, both the full length and carboxy terminus of Cidea caused a significant increase in triglyceride accumulation above the GFP control (an increase of 35% and 27%, respectively). Although overexpression of the carboxy terminus causes slightly less triglyceride accumulation, this is not statistically different from the full-length protein, suggesting that the carboxy terminus of Cidea is necessary and sufficient to inhibit lipolysis and promote triglyceride accumulation (Figure 3.5). This data also suggests that while the

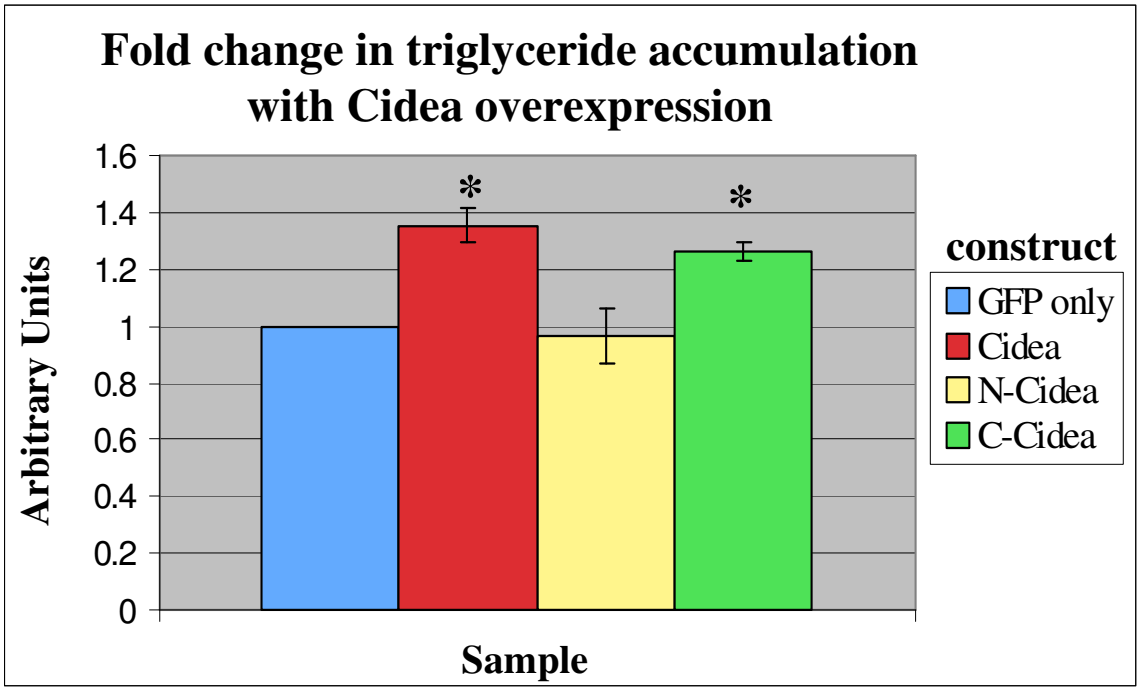


Figure 3.5. Cidea overexpression causes increased triglyceride accumulation. GFP only or the GFP-Cidea constructs were expressed in COS-7 for 24 hours in the presence of 400μM oleate and triglyceride levels were determined. The triglyceride levels with each condition were first normalized to cell number and then expressed as a fold change compared to the GFP only control. The values represent the average of three experiments. There is no significant difference in triglyceride in cells expressing the full length or C terminal domain. The asterisks denote a p value < 0.01.

amino terminus is required to promote the large lipid droplet formation seen with the full-length protein, this change in morphology is not required to promote triglyceride accumulation.

Cidea does not localize to the ER

A recent report claims that Cidea is localized to the ER in mouse brown adipocytes and may regulate lipid storage from this intracellular organelle (257). Since the ER is believed to be the site of lipid droplet biogenesis and Cidea is found both associated and dissociated from the lipid droplets in COS cells, Cidea may actually function in the ER rather than lipid droplets (65,68). In order to determine if Cidea localizes to the ER in COS cells, GFP-Cidea or the GFP-carboxy terminus were overexpressed in COS cells and the ER was labeled with calreticulin, an ER specific protein found throughout the smooth and rough ER. These experiments revealed clearly distinct labeling between Cidea and the ER, indicating very low, if any, colocalization of Cidea with the ER (Figure 3.6). Therefore, Cidea does not appear to function in the ER.

Discussion

Currently, published reports suggest that Cidea promotes lipid storage by inhibiting lipolysis, but the mechanism driving this function is still unknown (79,80). In this study, we show that the overexpression of human Cidea or the carboxy terminal 104 amino acids induced both lipid droplet targeting and triglyceride accumulation, identifying the carboxy terminus as both necessary and sufficient for these two functions.

Due to the anti-lipolytic role of Cidea, it is not unexpected that its overexpression increases triglyceride storage (Figure 3.5). Many studies have shown that inhibiting

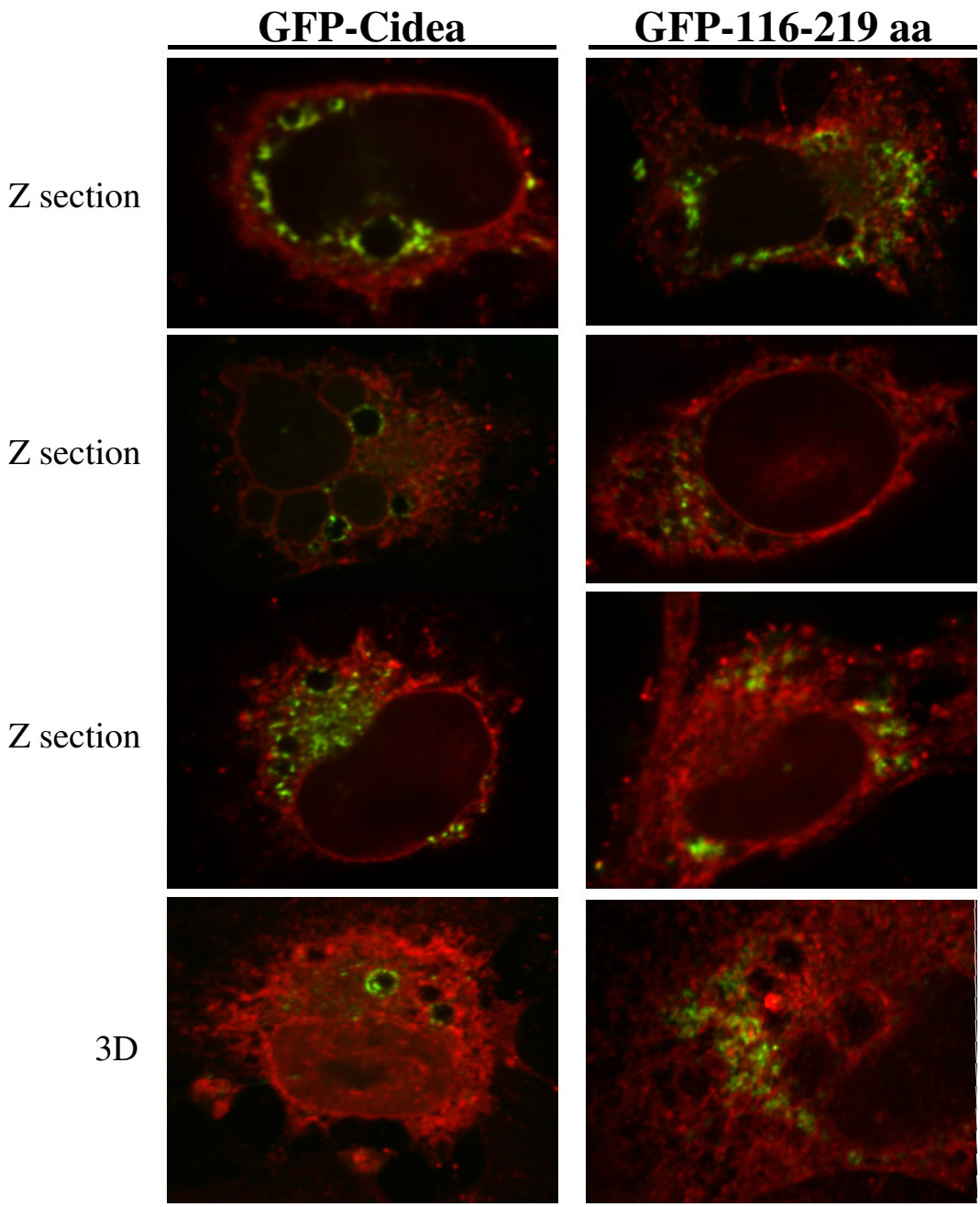
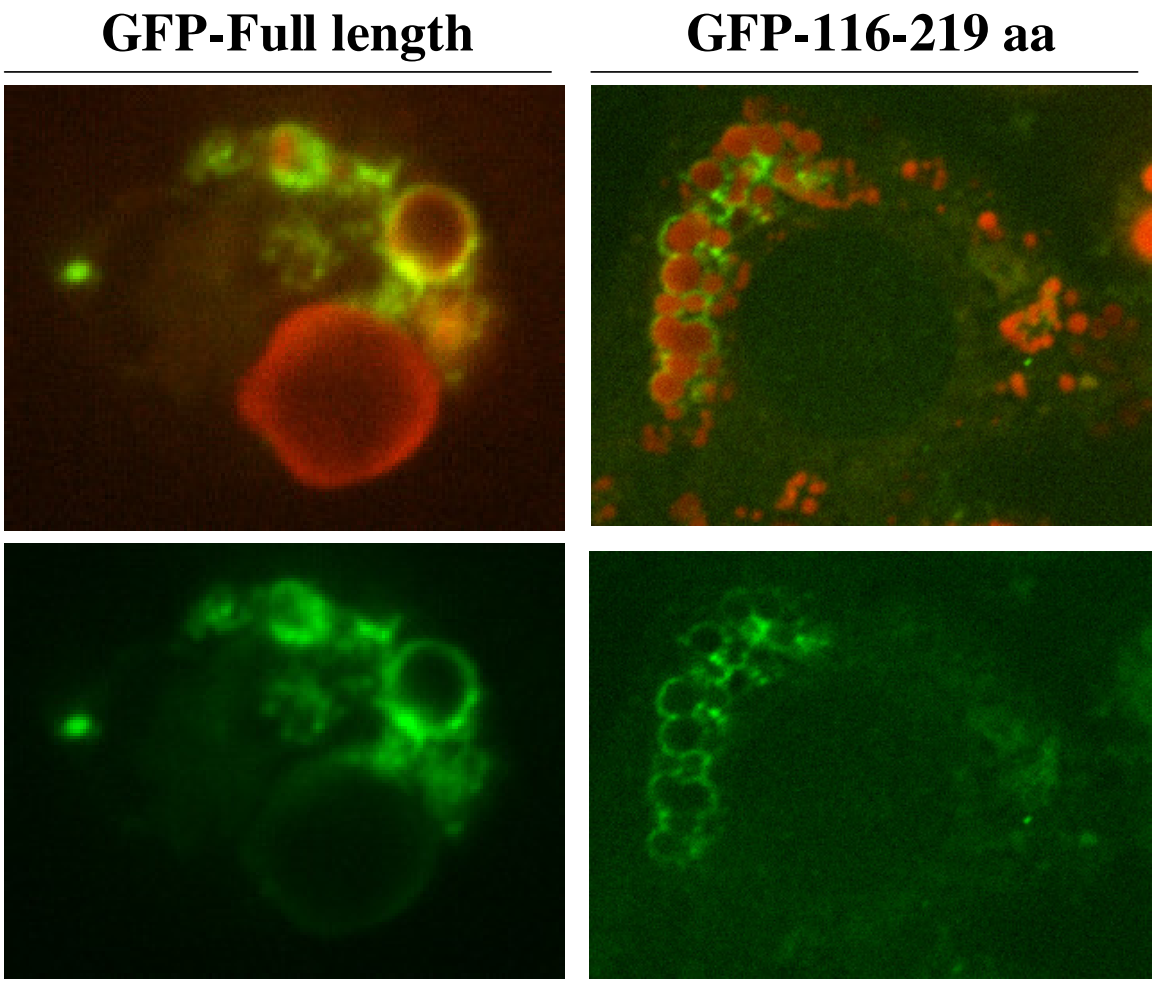
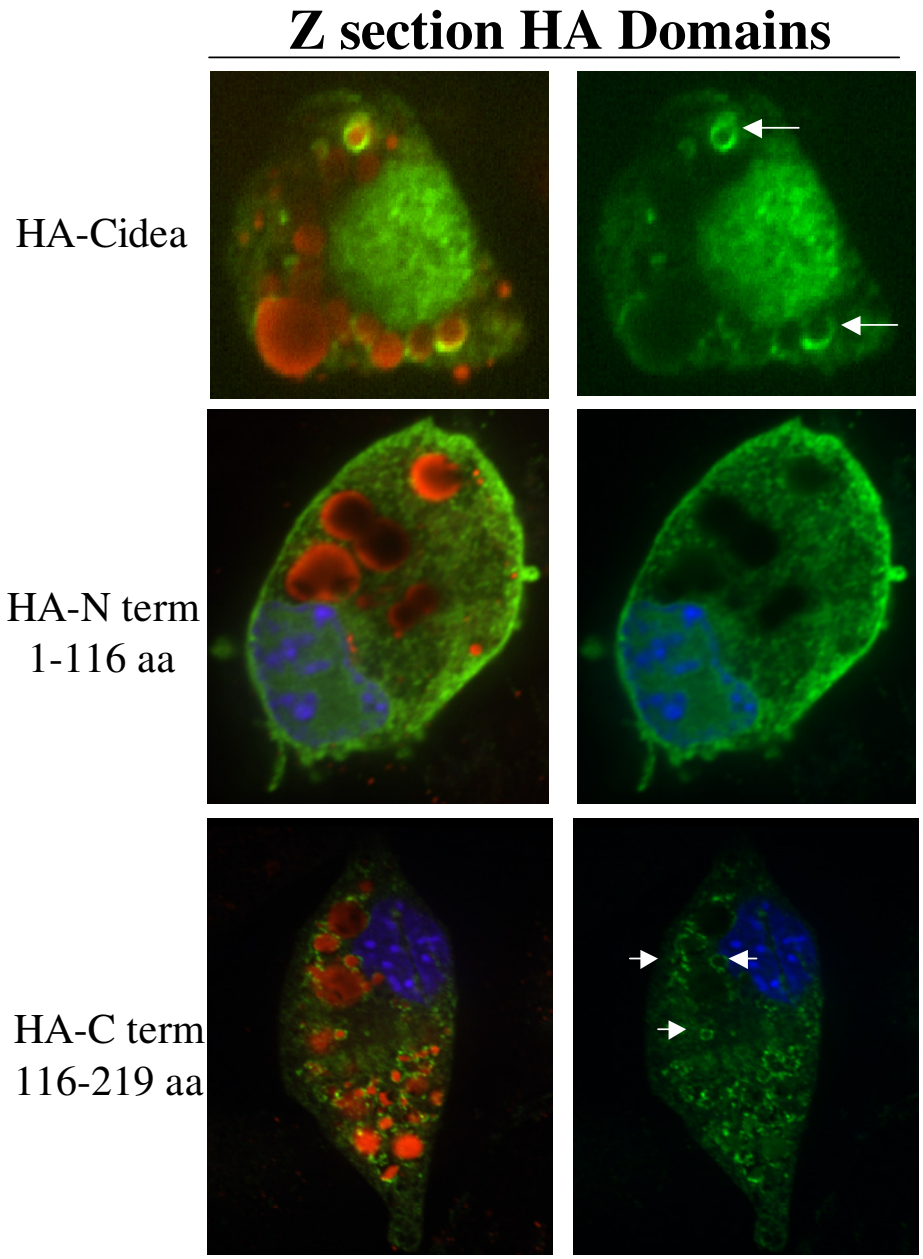


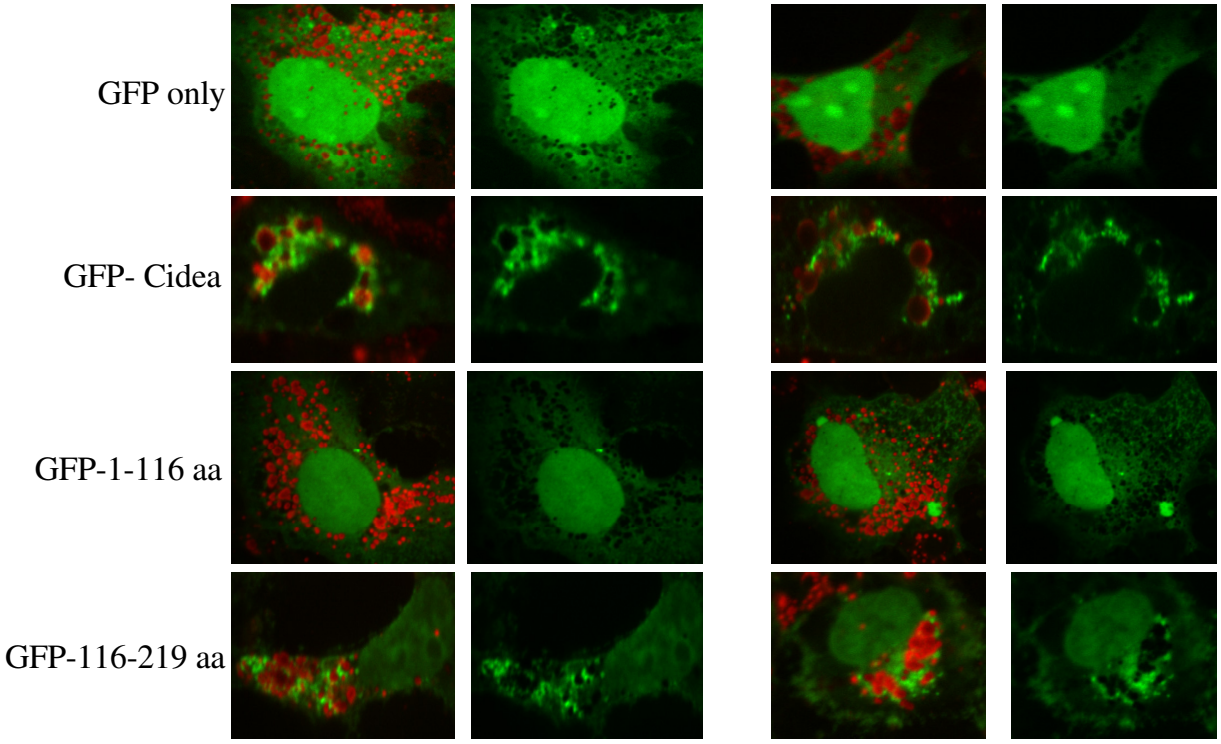
Figure 3.6. Cidea does not localize to the endoplasmic reticulum. GFP-Cidea or the GFP-116-219 amino acid construct was expressed in COS-7 for 24 hours in the presence of 400μM oleate, the cells were then fixed, and the endoplasmic reticulum was labeled with calreticulin, shown in the red channel. The left panel shows cells expressing full length Cidea and the right panel shows cells expressing the C terminus of Cidea. The top three panels are confocal Z sections, while the bottom panel is a 3D image. The images for each construct are from four different cells.



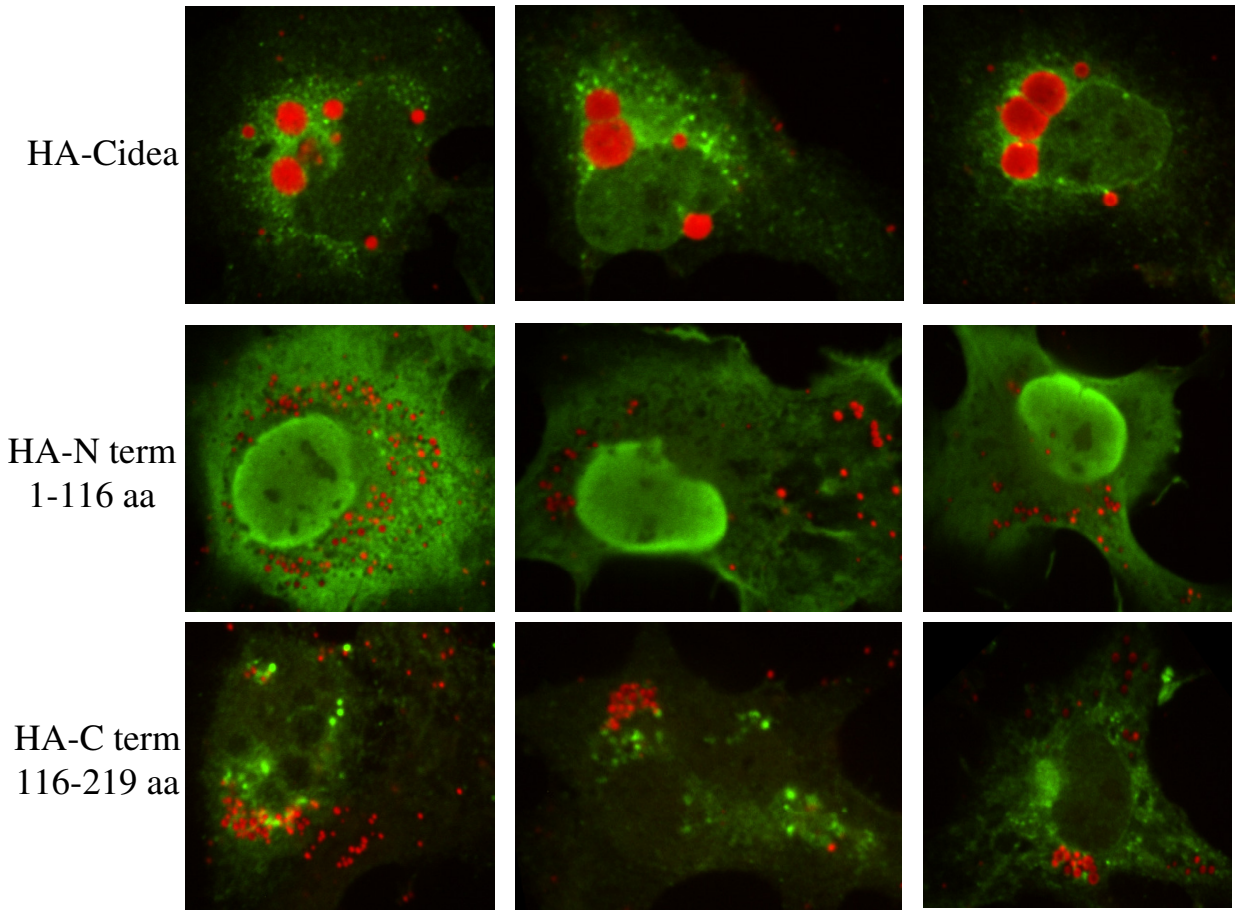
Supplementary Figure 3.1. Zoomed image of the full length or C terminal fragment of Cidea localized to lipid droplets in adipocytes. GFP-full length or GFP-C terminal Cidea constructs were expressed in day 5 adipocytes for 24 hours, the cells were then fixed, and the neutral lipid was labeled with oil red O. The top two panels show Z sections in the green and red channels together to visualize the Cidea localization to lipid droplets and the bottom two panels show the green channel only to visualize the cellular distribution of Cidea. All images are 100X.



Supplementary Figure 3.2. The C terminus of Cidea (116-219 amino acids) is necessary and sufficient for lipid droplet localization in adipocytes when fused to HA. The HA-Cidea constructs were expressed in day 5 adipocytes for 24 hours, the cells were then fixed, and the neutral lipid was labeled with oil red O. The left panel shows a confocal Z section in either the green and red channels together to visualize the Cidea localization to lipid droplets or the green channel only to visualize the cellular distribution of Cidea. All images are 100X.



Supplementary Figure 3.3. Cidea changes lipid droplet morphology in COS-7 cells, but does not tightly associate with lipid droplets. A. GFP only or the GFP-Cidea constructs were expressed in COS-7 for 24 hours in the presence of 400μM oleate, the cells were then fixed, and the neutral lipid was labeled with oil red O. The first and third panels show the green and red channels together to visualize Cidea localization to lipid droplets and the second and fourth show the green channel only to visualize the cellular distribution of Cidea. Shown are two different cells for each construct. All images are 100X confocal Z sections.



Supplementary Figure 3.4. Cidea changes lipid droplet morphology in COS-7 cells when fused to HA. HA-Cidea constructs were expressed in COS-7 for 24 hours in the presence of 400μM oleate, the cells were then fixed, and the neutral lipid was labeled with oil red O. The images for each construct are from three different cells. All images are 100X.

lipolysis by various means stimulates triglyceride accumulation. For example, depletion of ATGL expression with siRNA inhibits lipolysis resulting in increased triglyceride storage (258,259). Conversely, overexpression of shielding proteins, such as Perilipin, ADRP, or MLDP, also increases triglyceride storage (86,256,260,261). Another protein of the CIDE family, FSP27, binds lipid droplets and negatively regulates lipolysis (71,254). This protein is 65% homologous to Cidea and the two proteins show a similar phenotype when depleted or overexpressed. Interestingly, overexpression of FSP27 in 3T3-L1 preadipocytes increases triglyceride storage to a similar extent as Cidea overexpression, suggesting these two proteins may have redundant functions (73).

While Cidea clearly has a role in blocking lipolysis, it is also possible that it promotes triglyceride storage by enhancing synthesis. Although it has not been directly tested with Cidea, it has been shown that FSP27 does not promote triglyceride synthesis. Since Cidea and FSP27 are very similar, most likely Cidea also stimulates lipid storage through lipase inhibition rather than triglyceride synthesis (258).

It is not surprising the carboxy terminus of Cidea is responsible for both lipid droplet localization and triglyceride accumulation, since this region of Cidea shares sequence similarity to the carboxy terminal triglyceride shielding domain as well as to the targeting and anchoring domains of Perilipin (Figure 3.1) (75,86). Yet, we cannot conclude that these regions of Cidea are responsible for the targeting and anchoring to lipid droplets and triglyceride shielding without doing further analysis.

Despite the presence of a homologous motif in the amino terminus of Cidea to the amino terminal triglyceride shielding domain of Perilipin, this region is not required for

lipid droplet targeting or triglyceride accumulation (86). Nevertheless, the amino terminus is required to induce large lipid droplet formation and reduce lipid droplet number, which was not seen with the overexpression of the carboxy terminus alone, indicating that both termini are required for this function (Figure 3.4). Although further studies are required to determine if this morphological change is due to lipid droplet fusion, it is a likely possibility. Since lipid droplets can occupy much of the intracellular volume, especially in adipocytes, storing the lipids in few rather than several droplets would allow more cytoplasmic space for other cellular processes to occur. Since the adipocyte needs to maximize its triglyceride storage capacity, especially in response to increased fatty acid levels, this change in lipid droplet morphology could be very beneficial to the adipocyte. Interestingly, this change in lipid droplet morphology has not yet been reported for any other canonical lipid droplet associated protein and identifies a novel function for this class of proteins.

Due to the change in lipid droplet morphology, it was surprising that cells expressing full-length Cidea did not accumulate more triglyceride than cells expressing the carboxy terminus (Figure 3.5). Since a large droplet has less surface area to undergo lipolytic attack than a small droplet, forming larger droplets could reduce lipolysis and increase lipid storage. Experimental limitations may explain the lack in differences seen in triglyceride accumulation. For example, the experiments in this study were performed for 24 hours, since this timepoint yielded the optimal parameters for analysis: the cells had adequate GFP-Cidea expression, showed a phenotype, and are still healthy; longer timepoints result in increased cell toxicity. However, 24 hours may not be sufficient time

for the large lipid droplets to form and lipids to accumulate to a significant degree above the anti-lipolytic carboxy terminus. Perhaps if the experiments were carried out longer than 24 hours, a greater difference in triglyceride levels would be found between cells expressing the full-length and the carboxy terminal protein.

These studies outline the mechanism in which human Cidea regulates lipid storage and may be an important aspect in the maintenance of human adipocyte function and glucose homeostasis. This is because a number of studies in humans suggest that Cidea may have a beneficial role in regulating whole body insulin sensitivity. For instance, Puri et al showed a positive correlation of Cidea expression and insulin sensitivity in obese humans matched for BMI (72). Additionally, Nordstrom et al showed a positive correlation of Cidea expression in lean insulin sensitive patients compared to obese insulin resistant patients and an increase in Cidea expression in obese patients that had undergone bariatric surgery versus patients that did not have surgery. Since bariatric surgery increased insulin sensitivity in the obese patients, Cidea expression again correlates with increased insulin sensivity (79).

Perhaps Cidea regulates insulin sensitivity by controlling the rate of lipolysis in the adipose tissue and properly sequestering fatty acids as triglyceride within lipid droplets. This function would be particularly important during the obese state to prevent the elevation of free fatty acid levels in the serum, which would then accumulate in the liver and muscle, causing lipotoxicity and insulin resistance (4). Therefore, it is of extreme importance to continue these mechanistic studies into understanding Cidea

function to further our understanding of adipocyte biology and develop novel therapies for diseases, such type 2 diabetes.

Limitations and Future Perspectives

These studies provide mechanistic insight into the ability of Cidea to induce lipid storage by identifying the carboxy terminus as being both necessary and sufficient for lipid droplet targeting and triglyceride accumulation. Additionally, a novel function for Cidea was revealed in that it promotes a dramatic change in lipid droplet morphology: the formation of large and few lipid droplets from small and dispersed lipid droplets, suggesting that lipid droplet fusion may be occurring. However, many questions remain regarding the mechanism behind these findings.

For example, the carboxy terminus is equally efficient to the full-length protein in stimulating triglyceride accumulation within 24 hours. However, if the experiment were carried out for a longer time period, the full-length protein may induce significantly more triglyceride accumulation than the carboxy terminus. The triglyceride determination assay was performed at 24 hours because longer timepoints resulted in significant cell toxicity due to excessive GFP expression. Since these experiments require the use of a GFP tag to allow the sorting of cells expressing Cidea, they could only be performed for 24 hours. Perhaps expressing the constructs from a less efficient promoter would prevent reaching a toxic level of transcript too quickly by enabling a low rate of transcript expression, permitting a longer experimental duration. If the larger lipid droplets cause only a small reduction in lipolysis due to the reduced surface area accessible to lipases, it

might take longer than 24 hours to see a significant enhancement in triglyceride accumulation with the full-length protein. Therefore, any differences in triglyceride storage may require using a construct with a less efficient promoter to allow a longer experimental timecourse.

Although it seems unlikely that Cidea is promoting lipid storage by increasing triglcyeride synthesis, this is still a formal possibility and needs to be ruled out. Therefore, this could be accomplished using radiolabeled oleate and determining if the rate of oleate incorporation into triglyceride increases with Cidea overexpression.

Within the timecourse tested, it is evident that the carboxy terminus is required for lipid droplet localization, but the amino terminus is required for the formation of larger and fewer lipid droplets. However, it is not clear which region of the amino terminus holds this function. It would be interesting to generate GFP constructs expressing the carboxy terminus fused to various regions of the amino terminus to determine which motif facilitates the large lipid droplet formation. Likewise, it would be interesting to identify which region of the carboxy terminus is responsible for both the lipid droplet targeting and triglyceride storage. This could be accomplished by generating GFP constructs expressing various truncations of the carboxy terminus to determine which motifs are required for each function.

Additionally, it is important to determine if Cidea requires interaction with another protein to bind lipid droplets. This could be accomplished by overexpressing HA-Cidea and the HA-carboxy terminus and immunoprecipitating Cidea to identify the bound proteins by mass spectrometric analysis. The proteins that are bound to both the

full length and the carboxy terminus would be the candidates for targeting Cidea to lipid droplets. These proteins could then be depleted using siRNA to determine if GFP-Cidea localization to lipid droplets is inhibited.

Another important experiment that should be performed is to monitor the changes in gene expression with Cidea overexpression. Perhaps the changes in lipid droplet morphology are not directly due to Cidea, but rather a gene induced by Cidea overexpression. Since PPARγ, SREBP1, and LXR are known to promote lipid storage, genes regulated by these transcription factors could first be monitored for expression changes (262,263). Additionally, coexpression of a reporter plasmid for each of these transcription factors could also be used to monitor activity. An aggressive approach would be to do gene chip analysis to determine global changes in gene expression with Cidea overexpression.

Unfortunately, Cidea is not expressed in cultured human adipocytes or in mouse white adipose tissue. Therefore, it is very difficult to study the in vivo role of Cidea in the white adipose tissue. However, it may be possible to perform the overexpression studies in cultured human adipocytes to determine if Cidea behaves similarly in cultured primary human adipocytes as it does in 3T3-L1 adipocytes, preadipocytes, and COS cells. Additionally, perhaps rosiglitazone treatment increases Cidea expression in cultured human adipocytes and promotes lipid storage. If this is the case, then Cidea could be depleted using siRNA to determine if it is necessary for rosiglitazone induced triglyceride storage. These experiments would give additional insight into how Cidea mediates the

beneficial effects of PPARγ activation and provide a basis for better understanding the role of Cidea in regulating adipocyte function and whole body insulin sensitivity.

Chapter IV: Discussion

The adipose tissue functions as a regulator of whole body insulin sensitivity by safely storing excess fatty acid as triglyceride to prevent the accumulation of lipid in peripheral tissues, such as the liver and muscle, and by secreting factors called adipokines that enhance peripheral insulin sensitivity (4,6). Both of these important processes are absolutely dependent on the expression and activity of the nuclear receptor, PPARγ (6,203,264). This transcription factor is the master driver of adipogenesis and regulator of adipocyte gene expression in fully differentiated cells (98,203). Loss in function of PPARγ will result in a complete inhibition of the adipogenic process as well as decrease the ability of fully differentiated cells to store fatty acids as triglyceride and secrete insulin-sensitizing adipokines. Therefore, a loss in PPARγ function will result in serious metabolic disorders, such as insulin resistance and type 2 diabetes.

Regulation of PPARγ expression by SCD2

Due to the powerful regulation that PPARγ exerts on whole body energy metabolism, it is essential to understand the endogenous mechanisms that control PPARγ expression, activity, and function. In the first part of this thesis a novel and powerful regulator of PPARγ expression was identified in adipocytes. These studies revealed that the fatty acid Δ9 desaturase, Stearoyl CoA Desaturase 2 (SCD2) is required for PPARγ protein synthesis and therefore expression and function. Since SCD2 is required for PPARγ expression, loss in SCD2 expression inhibited adipogenesis and decreased

adipocyte gene expression. Although the phenotype resulting from the depletion of SCD2 may not be specifically due to the loss in PPARγ, this could fully explain the loss in adipocyte gene expression.

Since SCD2 is required to maintain general protein synthesis, these findings illustrate a novel role for endogenous fatty acid desaturation in adipocytes. Fatty acids are continually being recognized as powerful signaling molecules and the adipocyte has the potential to regulate many of its cellular processes through lipid signaling intermediates. Although it is not known whether SCD2 controls translation through the production of a desaturated fatty acid, it is interesting that the adipocyte, a cell that is specialized for the metabolism of fatty acids, controls the essential function of protein synthesis by a fat metabolizing enzyme.

Perhaps this is a mechanism for the adipocyte to communicate with the external environment. The saturated fatty acid, palmitate, is one of the most abundant free fatty acids present in the diet as well as in cellular membranes and triglyceride stores. Additionally, many membrane proteins are postranslationally palmitoylated, which may allow the proper folding of proteins in the endoplasmic reticulum, export from the Golgi, ability to assemble into protein platforms, acquire signaling capacity, or be endocytosed and sorted in the endocytic pathway (265). Therefore, palmitate has an immense effect on maintaining basic cellular functions. Interestingly, palmitate is a very efficient substrate of SCD2 (227). Thus, perhaps SCD2 functions as an indicator of palmitate levels in the adipocyte; when palmitate levels are low, SCD2 signals to the translational machinery to

slow protein synthesis and vice versa. This would prevent the synthesis of proteins that would not fold or function correctly.

Surprisingly, the highly homologous protein SCD1 does not regulate protein synthesis in the adipocyte. Finding this functional difference between the two SCD isoforms was unexpected because the proteins share 80% amino acid homology, are both present in the endoplasmic reticulum, and were both reported to utilize the same substrates with the same efficiency (227,229,232,233). Perhaps SCD1 and SCD2 associate with different proteins in the ER, which channels their products into different metabolic or signaling pathways. For example, the two isoforms may generate the same unsaturated fatty acid products, but the products of SCD2 are further metabolized in separate pathways from the products of SCD1. Although not much is known about the proteins associated with SCD2, enzymes involved in triglyceride synthesis have been shown to localize with SCD1 in the ER (266). Considering that most triglyceride molecules contain at least one unsaturated fatty acid moiety and the SCD1 knockout mouse has significantly reduced triglyceride levels, perhaps SCD1 channels fatty acids into triglyceride synthesis, whereas SCD2 channels fatty acids into other biosynthetic pathways, such as membrane and protein synthesis (229,235,267).

Since the discovery that SCD1 is a major driver of triglyceride synthesis, it has been a primary target for controlling obesity. Interestingly, mice have four highly homologous SCD isoforms, whereas humans have two SCD isoforms, showing approximately 80% and 60% homology (hSCD1 and hSCD5, respectively) to the mouse isoforms (229-231). Prior to the findings reported in this study, there was no data

regarding separate cellular functions among the mouse SCD isoforms. Given that human SCD1 is equally homologous to all four mouse isoforms, it may be that the human SCD1 shares functional homology to all four mouse desaturases. Therefore, SCD1 depletion in human adipocytes may provide insight into the function of this isoform and further understand its implications as an obesity target in humans.

Cidea regulation of triglyceride storage

Effective diabetic therapies currently on the market are the synthetic PPARγ agonists, called thiazolidinediones. These drugs improve glucose tolerance and serum lipid levels in diabetic patients, which is thought to be partially due to shifting lipid storage from peripheral tissues back into the adipose tissue by increasing triglyceride synthesis and storage in the adipocyte (193,194,268). This would relieve the liver and muscle from lipid overload and restore insulin sensitivity in these tissues. Interestingly, Cidea expression is highly regulated by PPARγ and thiazolidinedione treatment in mice induces a dramatic increase in Cidea expression (72). Since Cidea inhibits lipolysis in both mouse and human adipocytes, it may have a key role in regulating the beneficial effects of thiazolidinedione treatment in human diabetic patients by promoting lipid storage in the adipose tissue (79,80).

Previous findings from our lab identified Cidea as a lipid droplet associated protein in the mouse, suggesting that Cidea may promote lipid storage by binding to the lipid droplet and blocking lipase activity, thereby inhibiting lipolysis (72). However, prior to the findings reported in this study, nothing was known regarding the mechanism of Cidea association with lipid droplets and regulation of lipid storage. In this study, we

identified the carboxy 104 amino acids as being necessary and sufficient for lipid droplet binding and stimulation of triglyceride accumulation. This finding provides mechanistic detail into Cidea function and provides a basis for future studies in further defining this important aspect of adipocyte biology.

Interestingly, another member of the CIDE family, Cideb, was also recently described as targeting lipid droplets in the liver via the carboxy terminal amino acids 166-195 (255). Cidea and Cideb share approximately 62% amino acid homology and the corresponding region of Cidea to the lipid droplet targeting region of Cideb is predicted to also facilitate lipid droplet targeting based on homology to perilipin. In the liver, Cideb appears to be required for the assembly of triglyceride containing VLDL particles. This process requires the carboxy terminus to bind lipid droplets and the amino terminus to induce VLDL assembly and secretion (255). Reminiscent of these findings with Cideb were the findings in the present study regarding Cidea function. While the carboxy terminus of Cidea is necessary and sufficient for lipid droplet localization and triglyceride storage, the amino terminus is also required for a previously unrecognized function of Cidea: to form larger and fewer droplets from smaller, dispersed droplets. This change in lipid droplet morphology suggests that fusion of the lipid droplets may be occurring. Lipid droplet fusion has been reported to occur in muscle via the SNARE proteins, SNAP23, syntaxin 5, and VAMP4 (252). However, none of the canonical lipid droplet associated proteins of the PAT family or CIDE family have been reported to facilitate lipid droplet fusion. While these findings do not verify that fusion of the droplets is occurring, it is a likely possibility. Regardless of process causing the phenotype, Cidea is

causing a morphological change in lipid droplets and this change denotes a functional domain in the amino terminus.

Perhaps the formation of larger and fewer droplets allows large amounts of lipid to be stored efficiently in the intracellular environment. Supporting this idea is that under non-lipolytic conditions, primary adipocytes have unilocular droplets versus multilocular droplets. Formation of this large droplet may occur when the lipid droplets increase in size to the point of contact or it may occur through the facilitated trafficking and fusion via proteins, such as Cidea. Interestingly, another protein of the CIDE family, FSP27, may also regulate unilocular lipid droplet formation since the FSP27 knockout mice have multilocular droplets in the white adipose tissue (254). However, the multilocular droplet formation may also be due to increased lipolysis with FSP27 depletion, since lipolytic stimuli induces the same phenotype (269,270). One of the questions in the field is does a large lipid droplet reduce the efficiency of lipolysis by reducing the surface area accessible to lipases? Likewise, does the multilocular morphology result from lipolytic attack and breakdown of the lipid droplet core or is it a process induced to facilitate increased lipolytic efficiency. Since the carboxy terminus of Cidea is able to shield the lipid droplet, but not induce large lipid droplet formation, analysis of the lipolytic rates in cells expressing the carboxy terminus or full length protein may be a tool to answering these questions.

Many details remain to be discovered regarding lipid droplet biology and the impact these newly discovered organelles have on cellular functions. It is clear that dysregulated triglyceride storage in the adipocyte has detrimental consequences on whole

body energy metabolism and glucose homeostasis. Therefore, understanding the regulation of these dynamic intracellular storage sites is pertinent to understanding metabolic diseases, such as type 2 diabetes.

Appendix

Additional Experiments not discussed

1. Repeated siRNA knockdowns of EPHX1, EPHX2, NOS, CYP2F2, ELOVL 1,3,5, and 6 (alone and in combination) during differentiation of 3T3-L1 cells; No effect on PPARγ, Glut4, or PEPCK mRNA by real time PCR with any siRNA treatment.

2. Depleted SCD1 and SCD2 in fully differentiated adipocytes in the presence or absence of rosiglitazone; Partial restoration of PEPCK mRNA in SCD2 depleted cells with rosiglitazone treatment.

3. Depleted SCD2 in NIH-3T3 cells and monitored mRNA and protein of IRS1 and PTEN; IRS1 mRNA did not change, but protein decreased; PTEN protein did not change.

4. Monitored PPARγ mRNA in adipocytes with actinomycin treatment and found no change in mRNA half life with SCD2 depletion (mRNA half life ~ 2hours).

5. Treated preadipocytes with the differentiation media or a lipid extract from the media of differentiating cells, in the presence or absence of SCD2 depletion, to determine if SCD2 depletion affected PPARγ ligand production (ligand reported to be secreted in the media during differentiation); control media (never exposed to cells) also induced differentiation (presumably extracting IBMX) and found no difference in differentiation with media from SCD2 depleted cells.

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