

NOVEL ROLES OF STEROL REGULATORY ELEMENT-BINDING PROTEIN-1
IN LIVER

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Medical and Molecular Genetics,
Indiana University

June 2016

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

To my family, for their endless support, sacrifice, and love

ACKNOWLEDGEMENTS

This journey to obtaining my Ph.D. would not have been possible without the training and never-ending support from my mentors/teachers, my family, and friends. First and foremost, I would like to thank my mentor, Dr. Núria Morral, for allowing me the opportunity to pursue my Ph.D. under her tutelage. I have greatly expanded my expertise and have benefited from her immense knowledge of diabetes as well as gene therapy. Her guidance, support, and scientific insights have been invaluable to the success of my research studies and to attaining my doctorate. I truly appreciate her help in making sure I stayed the course and reached my research goals. I would also like to thank the members of my research committee, past and present, Dr. Kenneth Cornetta, Dr. Brenda Grimes, Dr. Jeffery Elmendorf, Dr. Robert Considine, Dr. Brittney-Shea Herbert, and Dr. Tamara Hannon for their constructive evaluations, guidance, knowledge, and ideas.

I would like to thank members of the Morral lab, past and present, including Dr. Miwon Ahn, Dr. Yongyong Hou, Dr. Sneha Surendran, and Aisha Gamble. It has been a privilege to work with them on projects and their help permitted me to learn many new techniques which I have since used in these studies. I am also grateful to the summer research students Viraj Maniar and Vanessa Ianson for their help with this work. I would like to thank Dr. Paul Herring and April Hoggatt for aiding in use of the luminometer for luciferase assays. I would like to thank Dr. Peter Roach, Dr. Ann Depoli-Roach, and Dyann

Segvich for allowing me to use their facilities and equipment. I would also like to thank Dr. Charlie Dong and Dr. Xiwen Xiong for use of their facilities and reagents which helped with this work. I would like to thank Dr. Amber Mosley and her lab members for use of reagents as well. I am grateful to the funding support I received to aid in my research studies through the R01-DK 07859-05S1 Research Supplements to Promote Diversity in Health-Related Research, and the President's Diversity Dissertation Fellowship.

I would like to thank the faculty and staff at the Center for Diabetes Research, as well as the faculty and staff of the Department of Medical and Molecular Genetics, past and present, especially Peggy Knople and Jean Good. These two women went above and beyond their duties, always providing help. I would like to thank the staff of the IUPUI Graduate Office and the Graduate Division of the Indiana University School of Medicine for their help during my Ph.D. studies.

Lastly, I would like to offer my deepest and heartfelt thanks to my family and friends for their constant support, encouragement, and unconditional love in the quest of my personal and professional goals. I am very thankful to my mother, Juliet, for her constant support and reassurance throughout this journey, my siblings for always being there for me, listening to me, and for helpful advice. I would not be the person I am today if it was not for my family. Their enthusiasm and motivation during this process helped me more than words can say and I am greatly and eternally thankful to them. I would like to genuinely thank all of my

friends and in-laws for their words of encouragement, support, and much needed cheer.

I would like express my unending gratitude to my husband, Matthew. This journey would not have been the same without his love, encouragement, wisdom, and sacrifice. Words cannot begin to express how much you mean to me. Matt and Frank, you have taught me so much and have brought me so much joy. Thank you both for always being there and for all the great memories. I look forward to many more journeys and adventures with you. Finally and most importantly, I would like to thank God for giving me this opportunity and the strength, day in and day out, and reminding me all things are possible through Him.

Victoria N. Jideonwo

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Sterol Regulatory Element Binding Protein-1 (SREBP-1) is a conserved transcription factor of the basic helix-loop-helix leucine zipper family (bHLH-Zip) that primarily regulates glycolytic and lipogenic enzymes such as L-pyruvate kinase, acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase 1, and mitochondrial glycerol-3-phosphate acyltransferase 1. SREBP-1c activity is higher in the liver of human obese patients, as well as *ob/ob* and *db/db* mouse models of obesity and type 2 diabetes, underscoring the role of this transcription factor as a contributor to hepatic steatosis and insulin resistance. Nonetheless, SREBP-1 deficient *ob/ob* mice, do not display improved glycemia despite a significant decrease in hepatic lipid accumulation, suggesting that SREBP-1 might play a role at regulating carbohydrate metabolism. By silencing SREBP-1 in the liver of normal and type 2 diabetes *db/db* mice, we showed that indeed, SREBP-1 is needed for appropriate regulation of glycogen synthesis and gluconeogenesis enzyme gene expression. Depleting SREBP-1 activity more than 90%, resulted in a significant loss of glycogen deposition and increased expression of *Pck1* and *G6pc*. Hence, the benefits of reducing *de novo* lipogenesis in *db/db* mice were offset by the negative impact on gluconeogenesis and glycogen synthesis. Some studies had also indicated that SREBP-1

regulates the insulin signaling pathway, through regulation of IRS2 and a subunit of the PI3K complex, p55g. To gain insight on the consequences of silencing SREBP-1 on insulin sensitivity, we analyzed the insulin signaling and mTOR pathways, as both are interconnected through feedback mechanisms. These studies suggest that SREBP-1 regulates S6K1, a downstream effector of mTORC1, and a key molecule to activate the synthesis of protein. Furthermore, these analyses revealed that depletion of SREBP-1 leads to reduced insulin sensitivity. Overall, our data indicates that SREBP-1 regulates pathways important for the fed state, including lipogenesis, glycogen and protein synthesis, while inhibiting gluconeogenesis. Therefore, SREBP-1 coordinates multiple aspects of the anabolic response in response to nutrient abundance. These results are in agreement with emerging studies showing that SREBP-1 regulates a complex network of genes to coordinate metabolic responses needed for cell survival and growth, including fatty acid metabolism; phagocytosis and membrane biosynthesis; insulin signaling; and cell proliferation.

Núria Morral, Ph.D., Chair

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LIST OF ABBREVIATIONS

°C	Degree Celsius
μl	Microliter
μM	Micromolar
ψ	Packaging signal
24.25-EC	24(S)-25-epoxycholesterol
² H ₂ O	Deuterium oxide
3T3-L1	Mouse 3T3-L1 pre-adipocytes
3'-UTR	3'-Untranslated region
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
ACAC	Acetyl-CoA carboxylase
ACACA	Acetyl-CoA carboxylase 1
ACACB	Acetyl-CoA carboxylase 2
Acat	Acetoacetyl-CoA thiolase
Acly	ATP-citrate lyase
Acox2	Acyl-CoA oxidase 2
Acss	Acetyl-CoAs synthetase
Akt	Protein kinase B
AMP	5' adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate

AU	Area under the curve
BHB-CoA	Beta-hydroxybutyrate
bHLH-ZIP	Basic helix loop helix-leucine zipper
bp	Base pair
BSA	Bovine serum albumin
BSL2	Biosafety level 2
C57BL/6J	C57 black 6 mice
C57BLKS/J	C57 black Kaliss mice
cAMP	Cyclic 5' adenosine monophosphate
CAR	Carboxylic acid reductase
Casp2	Caspase-2
CBP	CREB-binding protein
Cdkn1a	Cyclin-dependent kinase inhibitor p21
cDNA	Complementary DNA
CD44	CD44 Molecule
CDK8	Cyclin-dependent protein kinase 8
Chip-seq	Chromatin immunoprecipitation-sequencing
Chr 17p11.2	Chromosome 17, position 11 on short arm
Chr 22q13.2	Chromosome 22, position 13 on long arm
ChREBP	Carbohydrate-responsive element binding protein
CMV	Cytomegalovirus
COPII	Coat-protein complex II
CPT1A	Carnitine palmitoyl transferase 1A

CPT2	Carnitine palmitoyl transferase 2
Cre-loxP	Cre recombinase-lox P site
CsCl	Cesium Chloride
CUTL1	Cut-like homeobox 1
Cyc-40	Cyclophillin 40
Cyc-A	Cyclophillin A
CYP51	Lanosterol 14 α -demethylase
<i>Db/db</i>	Diabetic mouse model, homozygous for a point mutation in the leptin receptor gene, deficient in leptin receptor activity
dl	Deciliter
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNL	<i>De novo</i> lipogenesis
DGAT2	Diacylglycerol acyltransferase-2
E-box	Enhancer box
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EGR-1	Early growth response 1
EDTA	Ethylene diamine tetraacetic acid
EF1- α	Elongation factor 1 alpha
EGTA	Ethylene glycol tetraacetic acid
Elovl	Long-chain fatty acyl elongase

ENCODE	Encyclopedia of DNA Elements
ER α	Estrogen receptor alpha
ER	Endoplasmic reticulum
FAO	Fatty acid oxidation
Fasn	Fatty acid synthase
FBS	Fetal bovine serum
FFAs	Free fatty acids
FKHR	Forkhead box O1
FoxO1	Forkhead box O1
FPP	Farnesyl pyrophosphate
FXR	Farnesoid X receptor
g	Gram
G6pc	Glucose 6-phosphatase
gAd	Gutless adenovirus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G β L	G-protein beta-subunit-like
Gck	Glucokinase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
gp78	Glycoprotein 78
Gpm	mitochondrial glycerol-3-phosphate

GPAT	Glycerol-3-phosphate acyltransferase
GSK3 α	Glycogen synthase kinase 3 alpha
GSK3 β	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate
GTT	Glucose tolerance test
GYS2	Glycogen synthase 2
h	Hour/s
H14	Helper virus
HBSS	Hank's Balanced Salt solution
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HDL	High-density lipoprotein
Hepa1c1c7	Mouse hepatoma cell line
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Human hepatoma cells
HEK293	Human embryonic kidney 293 cells
HFD	High-fat diet
HI	Hyperinsulinemia
HL	Hyperlipidemia
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
Hmgcr	HMG-CoA reductase
Hmgcs	HMG-CoA synthase
Hmox1	Heme oxygenase 1

HNF4 α	Hepatocyte nuclear factor 4 alpha
HRP	Horse radish peroxidase
HSL	Hormone-sensitive lipase
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
INSIG-1	Insulin-induced gene 1
INSIG-2	Insulin-induced gene 2
Insig2a	Insulin-induced gene 2a
Insig2b	Insulin-induced gene 2b
IR	Insulin receptor
IRES	Internal ribosomal entry site
IRSs	Insulin receptor substrates
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
ITR	Inverted terminal repeat
ITT	Insulin tolerance test
JNK	c-Jun N-terminal kinase
Kb	Kilobase
KDa	Kilodalton
kg	Kilogram
L	Liter
LDL	Low density lipoprotein
Ldlr	LDL receptor

LPL	Lipoprotein lipase
LRH-1	Liver receptor homolog-1
Lss	Lanosterol synthase
LXR	Liver X receptor
LXREs	Liver X receptor elements
LXR α	Liver X receptor alpha
LXR β	Liver X receptor beta
MAPRE1	Microtubule-Associated Protein, RP/EB Family, Member 1
Mb	Megabase
MCS	Multiple cloning site
mg	Milligram
min	Minute/s
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
mRNA	messenger RNA
mSin1	Mammalian stress-activated map kinase-interacting protein 1
mTOR	Mammalian/mechanistic Target Of Rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
Mu	Mutant
Mut1	Mutant 1

Mut2	Mutant 2
MVA	Mevalonic acid
Mvk	Mevalonate kinase
MVB	Multivesicular bodies
ng	nanogram
nM	nanomolar
N/A	Not available
NaCl	Sodium chloride
NaF	Sodium fluoride
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEC	No expression cassette
NEFA	Non-esterified fatty acid
NF- κ B	Nuclear factor κ B
NH ₂ -	Amino group
n-SREBP-1	Nuclear SREBP-1
<i>Ob/ob</i>	Diabetic mouse model, recessive gene mutation causes mice to become incapable of producing leptin, resulting in lack of satiety
p300	E1A binding protein p300
PA	Palmitate
PAGE	Polyacrylamide gel electrophoresis
P/S	Penicillin/streptomycin

PCA	Principal component analysis
Pck1	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PDK1	3-phosphoinositide-dependent protein kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
Pfkl	Phosphofructokinase liver
PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol 3-kinase
PIK3R3	Phosphatidylinositol-3 kinase regulatory subunit p55 γ
PKC	Protein kinase C
Pklr	Liver pyruvate kinase
pmol	Picomolar
PMSF	Phenylmethane sulfonyl fluoride
PPAR- α	Peroxisome proliferator-activated receptor- α
PRAS40	Proline-rich Akt substrate 40kDa
pRes	Rescue plasmid
p-SREBP-1	Precursor SREBP-1
PTEN	Phosphatase and tensin homolog
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
QC	Quality control
qRT-PCR	Quantitative real-time polymerase chain reaction

Raptor	Regulatory-associated protein of mammalian target of rapamycin
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RNase	Ribonuclease
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RPS10	40S ribosomal protein S10
RT	Reverse transcription
RXR	Retinoid X receptors
S1P	Site-1 protease
S2P	Site-2 protease
S6K1	Ribosomal S6 kinase 1
Sar1	Secretion-associated RAS-related protein 1
SCAP	SREBP-cleavage activating protein
Scd 1	Stearoyl-Coa desaturase 1
Scd 2	Stearoyl-Coa desaturase 2
SCR	Scrambled
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Sec23	Protein transport protein 23
Sec24	Protein transport protein 24

Ser	Serine
SGK	Serum and glucocorticoid induced protein kinase 1
SHP	Small heterodimer partner
shRNA	Short hairpin RNA
siNC	Negative control siRNA
siRNA	Silencing RNA
siSRP1	SREBP-1 siRNA
SIRT1	Sirtuin 1
SNP	Single nucleotide polymorphism
Sp1	Specificity protein 1
SRE	Sterol regulatory element motif
SREBP	Sterol-regulatory element binding protein
STZ	Streptozotocin
Sqs	Squalene synthase
T0901317	LXR-selective agonist
T2DM	Type 2 diabetes mellitus
TBP	TATA binding protein
TBST	Tris-buffered saline and Tween 20
TG	Triglyceride
Thr	Threonine
TK	Thymidine kinase
TLC	Thin-layer chromatography
Top II	Type II topoisomerases

TR β	Thyroid hormone receptor beta
TSC1/2	Tuberous sclerosis 1 / 2
TSS	Transcription start site
Tyr	Tyrosine
U	Unit
Ub	Ubiquitin
UDP	Uridine diphosphate
VCP	Valosin-containing protein
VLDL	Very low-density lipoprotein
vp	Viral particles
WT	Wild type

INTRODUCTION

1. Control of carbohydrate and lipid metabolism by insulin

Maintaining glucose homeostasis under fed and fasting conditions is a vital physiological response. This balance is mainly orchestrated by the action of the hormones insulin and glucagon, which have opposite functions. Insulin is an anabolic hormone involved in promoting glucose uptake, glycogen synthesis, glucose conversion to lipid, and inhibition of glucose production. Under fed conditions, when plasma glucose levels are elevated, insulin is released from β -cells in the pancreas, facilitating glucose uptake by tissues, and clearing glucose from the circulation. In contrast, during fasting or after exercise, insulin secretion is diminished while glucagon is released from α -cells in the pancreas. Glucagon promotes glycogen breakdown and *de novo* glucose production, resulting in increased plasma glucose levels [1].

Insulin exerts its action in four main target tissues: skeletal and cardiac muscle, adipose tissue, and liver (Fig. 1). Insulin induces glucose uptake in the muscle by promoting the translocation of the GLUT4 glucose transporter to the cell membrane [2]. Insulin-mediated GLUT4 relocation enables glucose uptake by this tissue and is a main mechanism to reduce glucose levels from the circulation. During basal and hyperinsulinemic conditions, glucose uptake by the skeletal muscle accounts for 20 and 75% of insulin-dependent glucose disposal, respectively [3]. Under the fed state, glucose is oxidized into pyruvate through

glycolysis, which enters mitochondria and the Krebs cycle to produce ATP. In addition, glucose can be stored as glycogen [4].

Similarly, in the adipose tissue, insulin enhances glucose uptake by facilitating GLUT4 translocation to the cell membrane. Glucose is then metabolized to generate glycerol-3-phosphate, which provides the backbone for fatty acid esterification and triglyceride synthesis. In addition, insulin promotes transcription of the enzymes necessary for fatty acid and triglyceride synthesis. During fasting conditions, low insulin and high AMP/cAMP activate AMPK and PKA, blocking lipogenesis and activating the enzyme hormone sensitive lipase to reverse this process and release fatty acids into the circulation [4].

The liver is a versatile organ that has the ability to synthesize and breakdown glucose and fatty acids in response to nutrient/energy conditions, and is a critical tissue to maintain blood glucose levels within the normal range. Glucose uptake by the liver is mediated by the GLUT2 transporter and is not contingent on insulin [5]. However, in the transition from the fasted to the re-fed state, insulin stimulates gene expression and activity of the enzymes involved in the glycolysis, glycogen synthesis, and lipogenesis pathways. Glucose disposal to generate glycogen in the liver, represents ~17% of postprandial glucose [6]. Under fed conditions, glucose entering the hepatocyte is oxidized to generate ATP. When the levels are high, citrate and ATP inhibit phosphofructokinase-1, redirecting metabolites to the glycogen synthesis pathway as well as the pentose phosphate pathway and subsequently to palmitate synthesis [*de novo* lipogenesis (DNL) pathway] [4]. The newly synthesized palmitate molecule can

be desaturated and/or elongated, and used to produce triglycerides, phospholipids, ceramides, or other lipids. In addition, the liver receives fatty acids from the diet and from the circulation, which are released from triglyceride breakdown in the adipose tissue. Triglycerides generated in the liver remain in the cytoplasm as droplets, or are secreted into the circulation through very low-density lipoprotein (VLDL) for use by other tissues.

Insulin inhibits glycogen breakdown (glycogenolysis) and gluconeogenesis, preventing the production and release of glucose to the blood stream during fed conditions, when plasma glucose levels are high. However, under fasting conditions, glucose is produced from amino acids, glycerol, and lactate through the gluconeogenesis pathway and from glycogen breakdown. During fasting, hepatic glucose output is important for maintaining euglycemia. In addition, the liver receives a large supply of fatty acids from the adipose tissue, which are then oxidized to generate ketone bodies. These are secreted into the circulation and provide an alternative energy source for other organs, such as the brain and the skeletal muscle [4].

The liver also plays an important role at maintaining cholesterol homeostasis, and in the synthesis of bile salts. Furthermore, the liver synthesizes non-essential amino acids and makes most of the plasma proteins such as albumin and clotting factors. The liver also breaks down amino acids by deamination and transamination, and removes ammonia from the bloodstream, converting it into urea for excretion [4].

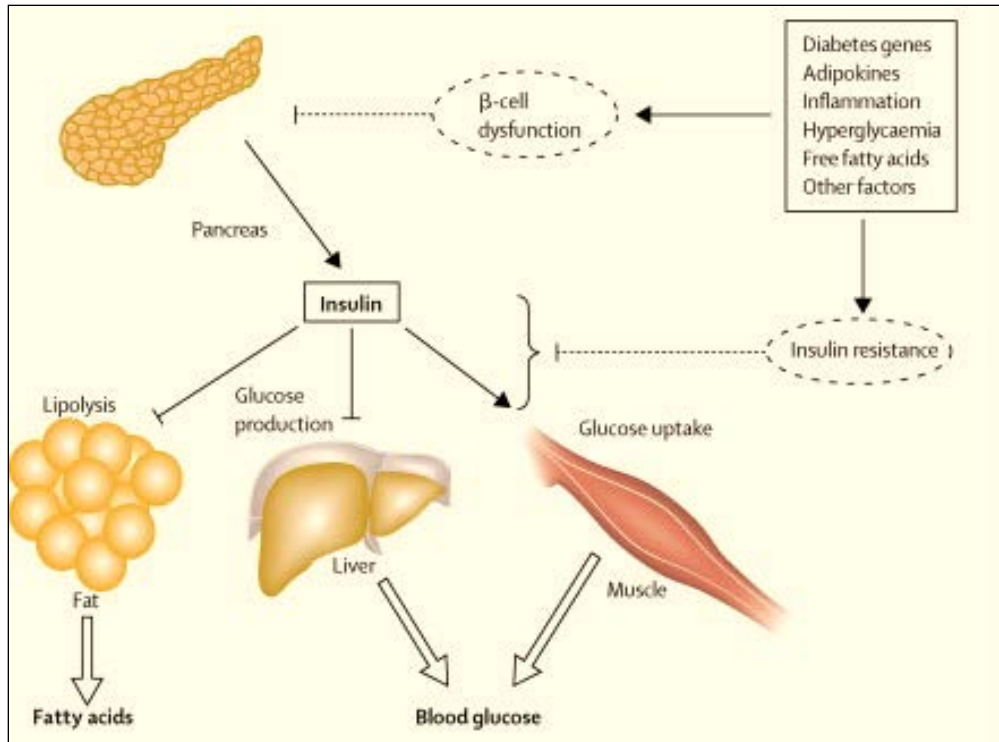


Figure 1. Insulin action in the principal target tissues. Insulin secreted from the pancreas, inhibits fatty acid release from the adipose tissue, decreases glucose output in the liver, and increases glucose uptake by the skeletal muscle. However, various contributing factors, such as insulin resistance/diabetes and hyperinsulinemia, lead to decreased insulin action in target tissues. As a result, hepatic glucose production increases and glucose disposal in the skeletal muscle is reduced, leading to increased blood glucose levels. In addition, lipolysis in adipose tissue is increased, raising the levels of circulating fatty acids. Both the increase in fatty acids and blood glucose exacerbate type 2 diabetes. Source: Image from [7].

2. Insulin resistance and type 2 diabetes

Insulin resistance is defined as decreased sensitivity or responsiveness to the action of insulin to exert its control on metabolism, resulting in multiple metabolic derangements in insulin-sensitive tissues [8]. Muscle, pancreatic β -cells, liver, and adipose tissue are contributors to the development of this pathology [9]. The causes of insulin resistance and hyperglycemia are complex and controversial. It is likely that insulin resistance starts simultaneously in more than one tissue. Nevertheless, multiple studies have provided evidence that adipose tissue dysfunction plays a critical role in the development of insulin resistance in other tissues. Insulin resistance in adipocytes leads to increased circulating free fatty acids (FFAs) due to up-regulation of lipolysis [10]. Excess circulating levels of FFAs causes lipid accumulation in liver, muscle, and β -cells, where it interferes with insulin signaling/action and causes lipotoxicity [11, 12]. Diacylglycerol and ceramide have been linked to insulin resistance by activating several isoforms of PKC [13] and protein phosphatase 2A, although the role of ceramides in the liver has been disputed [14]. PKC activation hinders IRS activity and downstream insulin signaling by phosphorylating IRSs at serine residues [15, 16]. In addition, free fatty acids activate c-Jun N-terminal kinase (JNK) [14, 17, 18], which interferes with IRS signaling. In the liver, JNK contributes to the development of insulin resistance and non-alcoholic steatohepatitis (NASH) [19, 20].

Insulin resistance leads to decreased glucose disposal in skeletal muscle as well as dysregulation of glucose transport and metabolism in adipose tissue,

and lack of inhibition of hepatic glucose production in the liver [8, 12]. The main consequence of lack of proper glucose metabolism in skeletal muscle and liver is high blood glucose levels, stimulating the pancreas to secrete insulin (Fig. 1). Hyperinsulinemia further exacerbates insulin resistance in target tissues.

The metabolic syndrome is a cluster of conditions that increase the risk of heart disease, stroke and diabetes. These conditions include: abdominal (central) obesity; elevated blood pressure and serum triglycerides; insulin resistance (elevated fasting glucose); and low high-density lipoprotein (HDL). Metabolic syndrome is becoming more common in the USA, due to the increase in obesity rates (more than 64% of the adult population is overweight). Without a change in lifestyle, metabolic syndrome may develop into type 2 diabetes (T2DM).

Non-insulin-dependent or type 2 diabetes mellitus is characterized by an elevated blood glucose concentration that derives from inadequate insulin action in target tissues and from abnormal insulin secretion [1]. The criteria for diagnosis of diabetes are: fasting plasma glucose ≥ 126 mg/dL (7.0 mmol/L); or 2-hour plasma glucose ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (with 75 g glucose dissolved in water); or HbA1c $\geq 6.5\%$ (48 mmol/mol); or random plasma glucose ≥ 200 mg/dL (in a patient with classic symptoms of hyperglycemia) [21]. The criteria for diagnosis of pre-diabetes are defined as: fasting plasma glucose of 100 mg/dL (5.6 mmol/L) to 125 mg/dL (6.9 mmol/L); or 2-hour plasma glucose of 140 mg/dl (7.8 mmol/L) to 199 mg/dL (11.0 mmol/L) during an oral glucose tolerance test; or HbA1c 5.7-6.4% (39-46 mmol/mol) [21].

Hyperinsulinemia is seen in the first stages of the disease due to β -cell compensation for the insulin resistant state. However, at later stages of the disease, loss of β -cell mass occurs, and the pancreas can no longer secrete enough insulin to compensate for the high plasma glucose levels. At this stage, individuals require the use of exogenous insulin to treat hyperglycemia [1]. Individuals with type 2 diabetes who do not practice a strict glucose control, develop complications at the long-term, including neuropathy, nephropathy, retinopathy, cardiovascular disease, stroke, and peripheral vascular disease, leading to lack of healing, gangrene, and amputation [22].

The prevalence of T2DM has escalated to alarming proportions, promoted by a dramatic increase in the incidence of obesity and a sedentary lifestyle. The Centers for Disease Control and Prevention have estimated that 29.1 million Americans (or 9.3% of the population) have diabetes [23]. In addition, the direct medical costs are currently estimated at \$176 billion, and another \$69 billion have been estimated as a result of disability, work loss, and premature death. The average medical expenses among people with diagnosed diabetes are 2.3 times higher than people without diabetes [23]. Given the economic burden of this disease, there is a pressing need to understand the mechanisms leading to the metabolic abnormalities characteristic of this condition and develop novel therapies.

3. Hepatic steatosis and non-alcoholic fatty liver disease (NAFLD)

Non-Alcoholic Fatty Liver Disease (NAFLD) is a hallmark of obesity and type 2 diabetes mellitus, and is characterized by the accumulation of lipids in the liver of individuals who consume little, if any, alcohol [24]. Among individuals with T2D, the prevalence of NAFLD is as high as 75%. NAFLD is also high in the general USA population, affecting approximately 30% of individuals [25, 26]. The NAFLD spectrum (Fig. 2) is comprised of a two hit theory in which the first hit alters the hepatic pathway of fatty acid uptake, synthesis, degradation, and secretion, making the liver more vulnerable to the second hit, where inflammation, oxidative stress, and mitochondrial dysfunction are present, leading to non-alcoholic steatohepatitis (NASH) [27]. This pro-inflammatory environment activates stellate cells, leading to fibrosis, and if exaggerated, to cirrhosis. Patients at this stage of the disease may require a liver transplant. In addition, the risk of hepatocellular carcinoma increases [28].

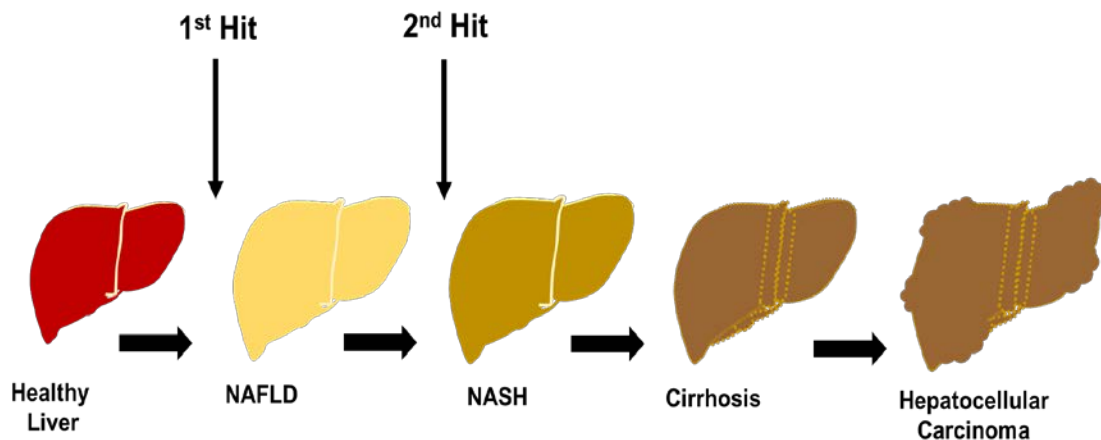


Figure 2. Non-alcoholic fatty liver disease spectrum.

Multiple factors contribute to the development of NAFLD. As mentioned above, in individuals with insulin resistance, lipolysis in the adipose tissue is increased due to enhanced activity of hormone-sensitive lipase (HSL). Excess circulating free fatty acids are taken up by the liver, leading to hepatic lipid accumulation and increasing very-low density lipoprotein production [29, 30]. In addition, the influx of fatty acids into the liver increases levels of various lipids such as diacylglycerols and ceramides, known inhibitors of the insulin signaling pathway [31]. Decreased insulin signaling leads to inadequate inhibition of gluconeogenesis, increasing hepatic glucose production. However, the *de novo* lipogenesis (DNL) pathway remains active, further exacerbating lipid accumulation. This phenomenon is known as selective insulin resistance, and it leads to a combination of hyperglycemia and hypertriglyceridemia. Hepatic insulin resistance and dyslipidemia contribute to increase the risk of cardiovascular disease and stroke [29, 32, 33].

An inverse relationship exists between DNL and fatty acid oxidation (FAO). In the conversion of glucose to palmitate, malonyl-CoA is produced by the enzyme acetyl-CoA carboxylase (ACAC, aka ACC1/2) from the carboxylation of acetyl-CoA. Malonyl-CoA is an allosteric inhibitor of the rate limiting enzyme carnitine palmitoyltransferase 1 A (CPT-1A). CPT-1A transfers the acyl group of acyl-CoA to carnitine, in the mitochondrial membrane. The acyl group is then translocated into mitochondria for oxidation [34]. Thus, under fed conditions, when DNL increases, malonyl-CoA prevents the oxidation of the newly synthesized fatty acids [35]. DNL is significantly increased in the liver of patients

with non-alcoholic fatty liver disease (NAFLD), and accounts for 26% of triglycerides relative to just ~5% in normal individuals [36]. Moreover, DNL is elevated in the fasted state and does not increase postprandially [36]. The higher rate of DNL in insulin resistant states leads to increased levels of malonyl-CoA synthesis and higher inactivation of CPT-1A, contributing further to heighten hepatic steatosis.

4. Sterol Regulatory Element Binding Proteins (SREBPs)

Two major transcription factors regulate expression of the enzymes implicated in *de novo* lipogenesis: Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) [37-39], whose expression is activated by insulin; and Carbohydrate-Responsive Element-Binding Protein (ChREBP) [40, 41], which becomes activated upon increased glucose oxidation and upon feeding a carbohydrate-rich diet. These master regulators increase the expression of enzymes in the DNL pathway such as acetyl-CoA carboxylase (*Acac*), fatty acid synthase (*Fasn*), and liver pyruvate kinase (*Pklr*). Notably, increased levels of SREBP-1c activity is linked to hepatic steatosis and insulin resistance in human obese patients [42]. Because of its dependence on insulin for transcription and for its processing to the active form, SREBP-1c has been the focus of intensive research to understand its contribution in hepatic insulin resistance, and it is a predicted candidate for therapeutic drug development.

4.1. SREBPs gene structure and isoforms

The SREBPs are transcription factors of the basic helix-loop-helix-leucine zipper family (bHLH-Zip) that are conserved from yeast to humans and play significant roles in the regulation of cholesterol, fatty acid and triglyceride synthesis. SREBPs are encoded by two well-characterized genes, SREBP-1 and SREBP-2. SREBP-1 is transcribed into two separate isoforms known as SREBP-1a and SREBP-1c [43-45]. Both SREBP-1 and -2 genes have been cloned and identified in several mammalian species, including human, mouse and rat.

In humans, the SREBP-1 gene is located in the short arm of chromosome 17 (17p11.2) and is 26-Kb long, containing 22 exons and 20 introns [46]. Due to the presence of an alternative promoter start site in this gene, two splice variants are generated yielding SREBP-1a and SREBP-1c (Fig. 3). The SREBP-1 isoforms differ in their first exon at the 5' end. In SREBP-1a, 12 negatively charged amino acids encoded from exons 1 and 2 are part of a 42 amino acid transcription activation domain (see Fig. 3 for structure and function of the different protein domains). Upon deletion of this activation domain, human SREBP-1a maintains the ability to bind to Sterol Regulatory Element motifs (SRE), but is unable to activate transcription. In contrast, the activation domain in SREBP-1c is only 24 amino acids long, with 7 negatively charged amino acids [47]. Unlike the mouse SREBP-1 mRNA, human SREBP-1a and -1c mRNA can undergo alternative splicing at the 3' ends [45]. In addition, exon 17 encodes the last amino acid shared between the two isoforms, with SREBP-1a having 113 additional amino acids within exons 18a and 19a, and SREBP-1c having 37

additional amino acids within exons 18c and 19c in humans [46]. The function and importance of the alternative splicing in the SREBP-1 regulatory domain is still unclear.

The human SREBP-2 gene is located in the long arm of chromosome 22 (22q13) and is 72-Kb long, containing 19 exons and 18 introns (Fig. 3) [46]. Furthermore, gene structure analysis revealed that SREBP-2 introns are larger than the corresponding introns in SREBP-1, resulting in SREBP-2 being 2.8 fold larger than SREBP-1 [48].

SREBP-1 and SREBP-2 proteins share roughly 47% of the amino acid sequence [43], and have three domains: 1) an NH₂- terminal domain of ~480 amino acids (also known as the transcription factor domain) responsible for DNA binding, as well as for homo- or heterodimerization, and for transcription activation; 2) a middle hydrophobic region of ~80 amino acids containing two hydrophobic transmembrane segments that allows the protein to anchor to the ER membrane and nuclear envelope; and 3) a long COOH-terminal domain of ~590 amino acids involved in posttranslational regulation, also known as the regulatory domain (Fig. 3). One structural difference in the SREBP-2 protein is the existence of a glutamine-rich region, making up to 27% of the 121 amino acids. This glutamine-rich domain interacts with specific co-activators, and may explain the functional differences between the SREBP-1 and -2 proteins [44, 49]. It should be noted that SREBPs are unique among the bHLH-Zip family proteins, due to their ability to bind to SRE motifs, in addition to E-boxes [50]. This dual

binding feature is due to the presence of a unique tyrosine in the conserved basic domain, in place of an arginine, the amino acid found in most bHLH proteins [50].

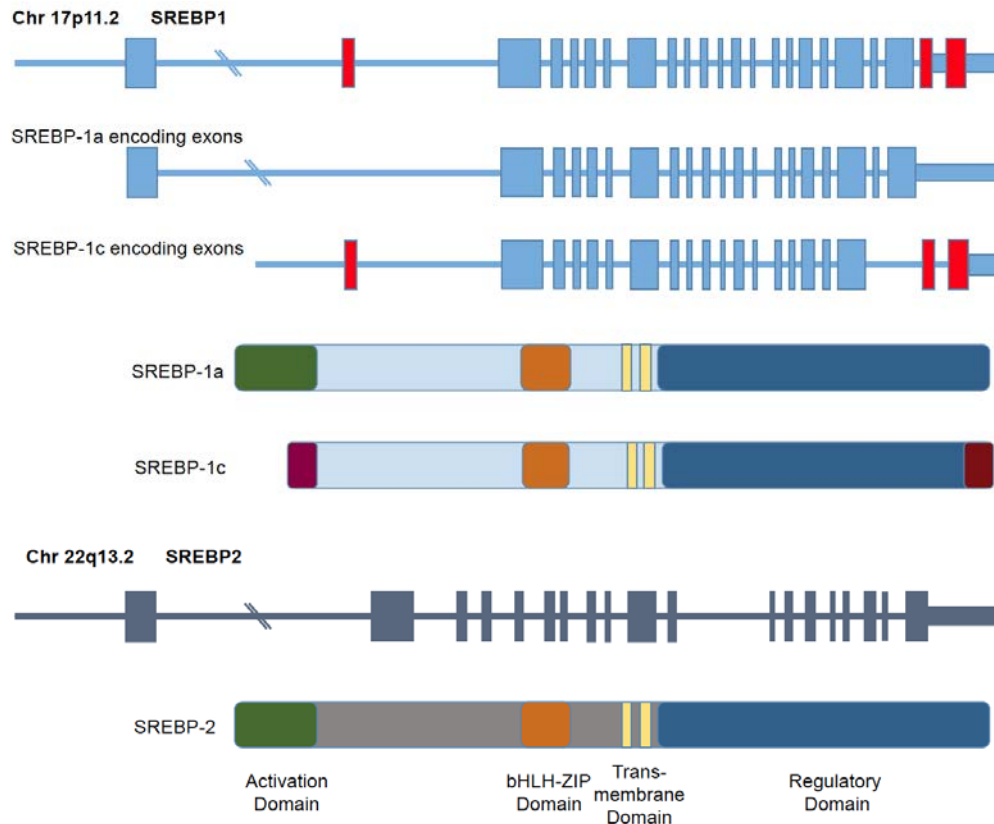


Figure 3. Graphic representation of genes encoding human SREBP isoforms. SREBP-1a and SREBP-1c are transcribed from SREBP-1 through alternative promoters and splicing. Each blue box denotes an exon in SREBP-1a or SREBP-1c. Red boxes denote the exons specific to the SREBP-1c isoform, with two main exons in the C terminus. SREBP-2 encodes one transcript, with exons denoted by grey boxes. Below the transcripts and encoding exons are the protein domains for SREBP-1a and 1c, and SREBP-2. The three proteins have an analogous NH₂-terminal domain, which includes the transactivation domain and bHLH-ZIP motif for DNA binding and dimerization. In addition, all have a transmembrane domain for localization to the endoplasmic reticulum membrane and a regulatory domain at the C terminus. Source: adapted from [51].

4.2. Transcription of SREBP genes

As mentioned above, the first exon of the SREBP-1c isoform is of different length and composition, suggesting SREBP-1c may be a weaker transcription activator than SREBP-1a. Indeed, transgenic mice overexpressing either SREBP-1a or SREBP-1c induce a different fold-level of transcription of genes involved in the fatty acid synthesis pathway. Fatty acid synthase and acetyl CoA carboxylase gene expression is increased 9- and 16-fold, respectively, in SREBP-1a animals, and only 2- and 4-fold in SREBP-1c mice [52]. In addition, expressing SREBP-1a or -1c in HEK293 cells under the control of the phosphoenolpyruvate carboxykinase (PEPCK), thymidine kinase (TK), or cytomegalovirus (CMV) promoters, leads to differential levels of target gene expression. Promoters inducing low (PEPCK) or moderate (TK) amounts of SREBP-1a and -1c demonstrated similar trends of transcription activation to those seen in mouse liver, i.e., SREBP-1a being a stronger activator than SREBP-1c. Overall, these results confirm that SREBP-1a is a stronger transcription factor than SREBP-1c under physiological conditions.

In addition, expression of SREBP-1 varies among tissues. Of note, the liver, white and brown fat, brain, and kidney of mice have higher levels of the SREBP-1 transcript than muscle or spleen [45]. Likewise, in humans, SREBP-1 expression is highest in liver, kidney, and white adipose tissue, while lower in the muscle. However, SREBP-1 expression is also lower in brain, differing from the mouse. The ratio between the two isoforms, SREBP-1c and -1a, are distinct in each tissue as well. Expression of SREBP-1c is highest in the liver of both

human and mouse, with a ratio of 1c:1a being 6:1 in humans and 9:1 in the mouse [45]. In mouse, SREBP-1c is also higher in white and brown fat, brain, and skeletal muscle. In the spleen, testis, intestine, and thymus, the ratio is flipped, and SREBP-1a is expressed at higher levels than -1c. In humans, the ratio is similar to that in the mouse, and SREBP-1c is more abundant than -1a in adrenal gland, ovary, brain, white adipose tissue, and muscle [45]. Although SREBP-1c seems to be the predominantly expressed transcript in animal tissues, the SREBP-1a isoform is primarily expressed in cell lines, including the HepG2 human hepatoma cell line, and mouse 3T3-L1 pre-adipocytes, with a 1a:1c ratio >2-fold [45].

In the liver, SREBP-1a is constitutively expressed, while expression of SREBP-1c is inducible and increases in the fed state, in response to insulin. Transcription activation of SREBP-1c is the primary determinant of the amount of active mature protein in the liver, although insulin also promotes its processing from precursor to mature form (described in detail in 4.4). Studies have shown that wild type mice fasted for 24 hours had lower SREBP-1c and SREBP-2 levels, 40% and 30% respectively, in comparison to non-fasted mice. Furthermore, re-feeding mice with a high carbohydrate/low fat diet for 12 hours, after a 24 hour fast, led to increased levels of SREBP-1 in the liver, with no change in SREBP-2 [53]. In addition, studies showed that rats treated with the drug streptozotocin (STZ), a known inhibitor of β -cell function in the pancreas, had decreased levels of SREBP-1c mRNA, due to insulin deficiency. However, treatment with insulin in these animals reversed this result. Moreover, treatment

with insulin did not affect SREBP-1a or SREBP-2 mRNA levels suggesting selective transcription activation of SREBP-1c by insulin in the liver [54].

Multiple key binding motifs are present in the SREBP-1c promoter, including liver X receptor (LXR), specificity protein 1 (Sp1), nuclear factor Y (NF-Y), and SREBP itself [55]. Insulin-dependent SREBP-1c transcription regulation requires LXR, although the mechanism has not been elucidated [37, 56]. LXRs bind to the SREBP-1c promoter as heterodimers with retinoid X receptors (RXRs), a family of nuclear receptors activated by their endogenous ligand 9-cis retinoic acid [57] and rexinoids (synthetic agonists) [58]. LXRs are activated by oxysterols (cholesterol derivatives). An important LXR ligand of note is the 24(S)-25-epoxycholesterol (24.25-EC), abundantly present in the liver [59, 60]. Evidence that LXRs are critical regulators of SREBP-1c came from studies in LXR α null mice. These animals have decreased levels of SREBP-1c and fatty acid synthesis gene expression in the liver [61]. In addition, animals treated with the synthetic LXR-selective agonist (T0901317) showed increased levels of plasma triglycerides, as well as increased fatty acid synthesis gene expression in the liver [62]. Upon studying the mouse SREBP-1 promoter, the binding sites for LXR/RXR complex, LXR elements (LXREs), were identified [56]. SREBP-1c activation through LXR is attenuated when both LXRE α and LXRE β motifs are deleted [63]. *In vitro* studies have demonstrated that SREBP-1c activity is suppressed by polyunsaturated fatty acids (PUFAs) due to competition with LXR ligands, which prevents binding of the LXR/RXR heterodimer to the LXRE site in the SREBP-1c promoter [64, 65]. In addition, studies performed on *ob/ob* mice

(an animal model of type 2 diabetes and obesity lacking leptin activity) fed a PUFA diet, showed a decrease in SREBP-1c and in triglycerides in the liver, as well as improved hepatic steatosis and insulin resistance, further validating the regulation of SREBP-1c through PUFAs [66].

One additional regulator of SREBP-1c is the transcription factor peroxisome proliferator-activated receptor α (PPAR- α). Similar to PUFAs, PPAR- α competes with LXR for RXR binding on the SREBP-1c promoter. *In vitro* experiments showed that PPAR- α overexpression hindered LXR/RXR-mediated SREBP-1c transcription [67]. Similarly, mice under fasting conditions and treated with a PPAR agonist resulted in reduced LXR-mediated SREBP-1c activation and decreased LXR/RXR heterodimer formation [67]. Remarkably, overexpressing LXR or LXR ligands decreases PPAR- α /RXR formation, resulting in diminished PPAR- α signaling [68]. It should be noted that PUFAs are well-known activators and PPAR- α ligands. Since PPAR- α activity is increased during fasting and decreased under fed conditions [69], while LXR is active during fed conditions, PPAR α and LXR may coordinate fatty acid metabolism during fasted and fed conditions by mutual inhibition.

The transcriptional regulation of SREBP-1a is much simpler than that of SREBP-1c. The promoter is small and has a minimal structure, consisting of two SP1 binding sites [70], and an early growth response protein 1 (EGR-1) [71]. *In vitro* promoter studies demonstrated that increased EGR-1 dislocates the SP1 bound transcription factor from the SREBP-1a promoter, suggesting that EGR-1 might act as an inhibitor for SREBP-1a transcription activity [71].

4.3. Gene targets of SREBPs

SREBP-1a and SREBP-1c transcriptionally upregulate genes encoding enzymes in the glycolysis and lipogenesis pathways, such as L-pyruvate kinase (*Pklr*), fatty acid synthase (*Fasn*), acetyl-CoA carboxylase (*Acac*), ATP-citrate lyase (*Acly*), acetyl-CoA synthetase (*Acss*), long-chain fatty acyl elongase (*Elovl*), stearoyl-CoA desaturase 1, 2 and 3 (*Scd*), mitochondrial glycerol-3-phosphate acyltransferase 1 (*Gpam*) (Fig. 4) [72]. In addition, SREBP-1a controls expression of some enzymes necessary for *de novo* synthesis of cholesterol such as 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase (*Hmgcs*), HMG-CoA reductase (*Hmgcr*), squalene synthase (*Sqs*), as was the LDL receptor (*Ldlr*) [72, 73]. In addition, SREBP-1 has been shown to directly bind to the promoters of insulin receptor substrate 2 (*Irs2*), phosphatidylinositol-3 kinase regulatory subunit p55 γ (PIK3R3), heme oxygenase 1 (*Hmox1*), caspase-2 (*Casp2*), glycogen synthase kinase 3 alpha (*Gsk3 α*), and cyclin-dependent kinase inhibitor p21 (*Cdkn1a*) [74-79], which indicates SREBP-1 plays a role at coordinating other cellular functions, including insulin signaling and the response to stress. SREBP-2, instead, largely regulates genes involved in *de novo* synthesis of cholesterol, including mevalonate kinase (*Mvk*), acetoacetyl-CoA thiolase (*Acat*), HMG-CoA synthase, HMG-CoA reductase, lanosterol synthase (*Lss*), LDL receptor (Fig. 4) [72].

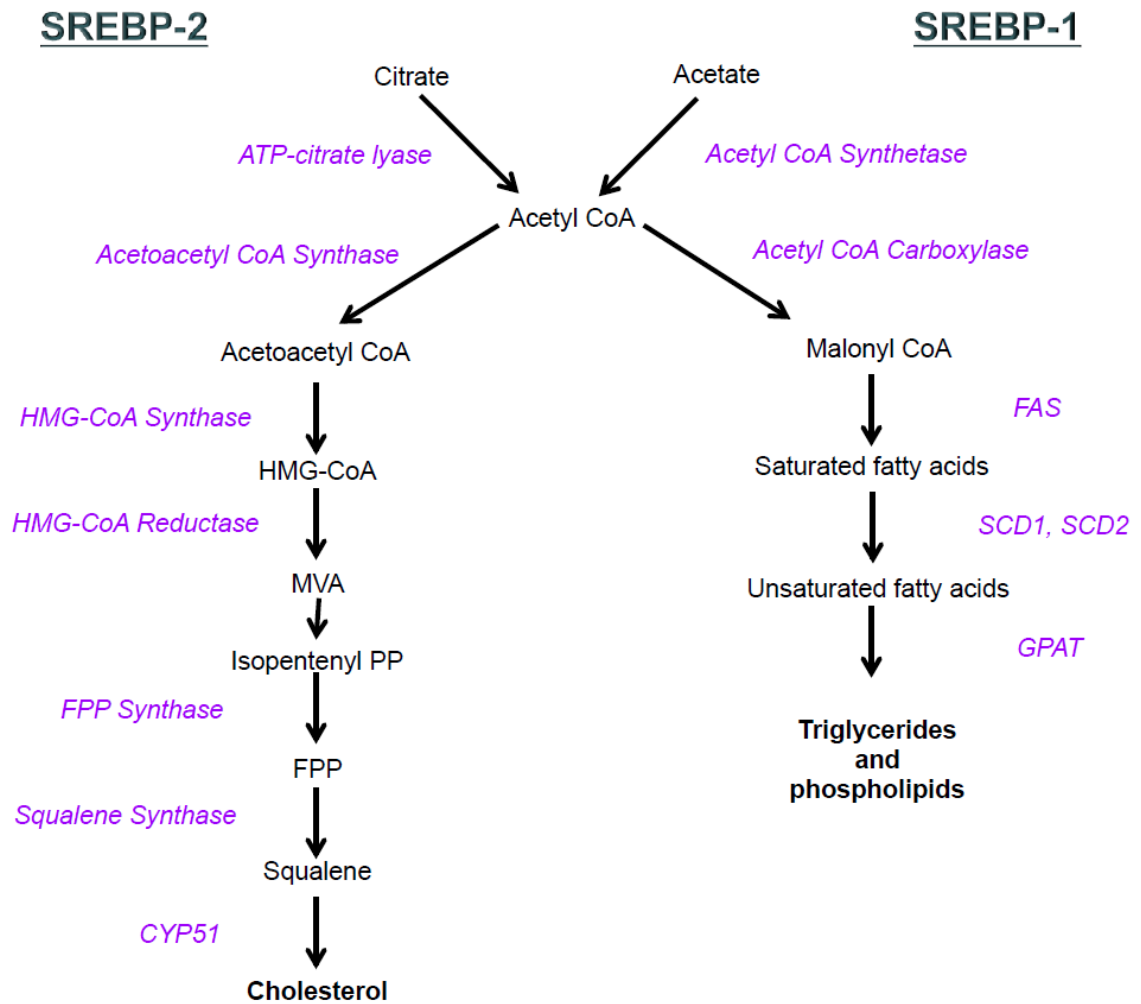


Figure 4. SREBP-1 and SREBP-2 gene targets. SREBP-1 mostly regulates genes of the glycolysis and fatty acid synthesis pathways, while SREBP-2 controls expression of *de novo* cholesterol synthesis.

4.4. Regulation, proteolysis, and processing of SREBPs

SREBPs play a critical role in fatty acid and cholesterol synthesis. Consequently, SREBP activity is tightly regulated to maintain lipid homeostasis and respond appropriately to nutrient stimuli. In addition to being regulated transcriptionally, SREBPs are regulated post-translationally by proteolytic processing. SREBP-1a, -1c, and -2 are generated as inactive 125-KDa precursors (~1,150 amino acids) bound to the endoplasmic reticulum (ER). Under low sterol conditions or high insulin, SREBPs are transported to the Golgi apparatus, where they are cleaved, releasing the active 60-KDa NH₂-terminal fragment (~470 amino acids). Finally, the active transcription activator fragment enters the nucleus, binding regulatory elements in gene promoters for transcription to ensue [49, 80, 81] (Fig. 5).

Several processing steps take place to cleave the SREBP precursor and transport it to the Golgi apparatus, and most of the details of this process has been studied for SREBP-2 and SREBP-1a, which regulate cholesterol biosynthesis. The SREBP-cleavage activating protein (SCAP) is bound to SREBPs and acts as a sterol sensor. The Insulin-Induced Genes (INSIGs) bind to the sterol-sensing domain of SCAP in the presence of sterols. There are two isoforms of INSIG, denoted as INSIG-1 and INSIG-2. Notably, INSIG-1 expression is high in human liver. Studies performed in SCAP deficient cell lines, showed that expressing SCAP protein increased processing of SREBP-1a and SREBP-2, in the absence of sterols [82]. However, under the same conditions, the process was inhibited when INSIG-1 was overexpressed. In addition,

transgenic mice overexpressing INSIG-1 in their livers have decreased SREBP-1c and SREBP-2 expression in the nucleus in comparison to control mice [83]. Taken together, this would suggest that sterols bind to the SCAP protein causing a modification that allows the INSIG-1 protein to bind to SCAP, hindering SREBP processing and trapping it in the ER [82]. In the absence of sterols, INSIG-1 frees SCAP, which chaperones SREBPs from the ER to the Golgi apparatus (Fig. 5).

Additional information revealed that the SREBP-SCAP complex, during sterol deficiency, assembles in coat-protein complex II (COPII) coated vesicles to allow exit from the ER to the Golgi. Specifically, Sec24, which binds to SCAP and is one of the COPII proteins required for COPII-coated vesicles, is part of the Sec23/Sec24 complex that imparts proper clustering of membrane proteins such as SREBP into budding vesicles for transport [84]. When INSIG-1 binds to SCAP in the presence of sterols, COPII protein, Sec24 is unable to bind to the hexapeptide sorting signal (MELADL) in SCAP, impeding any protein cargo (i.e. SREBP), to be transported out of the ER [85]. Thus, the role of INSIG-1 together with Sec24 is an important part of the regulation and proteolytic processing of SREBPs.

Once in the Golgi, SREBPs undergo two consecutive cleavages, facilitated by two Golgi-associated proteases, Site-1 (S1-P) and Site-2 (S2-P), releasing the NH₂-terminal fragment [86]. Proteolytic cleavage of the SREBP precursor in sterol-depleted cells generates a water soluble 68-KDa mature fragment, which is translocated to the nucleus, where it activates transcription of target genes (Fig. 5).

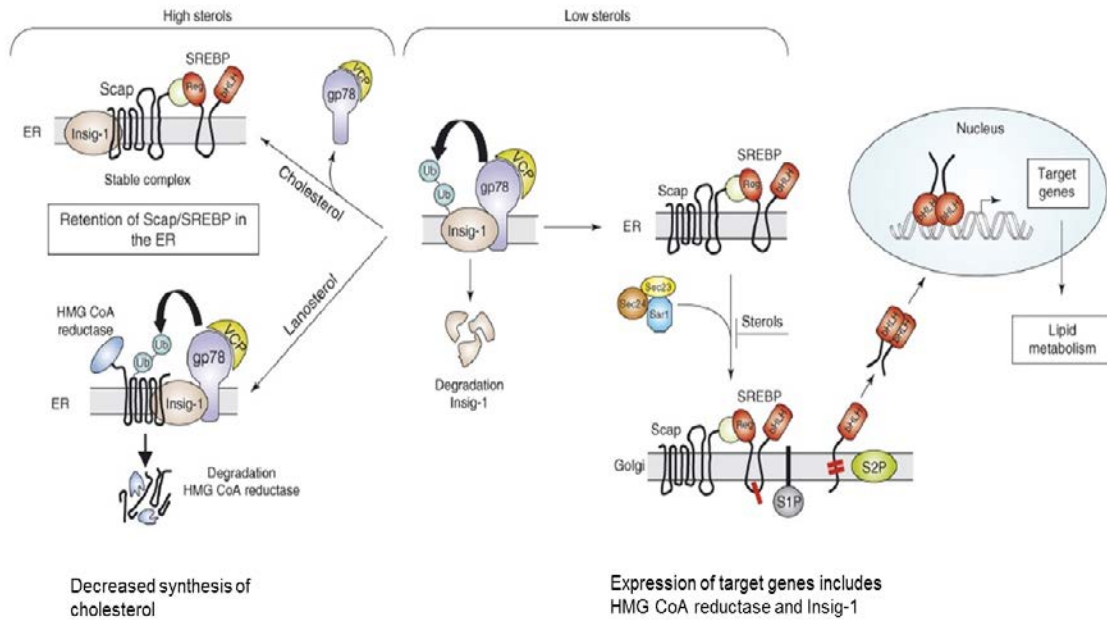


Figure 5. SREBP proteolytic processing. Increased sterol (cholesterol and lanosterol) levels in the ER membrane promotes the interaction between the Scap/SREBP and Insig-1, stabilizing the complex and retaining it in the endoplasmic reticulum (ER). HMG-CoA reductase is stimulated to bind to Insig-1, which is bound to the gp78/VCP complex for ubiquitination and degradation, preventing cholesterol synthesis. Under low sterol conditions, Insig-1 interacts with the gp78/VCP complex, undergoing ubiquitination and degradation, permitting the Scap/SREBP complex to exit the ER via the Sec23/Sec24/Sar1 complex. SREBP is further processed in the Golgi apparatus by the S1P and S2P proteases, releasing the mature form of SREBP. SREBP is then translocated to the nucleus and can then bind to promoters of its target genes. Abbreviations: Insig-1, Insulin-induced gene 1; bHLH, SREBP N-terminal transcription factor domain; Reg, SREBP C-terminal regulatory domain; Scap,

SREBP-cleavage activating protein; gp78/VCP, glycoprotein 78/valosin-containing protein complex; Ub, Ubiquitin; Sec23/Sec24/Sar1, protein transport protein 23/protein transport protein 23/secretion-associated RAS-related protein 1 complex; S1P, site-1 protease; S2P, site-2 protease. Source: combined image from [87].

One important downstream target of SREBP-1a and SREBP-2 is the low-density lipoprotein (LDL) receptor [88]. The LDL receptor binds and internalizes plasma LDL. During low cholesterol conditions, the LDL receptor is abundantly expressed (in addition to genes involved in *de novo* synthesis of cholesterol [81]), while under high cholesterol conditions, it is inhibited, reducing uptake of LDL. Thus the amount of cholesterol in cells is regulated through feedback inhibition of the LDL receptor gene, and SREBPs play a key role in that process [89].

Unlike SREBP-1a and SREBP-2, sterols do not play an important role in the regulation and processing of SREBP-1c. Both, *in vivo* and *in vitro* studies, have revealed that insulin and fatty acids are the essential players in the production of the mature form of SREBP-1c. Studies performed in a primary hepatocyte cell line (H2.35) treated with cholesterol revealed a decrease in SREBP-2 processing, but minimal effect on SREBP-1c processing from the ER [90]. However, when cells were treated with the monosaturated fatty acid oleate, SREBP-1c processing was decreased, without affecting SREBP-2 [90]. Furthermore, mice fed a PUFA-containing diet, had reduced SREBP-1c mature

form, with no effect on the precursor form, suggesting that PUFAs inhibit the processing of SREBP-1c [91].

Insulin is an important and the best characterized regulator of SREBP-1c processing, and INSIG-2 plays a significant role in this process. The Insig2a transcript is abundantly expressed in the liver, while the Insig2b isoform is ubiquitously expressed [92]. Yabe and colleagues uncovered that under fed conditions, insulin decreases Insig2a expression in the liver. In contrast, Insig2a increases under fasting and decreases again upon refeeding [93]. Notably, normal expression levels of INSIG-1 do not affect SREBP-1c processing in the liver under fed or refeed conditions [83]. Moreover, since Insig2a is decreased under fed conditions, this would permit SREBP-1c (which is needed under the fed state) to be processed from the ER to the nucleus [93]. Insulin also improves the stability of the mature form of SREBP-1c in the nucleus [94]. In addition, because Insig2a is repressed by insulin, and INSIG-1 has a minimal effect on SREBP-1c processing despite high levels of cholesterol, SREBP-1c is still able to be processed to mature form under fed conditions [93].

4.5. Degradation of SREBPs

In addition to transcription and processing, regulation of SREBPs also takes place through protein degradation. Multiple modifications influence its decay, including phosphorylation, ubiquitination and acetylation. GSK3 β directly phosphorylates SREBP-1a at Thr426 and Ser430, priming the molecule for ubiquitination by the ubiquitin ligase Fbw7 [95]. SREBP-1c binding to the DNA of

target genes enhances the interaction with GSK3 β [95]. Furthermore, CDK8 phosphorylates SREBP-1c, which enhances ubiquitination and degradation [96]. The energy sensor AMP-activated protein kinase (AMPK) also negatively regulates SREBP-1c and SREBP-2 by preventing their processing to the mature form [97]. Finally, SREBPs are acetylated in the DNA-binding region by p300 and CBP, which stabilizes the molecules [98]. On the contrary, SIRT1 deacetylates SREBP-1c, eliciting its degradation [99].

5. mTOR and insulin signaling pathways

The insulin signaling pathway is interconnected with other metabolically regulated networks, including the mammalian/mechanistic target of rapamycin (mTOR) pathway. Briefly, upon insulin binding to the insulin receptor, insulin receptor substrate 1 and 2 (IRS1/2) are phosphorylated on tyrosine residues. This sends a downstream signal that activates phosphatidylinositol 3-kinase (PI3K), consequently activating PDK1 (3-phosphoinositide-dependent protein kinase 1), which phosphorylates protein kinase B (also known as Akt) at the threonine 308 residue [100].

The mTOR pathway is activated by environmental signals in response to nutrient and energy demands, to regulate energy homeostasis as well as cellular growth and proliferation. The mTOR protein is a kinase belonging to the PI3K family. It exerts its function by participating in two major complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [101]. The mTORC1 complex contains Raptor (regulatory-associated protein of mammalian target of

rapamycin) and PRAS40 (proline-rich Akt substrate 40kDa), while mTORC2 is composed of Rictor (rapamycin-insensitive companion of mTOR) and mSin1 (mammalian stress-activated map kinase-interacting protein 1) [101]. mTORC1, but not mTORC2, is inhibited by rapamycin. The mTORC1 complex is activated by GTP-Rheb (Ras homolog enriched in brain) [101], an indirect result of Akt activation (Fig. 6). Upon activation of mTORC1, the Raptor subunit interacts with ribosomal S6 kinase 1 (S6K1) resulting in its phosphorylation and activation [102]. S6K1 is an important regulator of protein translation and synthesis [103, 104]. mTORC1 mediates insulin action to transcriptionally activate SREBP-1 expression during fed conditions [105]. In addition, by phosphorylating and inactivating Lipin1, mTORC1 promotes SREBP-1 nuclear retention [106]. Emerging evidence is showing that mTORC1-mediated SREBP-1 upregulation is strictly needed for cell growth and proliferation to provide lipids for cell membranes [101]. In fact, SREBP-1 is becoming a novel molecular target in cancer [107].

Although the upstream activators of mTORC2 remain unknown, this complex responds to growth factors and insulin. A main function of mTORC2 is to phosphorylate Akt on serine 473 [101, 108], and phosphorylation at this site is needed to phosphorylate and inactivate the transcription factor FoxO1, and thus, inhibit transcription of gluconeogenesis enzymes [109]. Akt^{Ser473} is not strictly needed to regulate GSK3 β and TSC2 [109]. In addition, mTORC2 influences cytoskeleton functions by phosphorylating SGK1 (serum- and glucocorticoid-induced protein kinase 1) and PKC (protein kinase C) [110, 111].

Upon Akt activation at Thr308 and Ser473, the transcription factor forkhead box O1 (FKHR or FoxO1) is inactivated through phosphorylation and excluded from the nucleus. In the cytoplasm, it is ubiquitinated and degraded [112]. FoxO1 phosphorylation results in down regulation of gluconeogenic gene expression and decreased hepatic glucose production. Akt activation also phosphorylates and inactivates glycogen synthase kinase 3 (GSK3). GSK3 is encoded by two genes, GSK3 α and GSK3 β , and among other functions, it inhibits glycogen synthase [113]. Thus, its phosphorylation by Akt activates glycogen synthase, resulting in increased glycogen biosynthesis. In addition, phosphorylated Akt inhibits the GTPase-activating protein heterodimer TSC1/2 (tuberous sclerosis 1/2), an inhibitor of the mTOR pathway [101] (Fig. 6). Thus, insulin signaling activates mTORC1. Notably, upon mTORC1 activation by Akt, the former elicits a negative feedback loop to block Akt activity [114]. On one side, S6K1 negatively regulates IRS-1 through serine phosphorylation, which impairs tyrosine phosphorylation and downstream signaling [103, 104]. In addition, S6K1 inhibits mTORC2/Rictor activity by phosphorylating Rictor at Thr1135 [115] (Fig. 6).

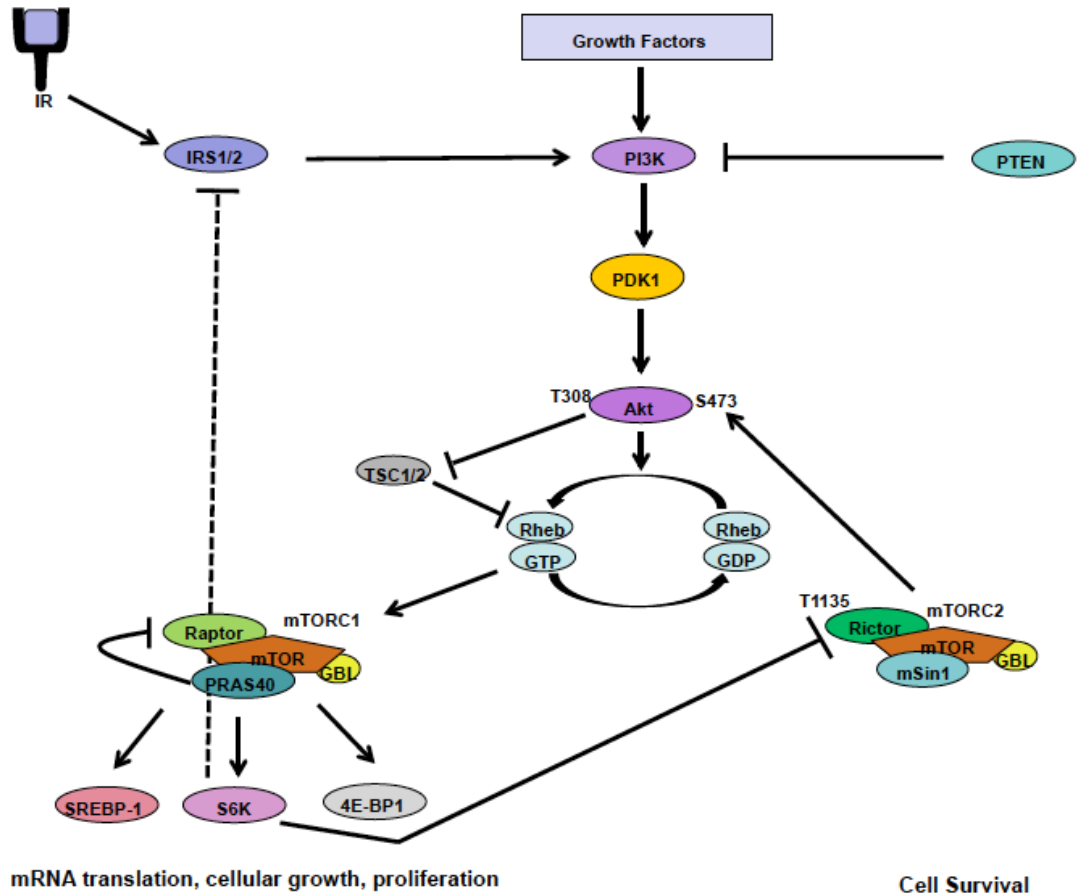


Figure 6. mTOR pathway. Insulin receptor binding triggers phosphorylation of Akt at T308 through PI3K and PDK1. PTEN inhibits PI3K activity. Akt indirectly activates mTORC1 through inhibition of TSC1/2. As a result, mTORC1 activates S6K and SREBP-1. In turn, S6K inhibits insulin signaling by blocking IRS1 tyrosine phosphorylation, through a negative feedback loop. In addition, S6K inhibits mTORC2 activity through phosphorylation of Rictor at the Thr1135 residue, resulting in reduced phosphorylation of Akt. These feedback mechanisms are important for proper signaling. Loss of this mechanism is seen in various cancers, with up-regulation of Akt. Source: adapted from [116].

6. Thesis hypothesis and research aims

The liver plays a significant role in glucose and lipid homeostasis. Proper regulation of multiple pathways, including glycolysis, lipogenesis, gluconeogenesis, and glycogen synthesis, are vital in preventing metabolic disorders associated with insulin resistance. As a result, the regulation of the associated gene networks must be appropriately controlled. The transcription factor SREBP-1 aids in enhancing expression of enzymes in the glycolysis and *de novo* lipogenesis pathways. Understanding the mechanisms by which SREBP regulates other pathways will be beneficial for the development of treatments of not only metabolic diseases, but also cancer. Here I proposed to knock-down SREBP-1 *in vivo* and evaluate the effects on lipid and carbohydrate metabolism as well as insulin signaling. My overall hypothesis was that SREBP-1 regulates other facets of liver function, thereby connecting metabolism to cellular growth and proliferation. In order to test this hypothesis, two aims were pursued:

- 1. Determine the impact of knocking-down SREBP-1 on glucose homeostasis in a type 2 diabetes mouse model.** SREBP is highly expressed in human obese patients as well as in mouse models of obesity and type 2 diabetes. Using helper-dependent adenoviral vectors, we depleted SREBP-1 in the liver of *db/db* mice and analyzed the impact on lipogenesis and carbohydrate metabolism.
- 2. Examine the effects of silencing SREBP-1 on the insulin signaling and mTOR pathway.** The working hypothesis was that SREBP-1 regulates several

molecules in this pathway, thereby influencing overall insulin signaling. In addition, studies resulting from the ENCODE project revealed that SREBP binds to the S6K1 gene. Given the role of S6K1 in regulating the insulin signaling pathway as well as protein synthesis and cellular growth, I depleted SREBP-1 in primary hepatocytes, and in the liver of normal and *db/db* mice, and evaluated the impact on mTOR and insulin signaling pathways.

Chapter 1: Sterol Regulatory Element Binding Protein-1 (SREBP-1) is required to regulate glycogen synthesis and gluconeogenic gene expression in mouse liver

Introduction

In the past couple of decades, the prevalence of non-alcoholic fatty liver disease has escalated to alarming levels. Currently, this condition affects > 30% of the general population [24, 117] and is as high as 75% in patients with type 2 diabetes [26]. Hepatic lipid accumulation is associated with impaired insulin signaling and lack of inhibition of hepatic glucose production [30]. However, insulin maintains *de novo* lipogenesis, in a process known as selective insulin resistance [118]. Reduced inhibition of hepatic glucose output and increased lipogenesis lead to a combination of hyperglycemia and hypertriglyceridemia.

The transcription factor SREBP-1c regulates *de novo* lipogenesis in the liver in response to increases in insulin. SREBPs are transcription factors of the basic helix-loop-helix leucine zipper family that are synthesized as precursors and bound to the endoplasmic reticulum membrane [43]. In the presence of the appropriate signals, SREBPs transition to the Golgi, where they are cleaved, releasing the mature form, which translocates to the nucleus and activates target gene expression [43]. SREBP-1a and SREBP-1c are isoforms of the same gene and transcriptionally up-regulate glycolytic and lipogenic enzymes such as L-pyruvate kinase (*Pfkfb*), fatty acid synthase (*Fasn*), stearyl-CoA desaturase 1 (*Scd1*), mitochondrial glycerol-3-phosphate acyltransferase 1 (*Gpam*), and

acetyl-CoA carboxylase (*Acac*) [43]. In human obese patients, increased levels of SREBP-1c correlate with hepatic steatosis and insulin resistance [42]. Secondary to the hyperinsulinemia, SREBP-1c activity is higher in the liver of *ob/ob* and *db/db* mice, mouse models of obesity and type 2 diabetes, underscoring the role of this transcription factor as a contributor to hepatic steatosis and insulin resistance [39]. These data suggest that strategies to reduce SREBP-1 activity have therapeutic potential to reduce hepatic lipid accumulation and improve insulin sensitivity to block gluconeogenesis and hepatic glucose output. However, *ob/ob* mice lacking SREBP-1 do not exhibit improved glucose levels despite a significant decrease in hepatic lipid accumulation [119]. Here we have used helper-dependent adenoviral vectors to acutely silence SREBP-1 in the liver, to test the hypothesis that SREBP-1 regulates carbohydrate metabolism, in addition to hepatic *de novo* lipogenesis. Our data suggest that SREBP-1 is necessary to regulate carbohydrate metabolism during fed conditions. Thus, its depletion is not beneficial as a strategy to improve hepatic glucose output in animal models with hepatic insulin resistance.

Materials and methods

Helper-dependent adenoviral vector production

Helper-dependent adenoviral vectors were generated using a Cre-loxP system developed by Merck Laboratories and Microbix (Toronto, Canada) [120, 121]. These vectors are the most advanced type of adenoviral vector; they are devoid of viral coding sequences, and only retain the inverted terminal repeats

and packaging signal (Fig. 7A). The lack of viral genes eliminates the possibility of inducing immune responses and toxic effects, therefore leading to transgene expression for years, in mice and non-human primates [122, 123]. Helper-dependent adenoviral have identical tropism to first generation adenoviral vectors, and predominantly transduce the liver [124, 125]. The construction of helper-dependent adenoviral vectors expressing a shRNA to silence SREBP-1 or a shRNA scrambled sequence (gAd.shSREBP1 and gAd.shSCR, respectively), as well as the efficacy of silencing SREBP-1 in the liver, have been previously described [126]. Vectors were stored at -80°C in 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 150 mM NaCl, 10% glycerol. Total particle counts were determined spectrophotometrically, as described [126].

Animals

SREBP-1 silencing in normal mice. Eight-to-nine-week old C57BLKS/J mice were given 1×10^{11} vp, and euthanized 7 days after adenovirus vector administration under *ad libitum* fed conditions, 24-h fasted and 24-h fasted followed by a 4-h re-feeding period.

SREBP-1 silencing in db/db mice. Male eight-week old C57BLKS/J and *db/db* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and acclimated to our facilities for a week before adenovirus administration. Animal care guidelines set forth by Indiana University School of Medicine were followed. All animals received humane care according to the criteria outlined in the Guide

for the Care and Use of Laboratory Animals (NIH). Mice were kept in a BSL2 facility and had free access to a standard chow diet and water. Mice were given 1×10^{11} viral particles (vp) by tail vein injection, and euthanized 3 weeks later under fed or 24-hour fasted conditions. Tissues were collected and snap frozen in liquid nitrogen or fixed in 10% buffered formalin for histology analysis.

Metabolic tracer study

Three weeks after gAd.shSREBP1 or gAd.shSCR adenoviral vector administration, *db/db* mice were given $^2\text{H}_2\text{O}$ intraperitoneally (21.4 $\mu\text{l/g}$ body weight; this dose enriches body water content to 3%), and fasted for four hours with free access to water. Animals were euthanized and serum and liver were collected, and sent to the Mouse Metabolic Phenotyping Center at Case Western Reserve University for analysis.

Glucose tolerance (GTT), insulin tolerance (ITT) tests

Adenovirus vectors were administered to *db/db* or C57BLKS/J mice as described above. The GTT was performed by intraperitoneal administration of a bolus of glucose (1 mg/g body weight), after an overnight fast. Blood glucose was monitored every 30 minutes for 2 hours. For the ITT, mice were fasted for 5 hours and given insulin intraperitoneally (1 U/kg, Human Regular Insulin, Eli Lilly). Blood glucose was monitored every 30 minutes for 2 hours.

Liver lipids

Liver lipid analyses were carried out using ~100 mg of frozen liver tissue. Total liver phospholipid, triglyceride, free fatty acid and cholesterol, were analyzed by TLC and gas chromatography at the Mouse Metabolic Phenotyping Center at Vanderbilt University. In the metabolic tracer analysis, total palmitate, *de novo* palmitate, malonyl-CoA, and BHB-CoA, were analyzed by the Mouse Metabolic Phenotyping Center at Case Western Reserve University School of Medicine, as previously described [127-130]. For BHB-CoA, data are expressed as pmol/mg tissue relative to the internal standard [²H₉]pentanoyl-CoA [129].

Serum and tissue biochemistries

Insulin levels were analyzed at the Vanderbilt Hormone Assay & Analytical Services Core. Blood glucose was measured every 5 days with an Ascencia Elite®XL meter (Bayer, Tarrytown, NY), from a drop collected from the tail vein. Total triglyceride in serum was determined by a triglyceride determination kit (Sigma Aldrich), free fatty acids with the NEFA-HR(2) kit (Wako Chemicals, Richmond, VA), and β-hydroxybutyrate with a β-hydroxybutyrate determination kit from Pointe Scientific (Canton, MI). All reactions were carried out in duplicate on uncoated, flat-bottomed 96-well plate, following the manufacturer's instructions. Total liver glycogen content in tissues was determined by measuring amyloglucosidase-released glucose from glycogen as previously described [131]. Glycogen synthase fractional activity with low (0.24mM) or high (10mM) (L/H)

glucose-6-phosphate was assessed in total homogenates by monitoring the incorporation of glucose from UDP-[U-¹⁴C]glucose, as described [132].

CPT1A activity and IC₅₀ assays

CPT activity and malonyl-CoA sensitivity was determined using the radiochemical forward assay as described in detail earlier [133]. This assay is optimized for measuring malonyl-CoA sensitive CPT activity. The assay is carried out with 10 µl of a 2% liver homogenate (in physiological saline) in absence (total CPT activity) and presence of 200 µM malonyl-CoA (malonyl-CoA insensitive activity) at 37°C. The difference in velocity represents CPT1A activity. The malonyl-CoA sensitivity was assessed by measuring the activity in the presence of 8.0 µM malonyl-CoA that under the experimental conditions corresponds to the approximate IC₅₀ value for malonyl-CoA in mouse liver homogenates. All values were corrected by subtracting the malonyl-CoA insensitive values. The remaining activity upon incubation with 8 µM malonyl-CoA is plotted.

Western blotting

To generate liver whole cell protein extracts, 100 mg of frozen liver was homogenized in lysis buffer with protease and phosphatase inhibitors (50 mM HEPES pH 7.5, 2 mM EGTA, 2 mM EDTA, 150 mM NaCl, 10 mM NaF, 10% glycerol, 0.1% Nonidet™ P-40, 20 mM β-glycerolphosphate, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, and TLCK 0.1 mM protease inhibitors, 2 mM benzamidine, 0.5 mM PMSF and 10 µg/ml leupeptin). Liver extracts were

centrifuged at 13,000 rpm, the fat layer carefully aspirated, and the supernatant collected for use in Western blotting. NE-PER (Thermo Scientific, Rockford, IL) extraction reagents and protease inhibitor cocktails (Thermo Scientific) were used to isolate liver nuclear and cytoplasmic proteins according to the manufacturer's recommendations. Proteins were separated by PAGE and transferred to 0.2 mm PVDF membranes (Bio-Rad). Membranes were blocked with 5% dry milk-TBST or 5% BSA-TBST for 1-2 hours and incubated with the following antibodies: SREBP1 and tubulin- α (Thermo Scientific), Lamin A/C, ACAC, phospho-ACAC, phospho-glycogen synthase^{S641} and glycogen synthase (Cell Signaling), Topo II (Abcam), CPT1A and CPT2 [kind gift from Dr. Carina Prip-Buus (INSERM, U1016, Institut Cochin, Paris, France)]. Blots were developed with Pierce ECL blotting substrate (Thermo Scientific) and exposed to ECL film (GE Healthcare, Piscataway, NJ).

Real-time RT-PCR

Total RNA was isolated from frozen liver samples (Qiagen). qRT-PCR was carried out in an ABI 7500 instrument (Applied Biosystems, Foster City, CA), using 50 ng of RNA and the QuantiTect SYBR Green RT-PCR Kit (Applied Biosystems). Primer pairs were purchased from Qiagen, or designed to bind different exons of the gene and amplify fragments of approximately 200 bp, and were first confirmed to yield a single band of the expected size by agarose gel electrophoresis, as well as a negative result in wells containing sample without

reverse transcriptase. Primer sequences are available upon request. The relative quantification of each gene was normalized with Cyclophilin A.

Statistics

Numerical values represent mean \pm SD. *P* values were calculated using unpaired two-tailed Student's *t*-tests. A *P* value of less than 0.05 was considered statistically significant.

Results

Silencing SREBP-1 increases expression of gluconeogenesis genes and reduced glycogen accumulation

To study the effects of silencing SREBP-1 on carbohydrate metabolism during the transition between the fed and fasted, helper-dependent adenoviral vectors expressing and shRNA to target SREBP-1 (gAd.shSREBP1) or a scrambled shRNA (gAd.shSCR) were injected into the tail vein of normal mice (Fig. 7, A and B). No alteration in blood glucose levels were observed between the two treatments (Fig. 7C). However, glycogen accumulation was reduced by 30% under fed conditions (Fig. 7C). In addition, normal mice that received gAd.shSREBP1 has lower levels of glycogen synthase 2 (GYS2) protein (Fig. 7D). Furthermore, SREBP-1 deficiency increased expression of the gluconeogenesis enzyme phosphoenolpyruvate carboxykinase (PCK1) (Fig. 7D). These data indicate that SREBP-1 is needed to appropriately regulate glycogen synthesis and to block *Pck1* expression.

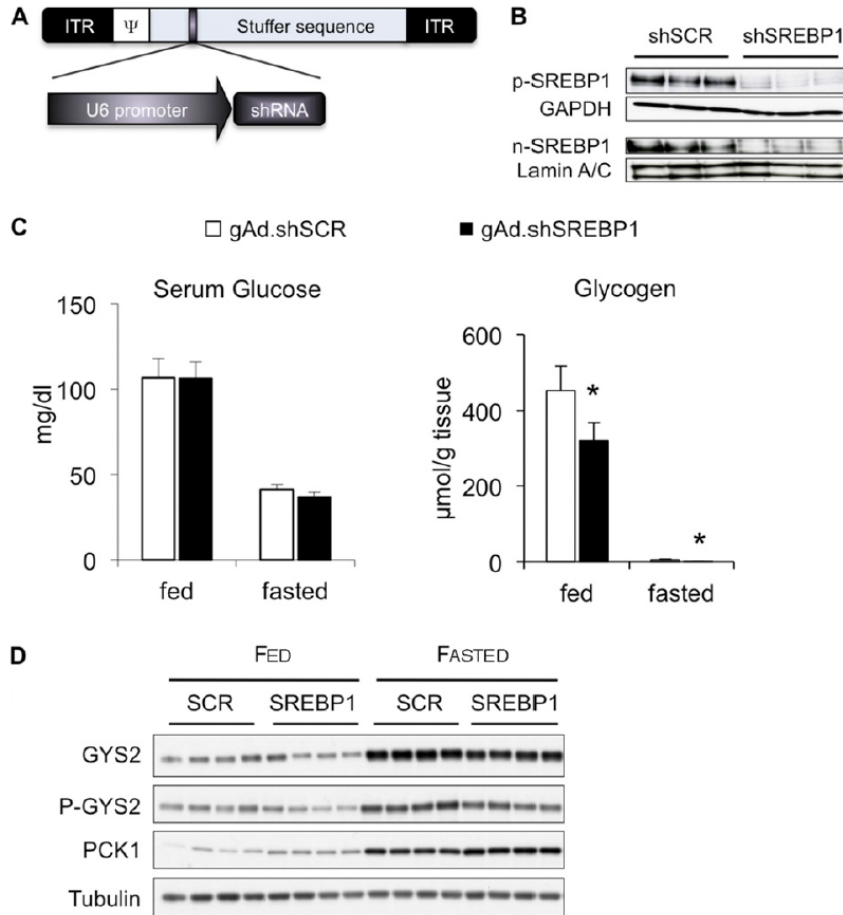


Figure 7. Impact of SREBP-1 knock-down on carbohydrate metabolism in normal mice. Groups of five C57BKS mice were given 1×10^{11} vp gAd.shSCR or gAd.shSREBP1 and euthanized one week later under fed or 24-h fasted conditions. **(A)** Helper-dependent adenovirus vector used to express shRNA. Hairpin RNA expression was driven by the U6 promoter. ITR, inverted terminal repeat; ψ , packaging signal. **(B)** SREBP-1 precursor (p-SREBP) or nuclear (n-SREBP) protein levels. **(C)** Blood glucose was not affected by SREBP-1 silencing. However, glycogen levels were significantly lower under fed and fasted conditions in mice treated with gAd.shSREBP1; * $p < 0.05$; $n = 5-6$. **(D)** Immunoblots to detect GYS2, P-GYS2, PCK1 and tubulin.

SREBP-1 knockdown reduced steatosis in an animal model of hepatic insulin resistance

SREBP-1 activity is higher in the liver of *db/db* mice, and animal model of obesity with hepatic steatosis and severe insulin resistance. Thus, depleting SREBP-1 was expected to diminish lipogenesis and improve steatosis and consequently, improve insulin resistance. As predicted, silencing SREBP-1 resulted in down-regulation of hepatic *de novo* lipogenesis genes *Fasn*, *Scd1*, and *Gpam* (Fig. 8A). Interestingly, *Acacb* mRNA and protein expression were dramatically blunted, whereas *Acaca* mRNA, protein, and protein phosphorylation were not changed relative to the *db/db* control group (Fig 8, A and B). These data indicate that SREBP-1 is an important regulator of ACACB and that SREBP-1 is not critical to maintain high ACACA levels in *db/db* mice.

Silencing SREBP-1 resulted in lower liver triglyceride levels in the *db/db* gAd.shSREBP1 group compared with the gAd.shSCR under fasting condition (Fig. 8C). Using $^2\text{H}_2\text{O}$ as a metabolic tracer, *de novo* liver palmitate and total liver palmitate were found to be reduced 70 and 30%, respectively, confirming that down-regulation of enzymes in the lipogenesis pathway resulted in reduced levels of glucose conversion into lipid (Fig. 9A) The decline in *de novo* lipogenesis (as well as in ACAC levels) was expected to decrease malonyl-CoA, an allosteric inhibitor of CPT1A. CPT1A is the enzyme that transfers an acyl group from CoA to carnitine, which is then translocated into mitochondria for oxidation. Lower malonyl-CoA levels were expected to increase CPT1A activity and subsequently, enhance fatty acid oxidation (FAO).

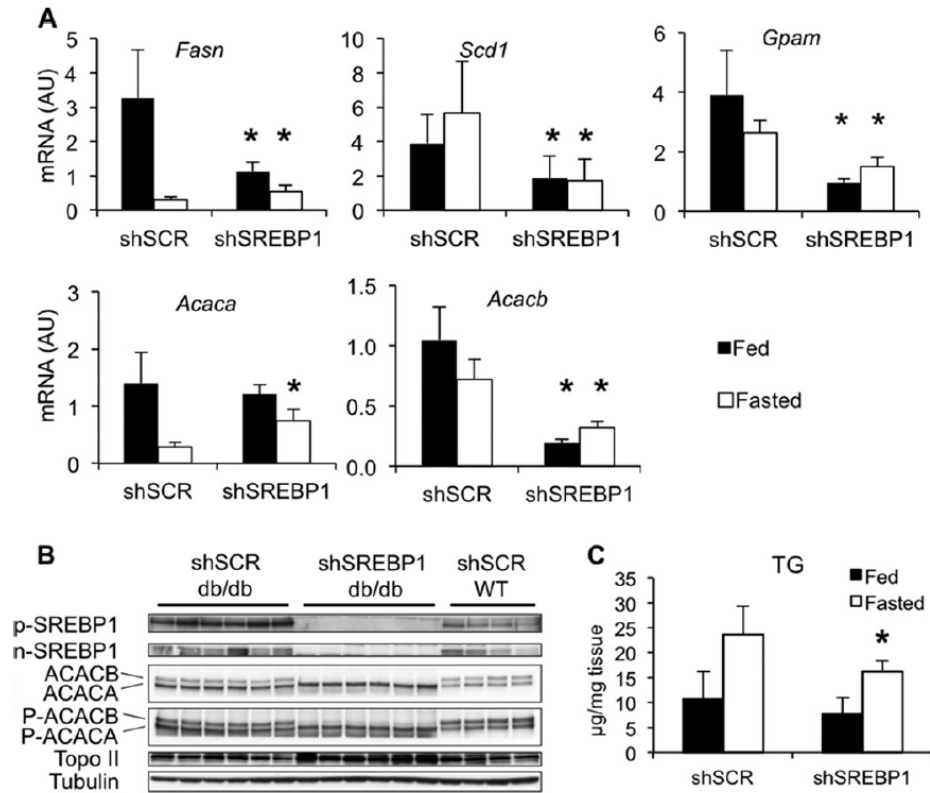


Figure 8. SREBP1 silencing in *db/db* mice. Groups of eight *db/db* mice were given 1×10^{11} vp gAd.shSCR or gAd.shSREBP1 through the tail vein, and euthanized three weeks later under fed (*ad libitum*) or 24-hour fasted conditions. Groups of four normal C57BLKS mice were used as controls and received the same dose of gAd.shSCR adenovirus. **(A)** Effect of silencing SREBP1 on lipogenic enzyme expression. Data are presented as mean \pm SD. * $p < 0.05$ versus control; $n = 7-8$. **(B)** Immunoblot analysis for SREBP-1 precursor (p-SREBP) or nuclear (n-SREBP) protein, ACAC and phosphorylated ACAC (P-ACAC), three weeks after gAd.shSCR or gAd.shSREBP1 administration. ACAC and P-ACAC panels show ACACA as a bottom 265 kDa band and ACACB as a top 280 kDa band. **(C)** Hepatic triglyceride levels in *db/db* mice; * $p < 0.05$ versus control; $n = 8$.

As anticipated, malonyl-CoA levels were depleted to near half the levels observed in control animals (Fig. 9A). We confirmed that CPT1A and CPT2 protein levels were not changed between gAd.shSCR and gAd.shSREBP1-treated mice, under fed or 24-h fasting conditions (Fig. 3B), and no difference was observed in CPT1A IC₅₀ values for malonyl-CoA between the two groups, suggesting similar capacity to be inhibited by this metabolite (Fig. 9B). Nevertheless, no evidence of increased FAO was observed in the gAd.shSREBP1-treated group, based on serum β -hydroxybutyrate and liver BHB-CoA levels (Fig. 9, A and C). Overall, these data suggest that SREBP-1 deficiency had a positive impact on hepatic steatosis, most likely as a result of reduced lipogenesis rather than increased FAO.

Serum triglyceride levels were found mildly elevated in SREBP-1 depleted mice compared with diabetic controls under fasting conditions (Table1), and this correlated with a significant reduction of LDL receptor mRNA (Fig. 9D), indicating decreased uptake of lipoproteins by the liver. The importance of SREBP-1a as a regulator of the low density lipoprotein receptor and its control of lipoprotein uptake/degradation in the liver has been highlighted in transgenic mice overexpressing SREBP-1a in this tissue, which display markedly increased levels of LDL receptor [134]. Whereas null *Ldlr* mice accumulate triglyceride and cholesterol in serum, mice overexpressing SREBP-1a do not accumulate these metabolites in plasma because they are degraded through the action of LDL receptors [134]. Furthermore, transgenic mice overexpressing SREBP-1a or SREBP-1c in liver have significantly higher levels of triglycerides in liver and

lower levels in serum [52]. Likewise, adenovirus-mediated SREBP-1c overexpression in liver induced a 2-fold increase in hepatic triglyceride levels and up-regulation of LDL-receptor, leading to reduced serum levels of triglycerides [135].

Table 1. Effect of SREBP-1 silencing on weight and serum chemistries

	<i>db/db</i>		WT
	gAd.shSCR	gAd.shSREBP	gAd.shSCR
Total body weight (g)	44.7 ± 1.92**	40.3 ± 3.65*,**	22.7 ± 1.32
Fed			
Liver weight (g)	2.63 ± 0.22	1.83 ± 0.18*	1.10 ± 0.06
Serum FFAs (mM)	1.55 ± 0.27**	2.23 ± 0.28***	0.96 ± 0.06
Serum TG (mg/ml)	1.17 ± 0.27**	1.41 ± 0.44**	0.75 ± 0.06
Fasted			
Liver weight (g)	2.18 ± 0.20	1.46 ± 0.16*	0.86 ± 0.05
Serum FFAs (mM)	1.32 ± 0.22	2.05 ± 0.14***	1.25 ± 0.1
Serum TG (mg/ml)	0.63 ± 0.08	1.01 ± 0.11***	0.74 ± 0.1

Mice were treated as described in the legend of Fig. 1. * $p < 0.05$ *db/db* gAd.shSREBP1 versus gAd.shSCR; ** $p < 0.05$ compared to WT. $n = 8$ *db/db*, $n = 4-5$ WT.

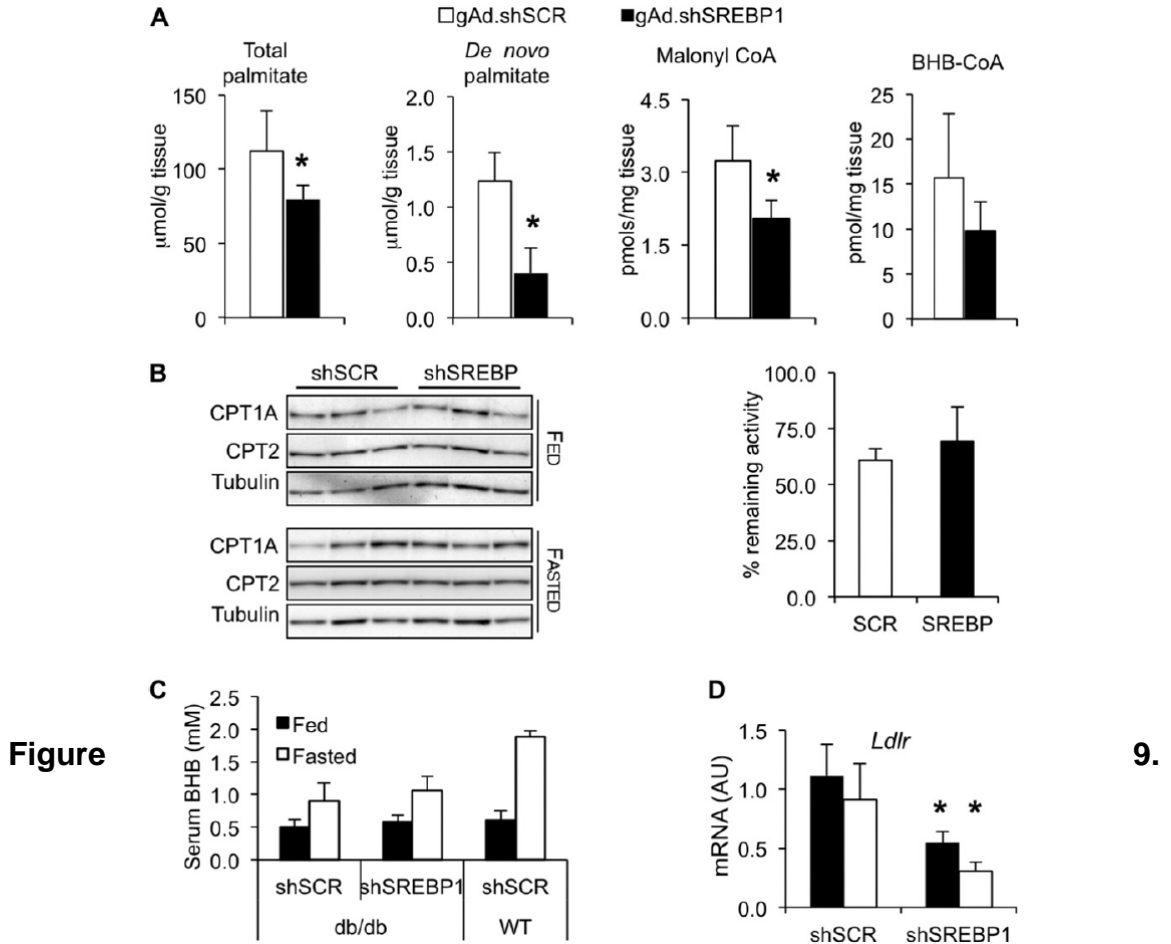


Figure 9. SREBP-1 silencing decreases *de novo* lipogenesis. (A) Groups of 8 *db/db* mice were administered 1×10^{11} vp gAd.shSCR or gAd.shSREBP1. Three weeks later, $^2\text{H}_2\text{O}$ was administered intraperitoneally. Mice were fasted for 4 hours and euthanized. Total palmitate, *de novo* synthesis of palmitate, malonyl-CoA and BHB-CoA were analyzed ($p < 0.05$ vs control; $n = 8$). (B) Mice were treated as described in Fig. 8. CPT1A and CPT2 protein levels under fed and fasted conditions, and CPT1A sensitivity to malonyl-CoA in livers of mice fasted for 24-hours; $n = 5$. (C) Mice were treated as described in Fig. 2. β -hydroxybutyrate serum levels; $n = 4-5$ WT, $n = 8$ *db/db*. (D) LDL receptor mRNA expression in *db/db* mice; $n = 7-8$.

Increased expression of gluconeogenesis genes and lower glycogen accumulation in SREBP-1 depleted db/db mice

Despite lower liver steatosis, acutely silencing SREBP-1 in *db/db* mice had no impact on glucose and insulin levels (Fig. 10A). Furthermore, mice were slightly more hyperglycemic upon a 24-h fast, suggesting a defect in the regulation of glucose homeostasis. To determine the impact of silencing hepatic SREBP-1 on glucose tolerance, glucose and insulin tolerance tests were performed. Consistent with the lack of glucose reduction, no differences between the gAd.shREBP1- and the gAd.shSCR- treated groups were observed (Fig. 10B). Thus, hepatic SREBP-1 silencing has no major impact on whole body glucose tolerance.

To investigate the basis of the lack of improvement in glucose homeostasis, glycogen accumulation was measured. Remarkably, SREBP-1 silencing resulted in ~ 50% lower glycogen levels under fed conditions, compared with *db/db* control mice, as previously seen in normal mice (Fig. 11A). In addition, glycogen synthase 2 (GYS2) mRNA, glycogen synthase activity, and protein levels were decreased, relative to diabetic control mice (Fig. 11, B-D). These observations were recapitulated in another animal model of obesity and type 2 diabetes, the *ob/ob* mouse (data not shown).

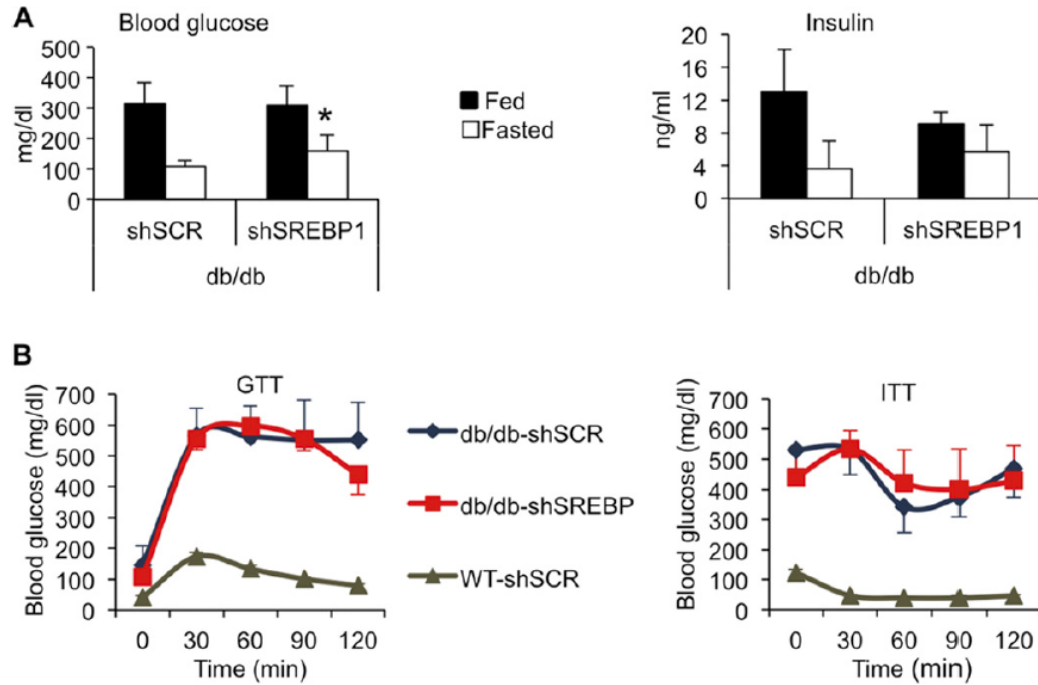


Figure 10. SREBP-1 silencing does not improve whole body glucose or insulin tolerance. Mice were treated as described in Fig. 2. **(A)** Glucose and insulin levels in serum; $n = 5-8$. **(B)** A glucose tolerance test (GTT) was conducted after overnight fast and intraperitoneal administration of a glucose bolus (1 mg/g body weight); an insulin tolerance test (ITT) was carried out after fasting animals for 5 hours and intraperitoneal administration of human regular insulin (1 U/kg); $n=7$ *db/db*, $n=5$ WT.

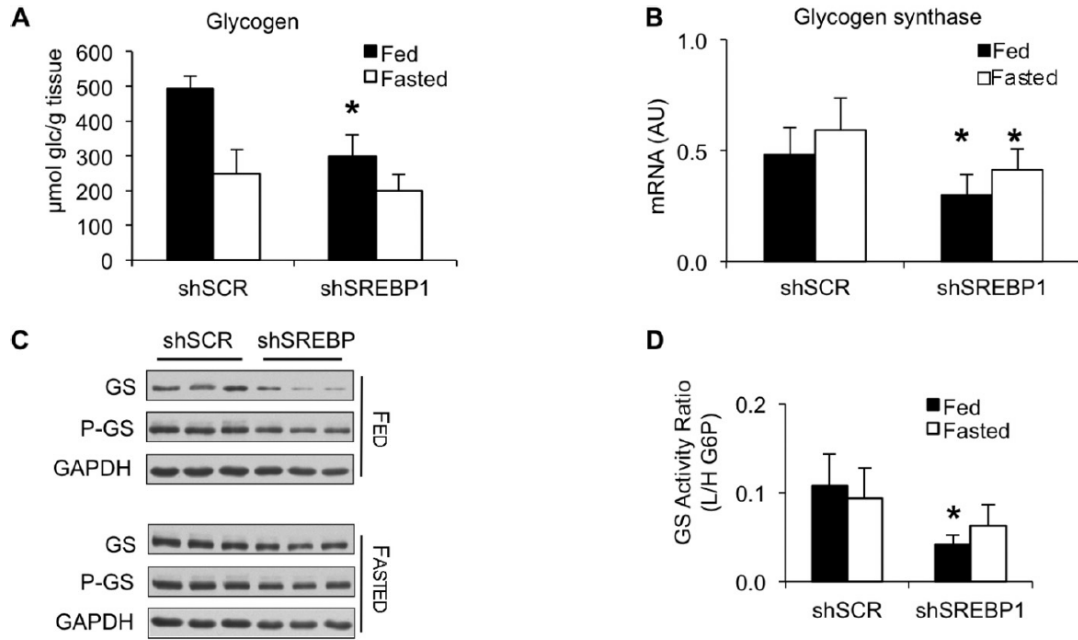


Figure 11. SREBP-1 silencing alters gluconeogenesis and glycogen synthesis. *db/db* mice were treated as described in Fig. 8. **(A)** Hepatic glycogen content in *db/db* mice; n=8. **(B)** Glycogen synthase 2 mRNA levels; n=7-8. **(C)** Immunoblot analysis of total and phosphorylated glycogen synthase. **(D)** Ratio of the glycogen synthase activity measured with low (L) over high (H) glucose-6-phosphate, under fed and fasted conditions; n=3; *p<0.05 vs control.

To further understand the role of SREBP-1 at regulating carbohydrate metabolism, we analyzed expression of limiting enzymes in the glycolysis and gluconeogenesis pathways, including glucokinase (*Gck*), liver-pyruvate kinase (*Pklr*), phosphoenolpyruvate carboxykinase (*Pck1*), and glucose-6-phosphatase (*G6pc*). SREBP-1 overexpression has been shown to induce glucokinase gene expression [135]. Thus, lack of SREBP-1 was expected to reduce its levels, which could have affected glycogen synthesis. *Gck* levels were not altered upon SREBP-1 silencing (Fig. 12A). However, *Pklr* gene expression was severely blunted compared with diabetic control animals, indicating reduce activity of the glycolysis pathway (Fig. 12A). In contrast, gluconeogenesis genes *Pck1* and *G6pc* were significantly higher in the gAd.shSREBP1- treated group. PCK1 mRNA levels were >2-fold higher, both under fed and fasting conditions, and protein levels were also increase (Fig. 12, A and B). Overall, our data suggest that increased PCK1 levels, reduced PKLR activity, and reduced glycogen storage capacity may have accounted for lack of improved glycemia in the gAd.shSREBP1- treated *db/db* animals (Fig. 10A).

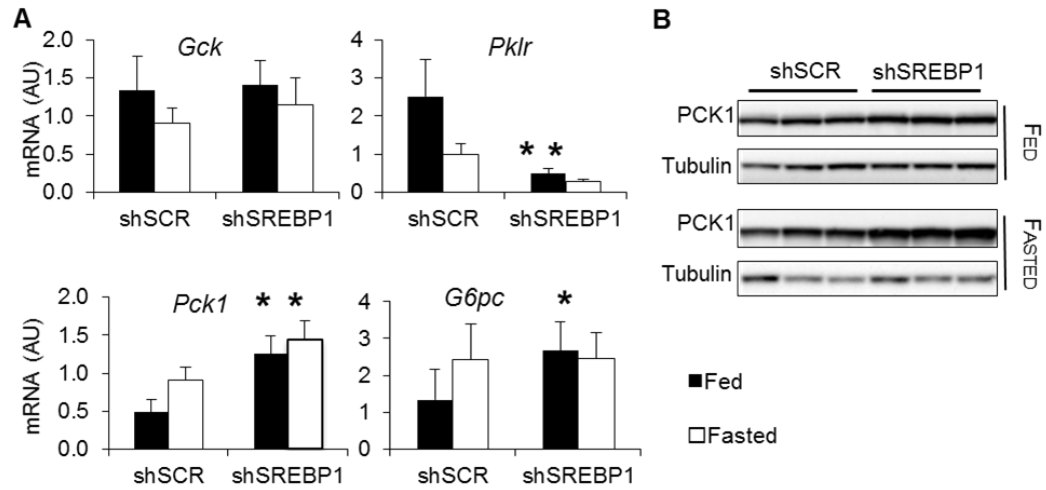


Figure 12. SREBP-1 silencing alters gluconeogenesis and glycogen synthesis. Mice were treated as described in Fig. 8. **(A)** *Gck*, *Pklr*, *Pck1*, *G6pc* mRNA expression; n=7-8. **(B)** PCK1 protein levels.

Discussion

Hepatic fat accumulation is strongly associated with insulin resistance and inappropriate inhibition of hepatic glucose output. A large amount of literature has linked high SREBP1 activity with insulin resistance and type 2 diabetes. A variety of gene ablation as well as insulin-sensitizing drug studies have shown concomitant reductions in SREBP-1 and insulin resistance. For example, it has been suggested that the beneficial effects of resveratrol and other polyphenols on hepatic steatosis and insulin sensitivity is mediated by AMPK, which phosphorylates and inactivates SREBP-1 [97]. Recently, it has been proposed that multiple nuclear receptors, including ER α , CAR, LXR-1, TR β , and FXR/SHP, exert their antidiabetic effects through down-regulation of SREBP-1 [136], placing this transcription factor at the crossroads between steatosis and hepatic insulin resistance. Nevertheless, these studies are in contrast with data generated in leptin-deficient (*ob/ob*) SREBP-1 whole body knock-out mice [119]. Lep^{ob/ob} x Srebp-1^{-/-} mice remain hyperglycemic despite a 50% decrease in hepatic lipid accumulation [119]. Given that this study did not provide a mechanism for the lack of improvement in insulin resistance, we hypothesized that SREBP-1 may play a role in other aspects of liver metabolism. Thus, we knocked down SREBP-1 in normal mice and in *db/db* mice to clarify the role of this transcription factor on carbohydrate metabolism as well as investigate the therapeutic value of silencing SREBP-1 in the liver of a mouse model with established steatosis and severe insulin resistance. This is the first comprehensive *in vivo* study addressing both

carbohydrate and lipid metabolism and the only adult model of hepatic SREBP-1 deficiency in normal and obese mice.

The results of this study have underscored the requirement for SREBP-1 in glycogen synthesis in the fed state. SREBP-1 deficiency in *db/db* mice decreased glycogen levels as well as glycogen synthase mRNA, protein, and enzyme activity during fed conditions, when glycogen synthesis take place. These observations were also observed in normal mice, implying that SREBP-1 is needed to regulate glycogen metabolism appropriately. The effects on glycogen were independent from glucokinase gene expression, which was unaffected by SREBP-1 silencing. Glucokinase gene expression has been shown to be up-regulated in response to SREBP-1c overexpression [135], although other studies have shown that increases in SREBP-1c were neither necessary nor sufficient to induce glucokinase [137, 138]. Our study confirms that *in vivo*, SREBP-1 is dispensable for expression of this gene.

In addition to alterations in glycogen synthesis, up-regulated *Pck1* and *G6Pase* gene expression was observed under fed and fasted conditions. *In vitro* studies have previously demonstrated that SREBP-1 negatively regulated *Pck1* gene expression by binding to two sterol regulatory elements present in the promoter of the gene [139]. SREBP-1 has also been shown to inhibit *Pck1* gene transcription by interacting with HNF4 α and interfering with recruitment of the transcription co-activator PGC-1 α to the *Pck1* promoter [140]. Here we show that *in vivo*, SREBP-1 deficiency results in lack of appropriate inhibition of *Pck1* and *G6pase* gene expression. Finally, *Pklr* was found severely diminished. Overall,

this study provides evidence that SREBP-1 controls carbohydrate metabolism during fed conditions by promoting glycogen synthesis, enhancing glycolysis, and inhibiting gluconeogenic gene expression.

The importance of the *de novo* lipogenesis pathway as a contributor of hepatic steatosis has been underscored in a study using metabolic tracers in human patients with non-alcoholic fatty liver disease [36]. In these individuals, as much as 26% of triglycerides are derived from this pathway [36]. In addition, *de novo* lipogenesis is elevated in the fasted state and does not increase postprandially, indicating that one of the underlying causes of hepatic steatosis in non-alcoholic fatty liver disease might be the inability of the liver to regulate this pathway appropriately [36]. As expected, SREBP-1 silencing resulted in decreased expression of *de novo* lipogenesis genes, including *Fasn*, *Acacb*, *Gpam*, and *Scd1*. Consistent with the decrease in gene expression, *de novo* synthesis of palmitate was 70% lower in livers of gAd.shSREBP1-treated animals relative to control *db/db* mice, and liver triglycerides were significantly decreased under fasting conditions. This indicates that, as observed in human with non-alcoholic fatty liver disease [36], enhanced *de novo* lipogenesis in the fasted state is one of the underlying causes of hepatic lipid accumulation in *db/db* mice and targeting SREBP-1 lowered steatosis.

ACACB co-localizes spatially with CPT1A on the mitochondrial membrane [141], and it has been suggested that the malonyl-CoA produced from this isoform inhibits CPT1A activity and FAO, whereas the malonyl-CoA produced by ACACA is used to generate palmitate [141]. Nevertheless, liver-specific *Acaca*-

deficient mice compensate for lack of ACACA by increasing ACACB expression and maintain physiological levels of *de novo* lipogenesis and FAO, suggesting that compartmentalization of malonyl-CoA produced by each ACAC isoform is not stringent [142]. Increased activities of transcription factors SREBP-1 and carbohydrate-responsive element-binding protein (ChREBP) have been observed in *db/db* and *ob/ob* mice, animal models of obesity and type 2 diabetes. Silencing ChREBP in liver of *ob/ob* mice resulted in significant reduction in ACACA and ACACB, *de novo* lipogenesis and triglyceride levels, as well as increased serum levels of β -hydroxybutyrate, which was attributed to higher FAO rates [40]. In contrast, SREBP-1 silencing in *db/db* mice largely reduced ACACB, but had no effect on ACACA, the predominant isoform in liver. Serum β -hydroxybutyrate levels were not elevated under fed or 24-h fasting conditions. Furthermore, the levels of liver BHB-CoA, an intermediate of fatty acid oxidation [130, 143], were not significantly different between the two groups. Given that SREBP-1 silencing did not reduce ACACA, these data suggest that both isoforms need to be diminished to reduced malonyl-CoA to levels that allow increasing CPT1A activity. Thus, depleting SREBP-1 in *db/db* mice reduced lipogenesis, but FAO was not enhanced.

A growing number of studies are reporting a dissociation between hepatic steatosis and insulin resistance [144-146]. Overexpression of diacylglycerol O-acyltransferase 2 (DGAT2) in the liver increased triglyceride, ceramide, and diacylglycerol without alteration on insulin or glucose tolerance [145]. *Gpam* deficiency in *ob/ob* mice diminished SREBP-1 levels with a concomitant

decrease in hepatic triglyceride accumulation, but no improvement in hepatic or peripheral insulin sensitivity [146]. Furthermore, fasting glucose was exacerbated, whereas the absence of *Gpam* in lean mice had no effect on glucose, similar to what we observed in gAd.shSREBP1-treated *db/db* and lean mice, respectively. SREBP-1 silencing is another example of dissociation between hepatic steatosis and glucose tolerance. Our data provide evidence of additional roles for SREBP-1 at regulating metabolism besides its established function at facilitating lipogenesis. Thus, SREBP-1 coordinates changes in gene expression physiologically important during the fed state: enhancing *de novo* lipogenesis and glycogen synthesis, facilitating glycolysis, and inhibiting gluconeogenesis.

In conclusion, this work provides evidence of novel roles for SREBP-1, a critical transcription factor that has been extensively associated with hepatic steatosis and insulin resistance. Our data indicate that SREBP-1 depletion has profound effects on expression of genes needed for carbohydrate metabolism. Hence, the benefits of reducing *de novo* lipogenesis in *db/db* mice were offset by the negative impact of gluconeogenesis and glycogen synthesis.

Chapter 2: Insulin resistance and hepatic steatosis in primary hepatocytes

Introduction

Due to the strong association between fatty acid accumulation and impairment of the insulin signaling pathway, multiple strategies are being pursued to reduce hepatic steatosis with the goal of improving insulin sensitivity. We have shown in Chapter 1 that silencing SREBP-1 in *db/db* mice reduces steatosis largely as the result of decreased DNL [147]. Contrary to our expectations, no evidence of increased fatty acid oxidation was observed, despite the fact that malonyl-CoA levels were significantly reduced [147]. Thus, additional studies are needed to investigate the underlying mechanisms leading to this observation, and to better understand whether CPT1A activity or the capacity of mitochondria to oxidize fatty acids is limiting in the *db/db* mouse model and in insulin resistant states. There is evidence showing that phosphorylation also influences CPT1A activity, although its regulation under pathophysiological conditions remains unknown [148]. It is also known that hyperinsulinemia reduces mitochondrial biosynthesis in hepatocytes [149]. Thus, we focused on setting up conditions to study whether increasing CPT1A activity by adenovirus-mediated overexpression of CPT1A would improve fatty acid oxidation under insulin resistance conditions.

There is evidence from the literature supporting the idea that CPT1A activity in insulin resistant states may be limiting and increasing it may be beneficial. Studies performed in adipocytes by Gao and colleagues demonstrated

that increasing human carnitine palmitoyl-transferase 1A (CPT1A) activity resulted in the down regulation of c-JNK as well as lipid derivatives [150]. Furthermore, insulin resistance was improved in both adipocytes and myocytes, suggesting that expressing CPT1A is beneficial to increase insulin sensitivity in a high fatty acid environment [150]. In the liver, two separate *in vivo* studies revealed diverging results regarding the use of CPT1A as a strategy to reduce hepatic steatosis and improve insulin resistance. Orellana-Gavaldà and colleagues demonstrated that high-fat fed mice overexpressing wild type CPT1A or a mutated form of CPT1A not sensitive to malonyl-CoA inhibition, decreases lipid accumulation and improves insulin resistance [151]. However, Monsénégo and colleagues showed that overexpressing malonyl-CoA-insensitive CPT1A, improved insulin signaling but failed to reduce triglyceride accretion in the liver of high-fat fed and *ob/ob* mouse models, suggesting a disconnection between insulin resistance and hepatic steatosis [152]. A couple of factors could have influenced the results of these studies that may explain the discrepancies between them. The Orellana-Gavaldà's group injected the vector expressing CPT1A two weeks after starting the high-fat diet treatment, which is unlikely to have been sufficient time to develop insulin resistance and hepatic steatosis. Mice were examined 3 months later [151]. Thus, this study focused on preventing insulin resistance, rather than treating it with CPT1A overexpression. On the contrary, Monsénégo's group conducted the study after the onset of steatosis and insulin resistance (20 weeks after high-fat feeding), which allowed testing whether CPT1A overexpression is a useful therapy for the treatment of steatosis

and insulin resistance [152]. Nevertheless, the authors concluded that the therapy improves insulin sensitivity but does not reduce steatosis. Given that their studies only lasted 2 weeks, this may have been insufficient time to reduce fatty acid buildup. These results suggest that CPT1A may have a fundamental role in the prevention of lipid accumulation and insulin resistance, while it may not be effective at treating this condition.

These data underscored the importance of defining a mechanism for how insulin resistance is prompted by hyperinsulinemia and lipid accumulation independent of one another. Studying hepatocytes under hyperinsulinemic or hyperlipidemic conditions separately may help further our knowledge on how each plays a role in the development of insulin resistance. Here, I evaluated the effects of overexpressing CPT1A using helper dependent adenoviral vectors in primary hepatocytes under each condition separately to better understand the mechanisms by which CPT1A may improve insulin signaling. The data indicate that insulin signaling was not improved by CPT1A overexpression regardless of the conditions used to induce insulin resistance. Although CPT1A alone cannot improve insulin signaling in primary hepatocytes, possibly due to the fact that primary cells can be cultured for only a short time, CPT1A overexpression may still be a viable therapeutic tool for NAFLD treatment. Moreover, the assays and techniques used to induce insulin resistance will be useful for future studies on mechanisms of insulin resistance development in the liver. This system could be used to further study the molecular mechanisms by which silencing SREBP-1

does not lead to improved fatty acid oxidation, and the possible impact of hyperinsulinemia on CPT1A phosphorylation and activity.

Materials and methods

Generation of adenoviral vectors expressing wild type or mutant CPT1A

Total rat liver RNA was used to generate CPT1A cDNA (High Capacity cDNA reverse transcription kit, Applied Biosystems, Life Technologies, Grand Island, NY). The following oligonucleotides were used to amplify CPT1A:

5'-TATGCAGAATTCCAATAGGTCCCCACTCAAGATGG-3' and 5'-AATCAGGATATCTTACTTTTTAGAATTGATGGTGAGGC-3'. Wild type CPT1A

(CPT1-WT) cDNA was cloned into the pBluescript II SK(+) plasmid (Agilent Technologies). Changing methionine 593, a critical residue for malonyl-CoA inhibition, to a serine, results in constitutively active CPT1A [153]. A constitutively active mutant CPT1A (CPT1A-Mu) was generated using these oligonucleotides:

5'-CTCACATATGAGGCCTCCTCAACCCGGCTCTTCCGAGAA-3' and 5'-TTCTCGGAAGAGCCGGGTTGAGGAGGCCTCATATGTGAG-3' with site

directed mutagenesis (Agilent Technologies, Quick Change II Site-directed Mutagenesis Kit, Santa Clara, CA). The cDNA sequence was confirmed by sequencing. Expression cassettes were then generated in which the cDNAs were under the control of the elongation factor 1- α (EF1- α) promoter. This promoter was used because it results in moderately high levels of expression and does not shut down in the liver, allowing long-term expression of CPT1A. The bovine growth hormone polyA signal was cloned downstream of the cDNA using the

following oligonucleotides: 5'-
TATGCAGATATCCTAGAGCTCGCTGATCAGCCTC-3' and 5'-
AATCAGAAGCTTCCATAGAGCCCACCGCATCCC-3'. Helper-dependent
adenoviral vectors were generated using a Cre-loxP system (Microbix
Biosystems Inc., Toronto, Canada) as previously described [154]. Briefly, the
expression cassettes were cloned into pRES, and the resulting plasmids were
digested with *Hind* III. Simultaneously, plasmid pC4HSU [121] was digested with
Asc I. *Escherichia coli* BJ5183 cells were co-transformed with the linearized
plasmid (100 ng) and each of the *Hind* III fragments (300 ng), as described [155].
Recombination between homologous regions in these plasmids generated the
helper-dependent adenovirus constructs containing the expression cassettes. To
rescue helper-dependent adenoviral vectors, plasmids containing expression
cassettes were digested with *Pme* I. HEK293Cre4 cells were transfected with 5
 μ g of *Pme* I-digested DNA. Twenty-four hours later, helper H14 was added at
MOI 3, and cells were collected after the onset of cytopathic effect (CPE).
Helper-dependent adenoviral vector amplification was achieved by passaging
lysates in 6-cm dishes (5×10^6 cells/dish). For large-scale vector amplification of
gAd.CPT1A and gAd.Mu-CPT1A, 16 x 500 cm² flasks ($\sim 1 \times 10^9$ cells total) were
used. The virus was purified by one CsCl step gradient centrifugation followed by
one CsCl isopycnic separation. The helper-dependent adenovirus band was
collected and dialyzed in TMN buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 150
mM NaCl, 10% glycerol], and stored at -80°C.

Primary hepatocyte isolation

Primary hepatocytes were isolated by collagenase digestion from male C57BL/6J mice, as previously described [98]. Briefly, mice were anesthetized by pentobarbital. The portal vein was cannulated with a 22-gauge intravenous catheter and the liver was perfused with Ca^{2+} , Mg^{2+} -free Hanks' buffer solution containing 0.5 mM ethylene glycol-bis-(2-aminoethylether) N,N,N',N'-tetracetic acid (EGTA), and 0.05 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, pH 7.4). This was followed by collagenase perfusion containing 0.05 M HEPES and 0.04% collagenase type IA. Primary hepatocytes were plated at a density of 6×10^5 cells/well in 2 mL of DMEM with 10% FBS, 1 nM dexamethasone, and 100 IU/mL penicillin and 100 ug/mL streptomycin (P/S) on a 6-well plate. After 4 hours medium was changed to 2 ml/well of DMEM with 5% FBS, 1 nM dexamethasone, and 100 IU/mL P/S.

Hyperinsulinemia-induced insulin resistance

Prevention studies: Primary hepatocytes were infected with gAd.CPT1A-WT, gAd.CPT1-Mu, or gAd.NEC virus at MOI 6, 4 hours after plating. Medium was changed to DMEM containing 4.5 g/L glucose, supplemented with 5% FBS, 1 nM dexamethasone, and 100 IU/mL / 100 ug/mL penicillin/streptomycin (1x P/S). Twenty-four hours later, cells were treated with 5 nM insulin to induce insulin resistance. An insulin-sensitive control group was infected with gAd.NEC and vehicle was added to the medium. Medium with/out insulin was replaced every 24 hours. Seventy-one hours after infection, all cells were cultured in medium

without serum and insulin for 30 minutes to fast cells. To evaluate insulin sensitivity, each group was divided in two and received either 20 nM of insulin or vehicle for 30 minutes and then harvested.

Treatment studies: Hepatocytes were treated with DMEM containing 4.5g/L glucose, supplemented with 5% FBS, 1 nM dexamethasone, and 1x P/S, supplemented with either 5 nM insulin or vehicle. Twenty-four hours after insulin or vehicle treatment, cells were infected with gAd.CPT1A-WT, gAd.CPT1A-Mu, or gAd.NEC virus at MOI 15 for 4 hours in regular medium (DMEM with 5% FBS, 1 nM dexamethasone, and 1x P/S). Medium was replaced with 5 nM insulin-containing medium or vehicle, and changed every 24 hours. Forty-seven hours post infection all cells were cultured in medium without serum and insulin for 30 minutes to fast cells. To evaluate insulin sensitivity, each group was divided in two and received 20 nM of insulin or vehicle for 30 minutes and then harvested.

Hyperlipidemia-induced insulin resistance

Prevention studies: Primary hepatocytes were infected with gAd.CPT1A-WT, gAd.CPT1A-Mu, or gAd.NEC virus at MOI 30. Medium was changed to serum-depleted DMEM containing 4.5 g/L of glucose, for 4 hours. To induce steatosis and insulin resistance, cells were incubated with serum depleted DMEM supplemented with 0.4 mM palmitate (PA) conjugated with BSA, for 6 hours at 37°C. One plate was infected with gAd.NEC and treated with medium containing only BSA as a control group (insulin-sensitive). Medium was replaced with

DMEM and 1x P/S after the 6 hours. Forty-seven hours after infection, each group was divided in two and received 20 nM of insulin or vehicle to evaluate insulin sensitivity. Cells were harvested 30 minutes later.

Treatment studies: Hepatocytes were incubated in serum depleted DMEM containing 4.5 g/L glucose and 1x P/S supplemented with 0.3 mM palmitate conjugated with BSA overnight to induce insulin resistance. Cells were infected with gAd.CPT1A-WT, gAd.CPT1A-Mu, or gAd.NEC virus at MOI 30 for 4 hours the next day, following another incubation with DMEM and 1x P/S supplemented with 0.3 mM/BSA for another 24 hours. One plate was used as a control and treated only with BSA. Twenty-eight hours post-infection, each group was divided in two and received 20 nM of insulin or vehicle to evaluate insulin sensitivity. Cells were harvested 30 minutes later.

Conjugation of BSA/palmitate

A 6:1 molar ration of palmitate:BSA solution was made (1 mM sodium palmitate/0.17 mM BSA). Fatty acid free BSA (Sigma-Aldrich, St. Louis, MO) was dissolved in 150 mM NaCl solution at 37°C, while sodium palmitate (Sigma-Aldrich, St. Louis, MO) was dissolved in 150 mM NaCl solution at 70°C. Half of the BSA solution was combined with the palmitate solution in equal parts (1:1) and incubated for 1 hour at 37°C to conjugate; pH was adjusted to 7.4. A 150 mM NaCl solution was added to the other half of the BSA solution in equal parts to make the BSA control solution.

Western blotting

Whole cell protein lysates were generated through homogenization in RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN). Proteins were separated by PAGE electrophoresis and transferred to 0.2-mm PVDF membranes (Bio-Rad). Membranes were blocked with 5% BSA-TBST or 5% dry milk-TBST for 1-2 h and incubated with the following antibodies: Akt^{Ser473} and Akt (Pan); Foxo1^{Ser256} and Foxo1 (Cell Signaling, Boston, MA); CPT1A [kind gift from Dr. Carina Prip-Buus, (INSERM, U1016, Institut Cochin, Paris, France)]; β -actin (Santa Cruz Biotechnology, Dallas, TX); and Tubulin- α (Thermo Scientific, Rockford, IL). Blots were developed using Pierce ECL blotting substrate (Thermo Scientific) and exposed to film (GE Healthcare).

Statistics

Numerical values represent mean \pm S.D. P values were calculated using unpaired two-tailed Student's T-tests. A P value of <0.05 was considered statistically significant.

Results

CPT1A overexpression in primary hepatocytes does not rescue insulin resistance induced by hyperinsulinemia

Helper-dependent adenoviral vectors expressing wild type CPT1A (gAd.CPT1A-WT) or mutated CPT1A (gAd.CPT1A-Mu) under the control of the elongation factor 1 α promoter, were generated as described in the Materials and methods section. HEK293 cells and primary hepatocytes were transduced with these vectors to confirm expression (Fig. 13).

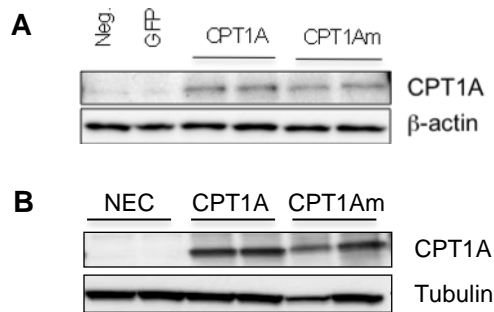


Figure 13. CPT1A-WT and CPT1A-Mu expression in primary hepatocytes.

(A) HEK293 cells were infected with gAd.CPT1A-WT (CPT1A) or gAd.CPT1A-Mu (CPT1Am), expressing wild type or mutant CPT1A, respectively. GFP, cells infected with a GFP-expressing virus. Neg., cells not infected with virus. Cells were harvested 30 h later. **(B)** Hepatocytes were infected with gAd.NEC (no expression cassette), gAd.CPT1A-WT or gAd.CPT1A-Mu at MOI 10 and harvested 48 h later.

It has been shown that culturing primary hepatocytes under hyperinsulinemic conditions leads to increased expression of lipogenesis genes and decreased expression of enzymes implicated in FAO, such as CPT1A, as well as genes necessary for mitochondria biogenesis/function [156]. Thus, experiments were set up in primary hepatocytes cultured under hyperinsulinemic conditions to determine the effects of overexpressing wild type or malonyl-CoA insensitive CPT1A. Primary hepatocytes were infected with gAd.CPT1A-WT, gAd.CPT1A-Mu, or gAd.NEC (no expression cassette). Insulin resistance was observed in cells cultured with 5 nM insulin, relative to cells cultured with vehicle, based on decreased phosphorylation of Akt^{Ser473} and its downstream target molecule FoxO1^{Ser256} (Fig. 14). Although CPT1A expression was seen in primary hepatocytes treated with either CPT1A-WT or CPT1A-Mu under hyperinsulinemic conditions, no improvement in insulin signaling was observed, based on lack of a significant increase in Akt^{Ser473} and FoxO1^{Ser256} (Fig 14).

I then analyzed whether CPT1A overexpression can rescue an established insulin resistance. Hepatocytes were treated with high insulin prior to being infected with gAd.CPT1A-WT, gAd.CPT1A-Mu, or gAd.NEC. Similarly to the prevention study, both Akt^{Ser473} and Foxo1^{Ser256} levels were attenuated in cells treated with high insulin. However, CPT1A overexpression did not rescue insulin sensitivity (Fig 15). Thus, overexpressing CPT1A before the onset of insulin resistance or once insulin resistance is established, did not have the desired outcome of improving insulin signaling.

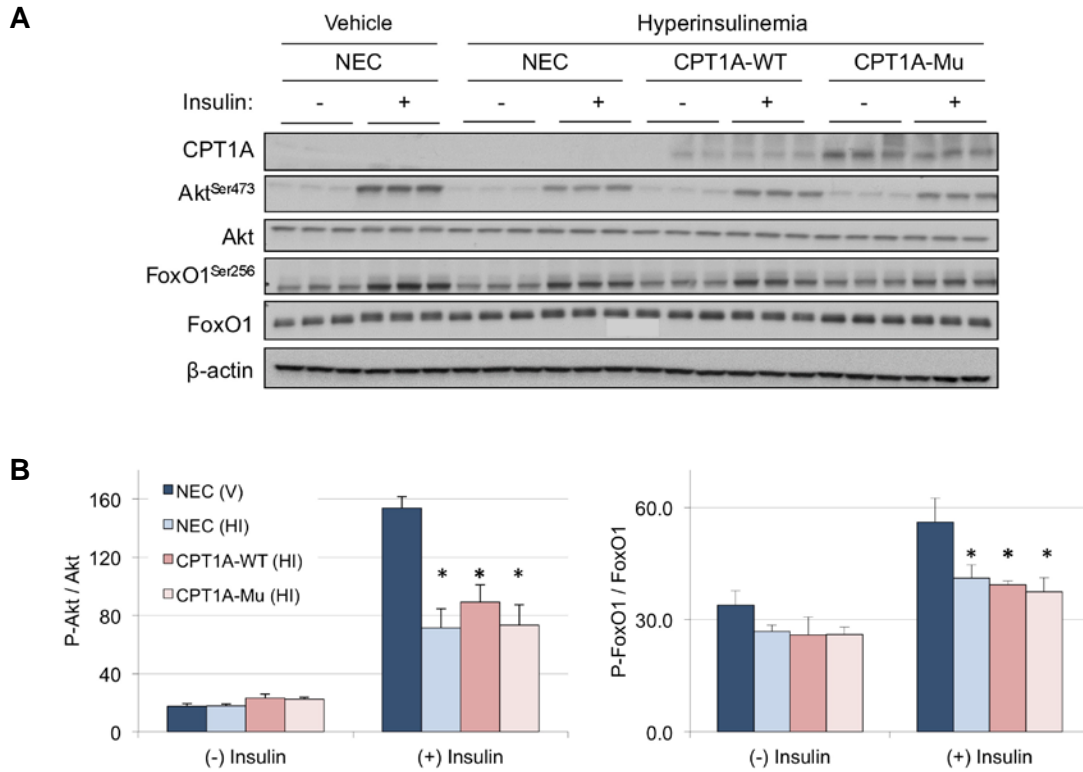


Figure 14. Prevention study under hyperinsulinemic conditions. (A) Primary hepatocytes were infected with gAd.NEC, gAd.CPT1A-WT, or gAd.CPT1A-Mu for a total of 72 hours. Cells were treated with 5 nM insulin for 48 hours, except for the insulin sensitive control group, which received vehicle. Insulin sensitivity was determined by culturing cells with vehicle or 20 nM insulin for 30 minutes. **(B)** Densitometry analysis of Akt^{Ser473} relative to total Akt, and of FoxO1^{Ser256} relative to total FoxO1. The experiment was repeated using primary hepatocytes from an independent isolation, and similar results were obtained. *P<0.05 relative to insulin-sensitive cells. Statistical analysis done only on insulin treated group.

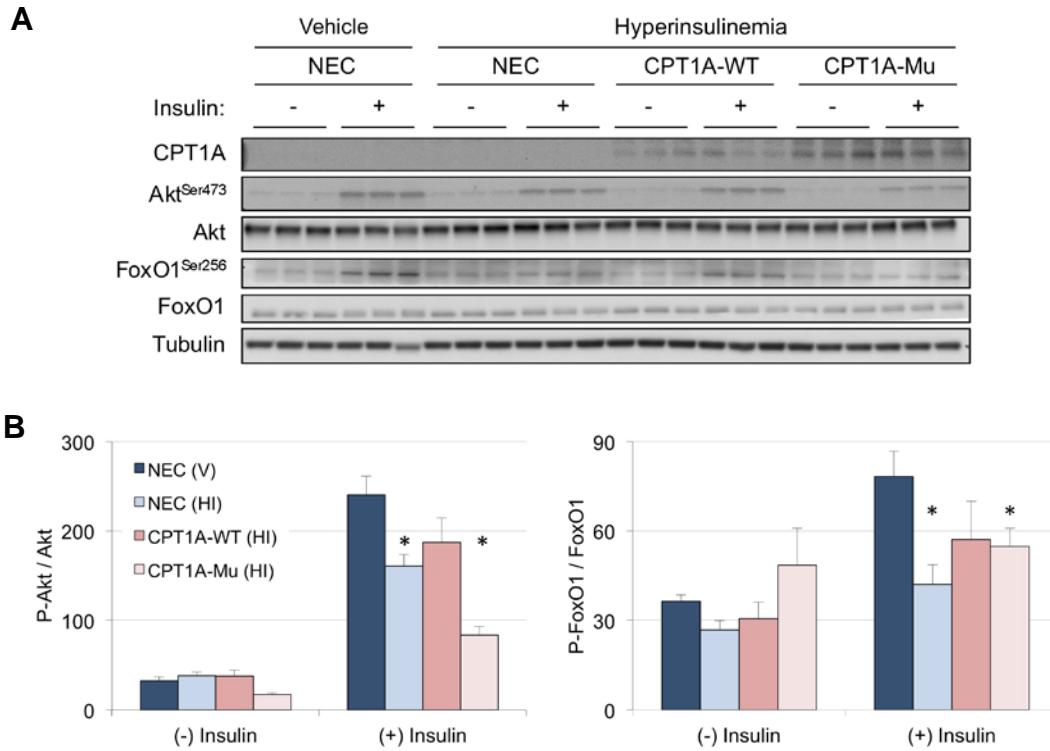


Figure 15. Treatment study under hyperinsulinemic conditions. (A) Primary hepatocytes were treated with 5 nM insulin for 20 hours then infected with gAd.NEC, gAd.CPT1A-WT, or gAd.CPT1A-Mu for 4 hours. Cells were treated again with 5 nM insulin for 48 hours. Insulin sensitive control group received vehicle. Insulin sensitivity assay was performed where the Basal group received vehicle and the Insulin group received 20 nM insulin for 30 minutes 52 hours post infection with virus. **(B)** Densitometry analysis of Akt^{Ser473} relative to total Akt, and of FoxO1^{Ser256} relative to total FoxO1. The experiment was repeated using primary hepatocytes from an independent isolation, and similar results were obtained. *P<0.05 relative to insulin-sensitive cells. Statistical analysis done only on insulin treated group.

CPT1A overexpression in primary hepatocytes does not rescue insulin resistance induced by hyperlipidemia

Studies have shown that exposing cells to large amounts of fatty acids, in particular palmitate, induces insulin resistance, impairing the insulin signaling pathway [157]. Thus, to induce insulin resistance, we used palmitate. Pilot studies were carried out in primary hepatocytes to determine the amount of palmitate (0.1-0.4 mM PA) conjugated to BSA that would induce insulin resistance in the absence of toxicity. A prevention or treatment set up were evaluated (data not shown). In addition to fatty acid concentration, several diluents, including ethanol, isopropanol, DMSO (dimethyl sulfoxide), and NaCl solution were tested. Ethanol, isopropanol and DMSO interfered with insulin signaling or caused toxicity (data not shown). Instead, dilution in NaCl solution did not induce toxicity, and this protocol was therefore used for all subsequent experiments (see Materials and methods for detailed description). Based on the results of these pilot experiments, the optimal amount of palmitate was 0.4 mM for the prevention study and 0.3 mM for the treatment study (Fig. 16).

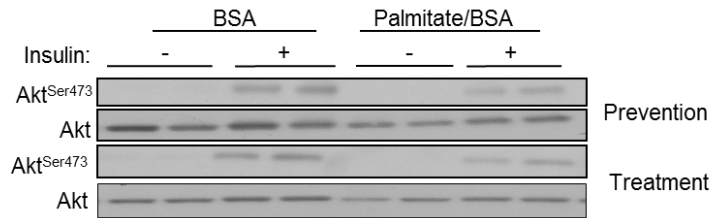


Figure 16. Palmitate induced insulin resistance in primary hepatocytes.

Primary hepatocytes were treated with either 0.4 mM (prevention) or 0.3 mM (treatment) palmitate (PA) conjugated to BSA for 24 hours. BSA alone was used for control cells. For the prevention setup, cells were cultured in medium without fatty acid for 24 hours, then cultured with fatty acid for 24 hours. In the treatment setup, cells were cultured with fatty acid for 24 hours, then cultured in fresh medium without fatty acid for another 24 hours. Cells were then treated with 20 nM insulin or vehicle for 30 minutes and then harvested at 48 hours.

Overexpressing CPT1A was expected to increase FAO and improve insulin signaling in primary hepatocytes. To evaluate if CPT1A overexpression could protect primary hepatocytes from developing insulin resistance, cells were treated with gAd.CPT1A-WT or gAd.CPT1A-Mu before adding PA (Fig. 17). As seen with the hyperinsulinemia conditions, palmitate induced a decrease in Akt^{Ser473} phosphorylation (Fig. 17). However, only a trend towards higher Akt^{Ser473} levels were observed in CPT1A-treated samples relative to the NEC control group (Fig. 17), as the data were not statistically significant. In order to determine if the lack of rescue was due to limiting levels of carnitine, cells were cultured with 1 mM of L-carnitine, under the same conditions described in 'Prevention studies' (material and methods section). Again, no increase in Akt^{Ser473} levels was observed in cells treated with CPT1A relative to the NEC control group (data not shown). Thus, we conclude that CPT1A overexpression does not improve insulin signaling in primary hepatocytes.

To study whether treating primary hepatocytes with CPT1A after insulin resistance had ensued, a similar experiment was set up, where primary hepatocytes were cultured with palmitate prior to transduction with gAd.CPT1A-WT or gAd.CPT1A-Mu. Similar to what was observed in the prevention study, no improvement in insulin signaling was observed in the CPT1A overexpressing cells relative to NEC control cells, based on Akt^{Ser473} phosphorylation (Fig. 18). Taken together, CPT1A overexpression did not improve insulin sensitivity under hyperlipidemic conditions in primary hepatocytes.

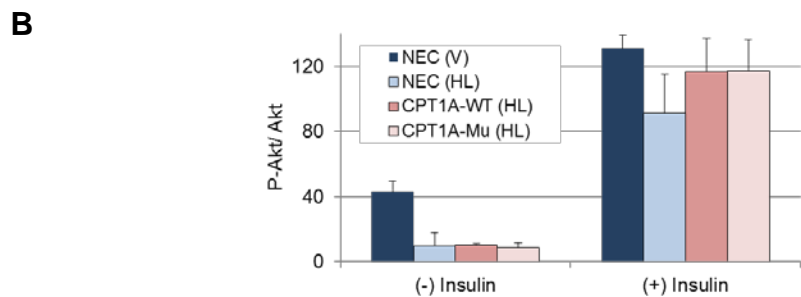
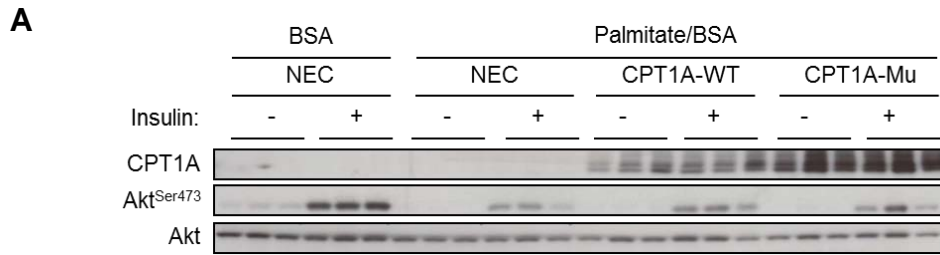


Figure 17. Prevention study under hyperlipidemia conditions. (A) Primary hepatocytes were infected with gAd.NEC, gAd.CPT1A-WT, or gAd.CPT1A-Mu for a total of 48 hours. Cells were treated with 0.4 mM palmitate for 6 hours, except for the insulin sensitive control group, which received BSA. Insulin sensitivity was determined by culturing cells with vehicle or 20 nM insulin for 30 minutes 48 hours post-virus infection. **(B)** Densitometry analysis of Akt^{Ser473} relative to total Akt. The experiment was repeated using primary hepatocytes from an independent isolation, and similar results were obtained. *P<0.05 relative to insulin-sensitive cells. Statistical analysis done only on insulin treated group.

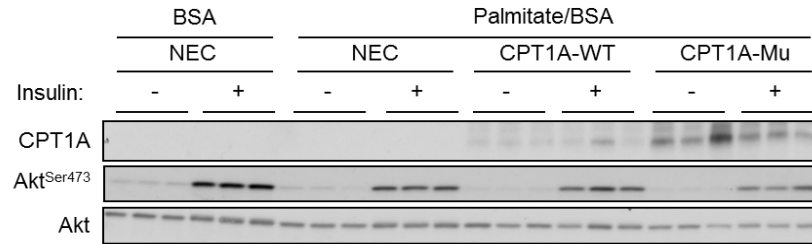
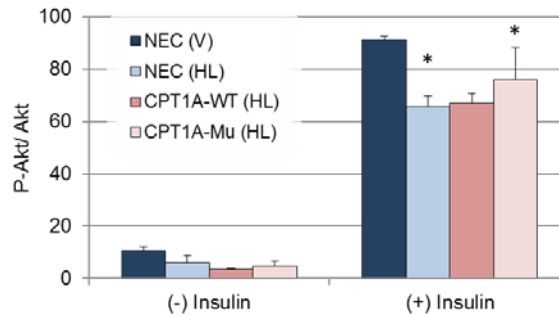
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Figure 18. Treatment study under hyperlipidemia conditions. (A) Primary hepatocytes were treated with 0.3 mM palmitate for 20 hours, washed and cultured with gAd.NEC, gAd.CPT1A-WT, or gAd.CPT1A-Mu for 4 hours. Cells were treated again with 0.3 mM palmitate for an additional 24 hours. The insulin-sensitive control group received BSA for the entire length of the experiment. Insulin sensitivity was determined by culturing cells with vehicle or 20 nM insulin for 30 minutes, 48 hours post-infection with adenovirus. **(B)** Densitometry analysis of Akt^{Ser473} relative to total Akt. The experiment was repeated using primary hepatocytes from an independent isolation, and similar results were obtained. *P<0.05 relative to insulin-sensitive cells. Statistical analysis done only on insulin treated group.

Discussion

Results from *in vitro* and *in vivo* studies have highlighted the importance of CPT1A in the pathophysiology of NAFLD and insulin resistance. Overexpressing CPT1A in obese animals lowered hepatic lipid levels, and increased the rate of fatty acid oxidation by approximately 36%, compared with β gal-treated rats, suggesting this might be a viable option to treat hepatic steatosis and the underlying insulin resistance [158]. Likewise, Orellana-Gavaldà and colleagues demonstrated that high-fat diet fed mice overexpressing wild type CPT1A or a mutated form of CPT1A (malonyl-CoA insensitive) had decreased lipid accumulation and improved insulin sensitivity [151]. However, Monsénégo and colleagues showed that increasing FAO using a first generation adenovirus expressing malonyl-CoA-insensitive CPT1A, improved insulin signaling but failed to decrease accumulation of triglycerides in the liver of both high-fat diet fed and *ob/ob* mouse models, suggesting a disconnection between insulin resistance and hepatic steatosis.

Here we demonstrated in primary hepatocytes that a similar prevention or treatment of insulin resistance approach does not lead to improvements in insulin sensitivity under hyperinsulinemia or hyperlipidemia conditions. This might be due the limitation of using primary cells, which only allow carrying out experiments for a few days, as cells become quickly senescent. Thus, it is possible that longer expression of CPT1A is needed to overcome the effects of high insulin or high fatty acids on insulin signaling. It is also possible that CPT1A treatment reduced the amount of fatty acid buildup without improving insulin

sensitivity, and future work is needed to address this possibility. In addition, there could be other factors controlling insulin signaling in primary hepatocytes and CPT1A overexpression was not enough to improve insulin sensitivity on its own.

Even though CPT1A overexpression did not have a positive outcome on insulin sensitivity, the system can be used in future studies to address the impact of exposure to hyperinsulinemia or to high glucose/high fatty acids on CPT1A activity, as well as on mitochondrial dysfunction. Furthermore, studies looking at genes involved in mitochondrial biogenesis, FAO, gluconeogenesis, and lipogenesis can also be evaluated. Impaired mitochondrial function plays a pivotal role in the development of hepatic steatosis [159, 160]. Recent findings suggest that mitochondrial dysfunction leads to impaired FAO, raising the idea that NAFLD is actually a result of defective mitochondria, and implying that decreased FAO is merely a consequence [161]. Some of the aspects leading to mitochondria dysfunction could be studied under controlled conditions in primary hepatocytes. Finally, *in vitro* studies to measure rates of FAO can be developed based on oxygen consumption and CO₂ production rates using a Seahorse XF analyzer (Agilent Technologies, MA).

In conclusion, I was able to induce insulin resistance in primary hepatocytes using two different approaches and under two different conditions. In addition, I showed that insulin resistance can occur separately regardless of fatty acid induction or chronic insulin induction and that overexpression of CPT1A alone cannot improve this phenotype.

Chapter 3: Silencing Sterol Regulatory Element Binding Protein-1 (SREBP-1) reduces insulin signaling in mouse liver

Introduction

Sterol Regulatory Element Binding Protein-1 (SREBP-1) is a conserved transcription factor of the basic helix-loop-helix leucine zipper family (bHLH-Zip) that primarily regulates expression of genes of fatty acid metabolism. SREBPs are bound to the endoplasmic reticulum membrane. In response to specific signals, SREBPs transition to the Golgi, where they are cleaved, releasing the mature form, which subsequently translocates to the nucleus [43]. SREBP-1a and SREBP-1c are isoforms of the same gene, and their role as transcription regulators of enzymes in the glycolysis and *de novo* lipogenesis (DNL) pathways, is well established [43]. Additional functions are emerging from recent studies, which have revealed that SREBP-1 regulates a complex gene network to coordinate metabolic responses needed for cell survival and growth, including fatty acid metabolism [43]; glycogen synthesis and gluconeogenesis [147]; phagocytosis and membrane biosynthesis[162]; pro-inflammatory response [163]; and cell proliferation [75]. In addition, SREBP-1 is implicated in the control of the insulin signaling pathway through feedback regulation of key molecules like IRS2 and PIK3R3 [74, 76].

Cell growth is linked to the availability of nutrients and energy to generate new lipids, cell membranes, and nucleic acids. During periods of fasting, low energy and nutrient availability are sensed by a complex network of molecules,

shutting down biosynthetic programs that consume energy. The kinase target of rapamycin (TOR) is a highly conserved protein from yeast to mammals, and plays a key role at orchestrating fundamental aspects of metabolism, cellular growth, proliferation and survival. Two complexes exist, TORC1 and TORC2, with distinct functions. In mammals, mTORC1 regulates ribosome biogenesis and protein synthesis [164], and mTORC2 regulates actin cytoskeleton organization [165, 166]. Unique protein subunits participate in the specific functions of each complex. The major partner of mTORC1 is regulatory-associated protein of mTOR (Raptor) [167, 168], while mTORC2 associates with rapamycin-insensitive companion of mTOR (Rictor) [166]. The 70 kDa ribosomal protein S6 kinase (RPS6KB1 or S6K1) is a serine/threonine kinase that becomes activated in response to nutrients and growth factors. S6K1 lies downstream of the mTORC1 complex, and its activity is enhanced by insulin signaling [104]. The major biological effect resulting from phosphorylation and activation of S6K, is protein synthesis. S6K1 also regulates the insulin signaling pathway by phosphorylating serine residues in IRS1 [169], thereby impairing IRS1 mobility and contact with the insulin receptor for tyrosine phosphorylation [170]. In fact, high nutrient intake has been associated with overactivation of S6K1 in the liver, with subsequent inhibition of insulin signaling [169, 171]. Insulin-induced SREBP-1 transcription activation is mediated by mTORC1 [172-174], and several lines of evidence have shown that mTORC1 is also implicated in the processing from precursor to the mature form [174-176]. Nevertheless, there are conflicting results on whether S6K1 mediates mTORC1 effects on the transcriptional

activation of SREBP-1 [172, 177]. In contrast, there is substantial evidence that S6K1 promotes SREBP-1 processing [175].

Given the role of mTORC1 in promoting protein and lipid biosynthesis needed to support cell growth and survival, and the role of SREBP-1 in sustaining anabolic processes, we questioned whether SREBP-1 regulates the mTOR pathway in a regulatory feedback mechanism. Our data indicates that silencing SREBP-1 has important consequences for S6K1 expression and mTORC1/2 function. In addition, lack of SREBP-1 impacts insulin signaling, underscoring the importance of this transcription factor as coordinator of anabolic processes in response to nutrient availability.

Materials and methods

Animals

All animal studies were in accordance with the National Institutes of Health guidelines and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. Male eight-week old C57BLKS/J and *db/db* mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and allowed to acclimate for at least a week before experimentation. A standard 12 h light/12 h dark cycle (7 AM/7 PM) was maintained throughout the experiments. Mice were maintained in a BSL2-certified room and were fed rodent chow *ad libitum* and allowed free access of water. C57BLKS/J mice were given 1×10^{11} viral particles (vp) by tail vein injection, and euthanized 7 days after adenovirus vector administration under *ad libitum* fed conditions, 24-h fasted or

24-h fasted followed by a 4-h re-feeding period. *db/db* mice were given 1×10^{11} vp, and euthanized 3 weeks later under fed or 24-h fasted conditions. Tissues were collected and snap frozen in liquid nitrogen or fixed in 10% buffered formalin for histology analysis. Male C57BL/6J mice (24 to 30g) were used for isolation of primary hepatocytes.

Helper-dependent adenoviral vector production

The development of helper-dependent adenoviral vectors to target SREBP-1 or expressing a scrambled sequenced has been previously described [178]. Vectors were stored at -80°C in 10 mM Tris-HCl (pH 7.5), 1 mM MgCl_2 , 150 mM NaCl, 10% glycerol. Total particle counts were determined spectrophotometrically, as described [178].

Primary hepatocyte isolation and cell culture

Primary hepatocytes were isolated from C57BL/6J mice using a two-step collagenase procedure followed by Percoll gradient centrifugation, to separate hepatocytes from non-parenchymal cells, as previously described [98]. Cell viability was assessed by trypan blue staining exclusion (>80% viability). Cells were seeded at a density of $4\text{-}6 \times 10^5$ cells per well or 35-mm dish in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin (P/S), 3 nM insulin and 1 nM dexamethasone. Cells were incubated at 37°C , 5% CO_2 in a humidified incubator and allowed to attach for 4 hours. Media was then replaced with fresh media.

For experiments addressing the response to insulin, primary hepatocytes were isolated and infected with gAd.shSCR or gAd.shSREBP-1 adenovirus at MOI 4. Media was changed the next day. Cells were incubated at 37°C for 71 hours and then washed twice with 1xPBS. Cells were cultured in DMEM supplemented with 5 mM glucose and 1% P/S for 1 hour. Cells were then incubated with 20 nM insulin for 5, 15, 30, 45, and 60 minutes. A subset of wells was incubated with 20 nM insulin for 15 minutes, and then insulin was removed. Cells were harvested after 5, 15, 30, and 45 minutes.

Hepa1c1c7 cells (American Type Culture Collection, Manassas, VA) were cultured in MEM- α supplemented with 10% FBS and 1% (v/v) P/S. Cells were seeded at 4×10^5 cells per well.

Cell transfection

Mouse primary hepatocytes, mouse hepatoma cell line (Hepa1c1c7) were transfected with 1-2 μ g plasmids, or 1.5 μ g mouse SREBP1 siRNA or non-targeting control siRNA (Dharmacon, Lafayette, CO). Transfection was performed with Metafectene-Pro (Biontex, Munich, Germany), as described [98]. After overnight incubation, media was replaced with fresh media. Cells were harvest between 48 and 72 hours.

qRT-PCR analysis

Total RNA was isolated from primary hepatocytes (Qiagen). qRT-PCR was carried out in an ABI 7500 instrument (Applied Biosystems, Foster City, CA) using the SYBR Green Qiagen One-Step reverse transcription-PCR kit (Qiagen, Valencia, CA) and the following primer pairs: RPS6KB1, 5'-CTCAGTGGAGGAGAACTATTTA-3' and 5'-CCAGGCTCCACCAGTCCAC-3'; β -actin, 5'-CTACAATGAGCTGCGTGTGGC-3' and 5'-ATGGCTGGGGTGTGAAGGTC-3'. Primer pairs were designed to bind different exons of the gene and to generate fragments of approximately 200 bp. The reaction was first confirmed to yield a single band of the expected size by agarose gel electrophoresis, and lack of amplification in wells containing sample without reverse transcriptase. The relative quantification of the gene was normalized with β -actin.

Western blotting

Primary hepatocytes and hepatocellular carcinoma cell lines were lysed in RIPA buffer (Thermo Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN). Protein concentration was determined using the BCA kit from Pierce (Rockford, IL). Proteins (7-30 μ g) were separated in 10% or 4-20% Tris-HCl SDS PAGE Criterion gel (Bio-Rad, Hercules, CA) and transferred to 0.2-mm PVDF membrane (Bio-Rad). Membranes were blocked with 5% BSA-TBST or 5% dry milk-TBST for 1-2 h and incubated with the following antibodies: Akt^{Ser473}, Akt^{Thr308} and Akt (Pan);

Foxo1^{Ser256} and Foxo1; GSK3 α ^{Ser21} and GSK3 α ; Irs1^{Ser302} and IRS1; mTOR^{Ser2448} and mTOR; Rictor^{Thr1135} and Rictor, Raptor, p70 S6 kinase (S6K), Acetyl-Coa Carboxylase (ACC1/2) (Cell Signaling, Boston, MA), α -tubulin; SREBP1 (Thermo Scientific, Rockford, IL), β -actin (Santa Cruz Biotechnology, Dallas, TX), Cyclophilin-40, (Abcam, Cambridge, MA), IRS2 (EMD Millipore, Billerica, Massachusetts), and IRS1^{Tyr612} (Invitrogen, Life Technologies, Grand Island, NY). HRP-conjugated secondary antibody was added and incubated at room temperature for 1 hour. Blots were developed with Pierce ECL kit (Thermo Scientific) and exposed to enhanced chemiluminescence (ECL) film (GE Healthcare, Piscataway, NJ).

Luciferase constructs and assay

pGL4.10-S6K-WT construct was generated by cloning a portion of the S6K promoter followed by an internal ribosomal entry site (IRES) upstream from the firefly luciferase gene in plasmid pGL4.10 (luc2) (Promega, Madison, WI). IRES was amplified from plasmid pT7CFE1-NHis (Fisher Thermo Scientific, Rockford, IL) using the following oligonucleotides: 5'-TATGCAGATATCGGTTATTTTCCACCATATTG-3' and 5'-AATCAGAGATCTTTTCAAAGGAAAACCGTC-3', and inserted into the EcoRV-BglII sites using Quick ligation kit (New England Biolabs, Ipswich, MA). The S6K1 promoter plus part of the first intron containing two SRE binding sites (positions -589 to +709 from the transcription start site) was amplified from mouse genomic DNA using the following oligonucleotides: 5'-

TATGCAGCTAGCAAAGGAGCAGAGGCTTGTGAA-3' and 5'-AATCAGCTCGCGTCCGCATCTCACCTGCACTAA-3'. The fragment was inserted into the NheI and XhoI sites in pGL4.10(luc2). Mutations of both SRE sites were made using site directed mutagenesis (Agilent Technologies, Quick Change II Site-directed Mutagenesis Kit, Santa Clara, CA) and the following primers: 5'-CTGAGGATGAGCTGGAGGATTTTGTGAGGCCCGGGGTCCC-3' and 5'-GGGACCCCGGGCCTCACAAAATCCTCCAGCTCATCCTCAG-3' for the first SRE site and 5'-CTTGAGTGCGGCCCGGGTGAAAAAGCGTGTGCTGGGGGTGG-3' and 5'-CCACCCCGAGCACACGCTTTTTTTCACCCGGGCCGCACTCAAG-3' for the second site to generate pGL4.10-S6K-Mut1 and pGL4.10-S6K-Mut2 constructs. All clones were sequenced prior to using them in luciferase assays. Low passage 293 cells (Microbix Biosystems, Inc., Ontario, Canada) were cultured in MEM- α supplemented with 10% FBS and 1% P/S. Cells were co-transfected with 225 ng of SREBP-1a, SREBP-1c, or GFP plasmids; 675 ng of pGL4.10-S6K-Wt, pGL4.10-S6K-Mut1, or pGL4.10-S6K-Mut2 plasmids; and 150 ng of pRES-EIF α - β -gal plasmid. Cells were incubated at 37°C in a 5% CO₂ incubator. Cells were harvested 18 hours later and analyzed for luciferase activity using the Luciferase® reporter assay system (Promega) and a luminometer (Centro LB 960 microplate luminometer, Berthold Technologies). β -galactosidase enzyme assay system (Promega) was used to normalize firefly luciferase activity.

Statistical analysis

Numerical values represent mean \pm SD. *P* values were calculated using unpaired two-tailed Student's *t*-tests. A *P* value of less than 0.05 was considered statistically significant.

Results

SREBP-1 regulates S6K1

Data generated from the ENCODE project (<http://genome.ucsc.edu/>) indicates that the promoter of the S6K1 gene is protected from DNase digestion, and the protection extends into the first intron of the gene, suggesting the presence of regulatory proteins in this area. Furthermore, genome-wide ChIP-seq studies have shown that SREBP-1 binds to the S6K1 gene in the human hepatoma cell line HepG2 [179] and in mouse liver [78]. Using TRANSFAC (Waltham, MA) we identified two putative SRE-binding sites in the first intron of the S6K1 gene. Furthermore, this area is highly conserved between mouse and human (Fig. 19A). Both sites are in reverse orientation. To test the functionality of these sites, luciferase reporter constructs were generated containing a fragment from -589 to +709 of the transcription start site (TSS), with or without mutated SRE binding sites (Fig. 19A). HEK293 cells were co-transfected with these plasmids and a plasmid expressing the nuclear form of SREBP-1a or SREBP-1c. Luciferase expression was significantly reduced in cells with the mutated binding sites relative to those with the wild-type sequence (Fig. 19B).

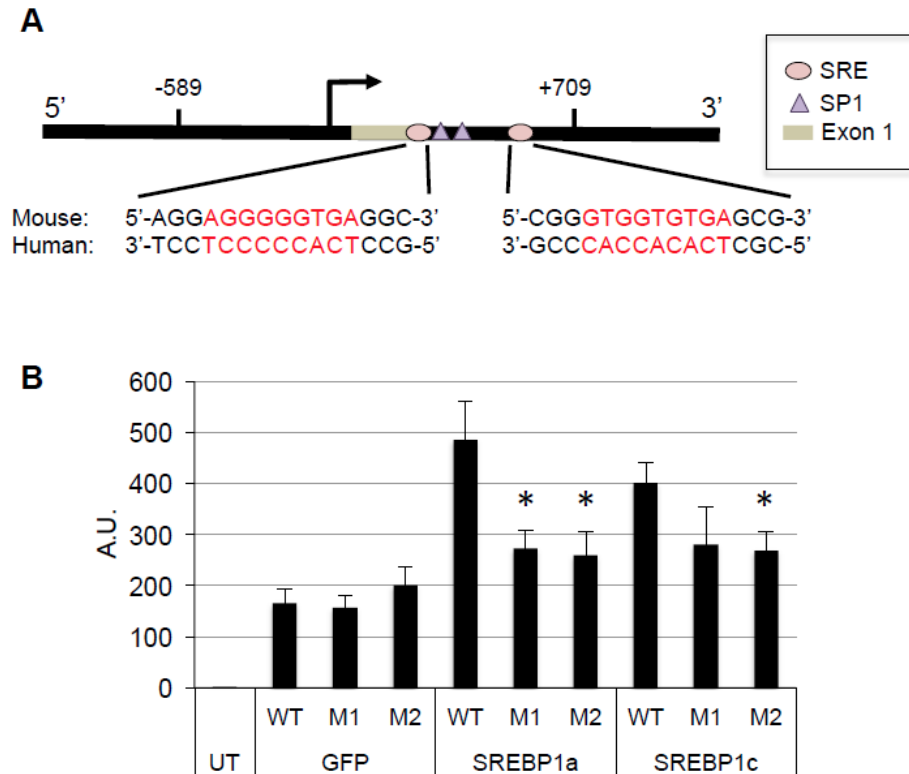


Figure 19. SREBP-1 binds to SRE motifs present in the first intron of the S6K1 promoter. (A) Schematic representation of the portion of the S6K1 promoter cloned with relevant SRE binding sites. **(B)** HEK293 cells were co-transfected with pGL4.10-S6K-WT(WT), pGL4.10-S6K-Mut1 (M1) or pGL4.10-S6K-Mut2 (M2) constructs and SREBP-1a, SREBP-1c, or GFP plasmids. A total of 1 ug of DNA was used. Eighteen hours later, cells were harvested and luciferase was analyzed. * $p < 0.05$; $n = 3$. Abbreviations: UT, untransfected; Sp1, Specificity protein 1; SRE, Sterol regulatory element motif.

To validate these results, primary hepatocytes were depleted of SREBP-1 via adenovirus-mediated shRNA expression or transfection using siRNA. As shown in Fig. 20A and 20B, levels of S6K1 protein and mRNA were significantly lower in primary hepatocytes treated with shSREBP1. Likewise, siRNA against SREBP-1 induced the same effects on S6K1 protein (Fig. 20C). Furthermore, the reduction in S6K1 expression was rescued by overexpression of the nuclear form of human SREBP-1c, which is not targeted by the shRNA against the mouse transcript (Fig. 20D). Similar data were observed using shRNA or siRNA in mouse hepatoma Hepa1c1c cells (data not shown). Overall, these data suggest that S6K1 is a direct target of SREBP-1.

Positive feedback between SREBP-1 and mTORC1

The results in primary hepatocytes suggested that SREBP-1 is needed for regulation of S6K1. We then analyzed the implications of silencing SREBP-1 *in vivo*, on mTOR and the insulin signaling pathway. Depleting SREBP-1 in normal mice resulted in a moderate decrease in S6K1, under fed conditions. It has been previously described that silencing SREBP-1 in hepatocellular carcinoma cell lines leads to a significant decrease in Akt^{Ser473} phosphorylation, while overexpression of SREBP-1 results in increased phosphorylation [115]. Thus, we questioned whether this would be the case in normal liver. Interestingly, a moderate decrease in IRS1^{Y612}, Akt^{Ser473} and GSK3 α ^{Ser21} phosphorylation was observed, under fast and re-fed conditions, suggesting lower insulin sensitivity (Fig. 21).

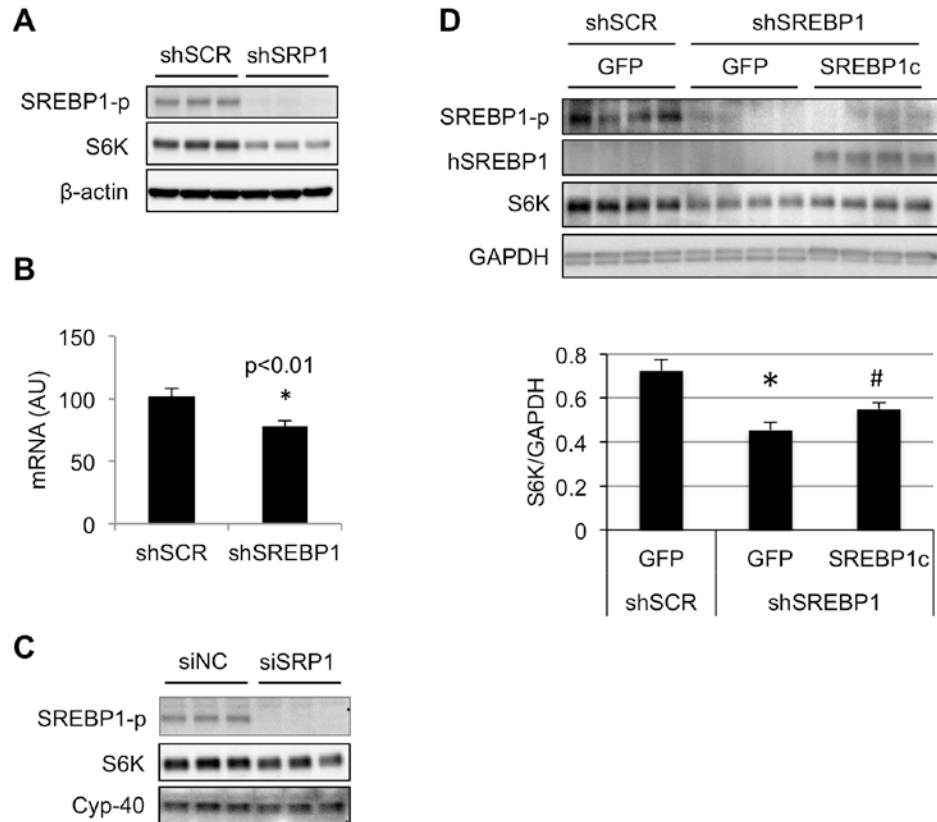


Figure 20. Silencing SREBP-1 downregulates S6K1 expression. **(A)** Primary hepatocytes were infected with gAd.shSCR (shSCR) or gAd.shSREBP-1 (SRP1) at MOI 3 for 72 hours, and protein lysates were generated for WB analysis **(B)** Primary hepatocytes were infected with gAd.shSCR or gAd.shSREBP-1 at MOI 3 for 72 hours. RNA lysates were generated and qRT-PCR for S6K1 was performed. **(C)** Primary hepatocytes were transfected with 1.5 ug non-targeting control siRNA (siNC) or siSREBP-1 (siSRP1). Cells were harvested 72 hours later, and protein lysates were generated. **(D)** Primary hepatocytes were infected with gAd.shSCR or gAd.shSREBP-1 at MOI 3, 4 hours post plating. Medium was changed the next day and cells were transfected with SREBP-1c or GFP plasmids (1.5 ug). Cells were harvested 72 hours post-infection.

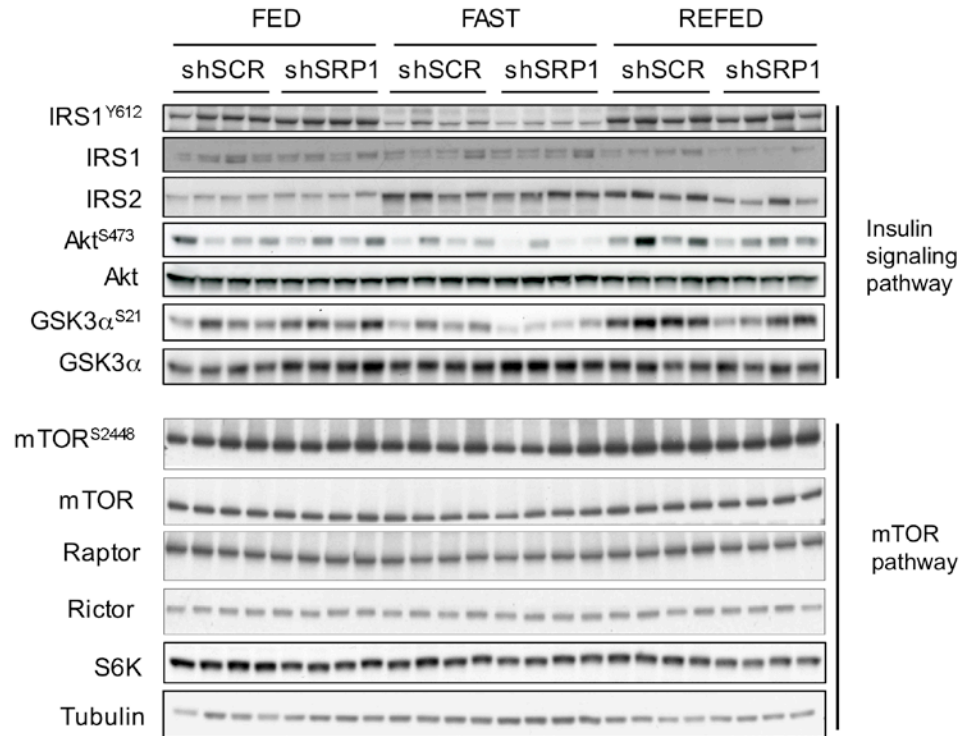


Figure 21. Silencing SREBP-1 downregulates S6K1 expression in normal mice, and reduces insulin signaling. Groups of five C57BKS mice were given 1×10^{11} vp gAd.shSCR or gAd.shSREBP1 and euthanized one week later under fed, 24-hour fasted, and 24-hour fasted followed by 5 hour re-feeding conditions. Protein lysates were generated from liver samples. Abbreviations: shSCR, gAd.shSCR; SRP1, gAd.shSREBP-1.

Although the mechanisms by which the decrease occurs are unknown, it is possible that SREBP-1 silencing triggers an attenuated response to insulin signaling due to changes in the composition of lipid rafts [180, 181]. The PI3K-Akt-mTORC1 axis is important to determine appropriate levels of fatty acids and cholesterol and for the integrity of lipid rafts [182].

SREBP-1 activity is higher in diet-induced obesity, *db/db* [183] and *ob/ob* mice [184], underscoring the role of this transcription factor in the etiology of hepatic insulin resistance. Similarly, increased S6K1 activity has also been reported in diet-induced obesity and insulin resistance [171]. Remarkably, silencing SREBP-1 in *db/db* mice resulted in significantly reduced S6K1 protein levels under fed and fasted conditions (Fig. 22A and 23), suggesting SREBP-1 is needed to maintain the high levels of S6K1 in this animal model. Depleting SREBP-1 did not induce changes in mTOR levels, although its activity was lower than in control animals, based on phosphorylation at Ser2448 [185]. Despite no alterations in total levels of mTOR, a moderate increase in Rictor and a decrease in Raptor were also observed (Fig. 22A), suggesting that mTORC1 activity was reduced while mTORC2 was more active. Furthermore, AMPK, a known mTORC1 inhibitor, was found activated under fed conditions (Fig. 22C). Altogether, these data suggest that silencing SREBP-1 in *db/db* mice leads to an increase in mTORC2 activity while decreasing mTORC1 activity.

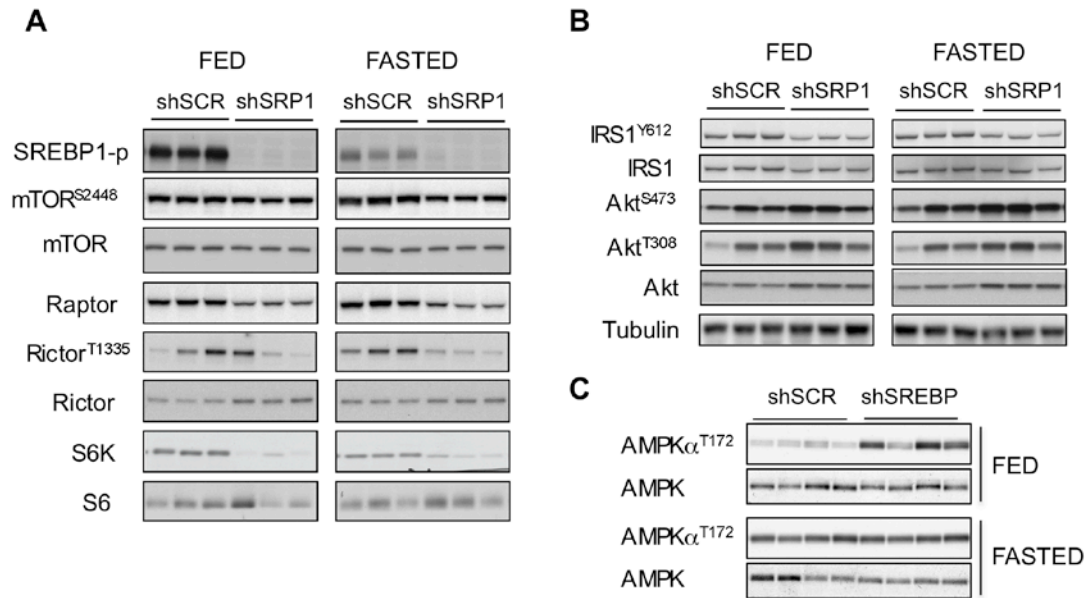


Figure 22. Silencing SREBP-1 downregulates S6K1 expression in *db/db* mice. Groups of eight *db/db* mice were given 1×10^{11} vp gAd.shSCR or gAd.shSREBP1 through the tail vein, and euthanized three weeks later under fed (*ad libitum*) or 24-hour fasted conditions. Protein lysates were generated from liver samples. **(A)** Effects of silencing SREBP-1 on molecules in the mTOR pathway. **(B)** Effect of silencing SREBP-1 on molecules in the insulin signaling pathway. **(C)** Under fed conditions, silencing SREBP-1 activates AMPK, a molecule involved in cellular energy homeostasis.

Silencing SREBP-1 in *db/db* animals resulted in increased Akt^{S473} phosphorylation under fed conditions (Fig. 22B). Furthermore, in an insulin sensitivity test, fasting mice for 5 hours was not sufficient to reduce Akt^{S473} to basal levels (Fig. 23), suggesting a defect in the inhibition of insulin signaling. Insulin administration did not further increase phosphorylation of Akt^{S473}. mTORC1 negatively regulates the response to insulin and insulin-like growth factor 1 by inhibiting mTORC2, and this effect is mediated by S6K1 [39, 186]. By phosphorylating Rictor, S6K1 inhibits mTORC2, preventing phosphorylation of Akt at Ser473 [39, 186]. Thus, we questioned whether the decrease in S6K1 would be associated with a decrease in phosphorylation of Rictor^{Thr1135} and as a result, Akt^{Ser473} phosphorylation would be increased. As shown in Fig. 22 and 23, Rictor^{Thr1135} was lower under fasting conditions. Thus, inhibiting SREBP-1 reduces S6K1, which possibly fails to inhibit mTORC2, resulting in enhanced Akt^{Ser473} phosphorylation. No significant differences on insulin signaling were observed in adipose tissue or skeletal muscle, as expected (Fig. 24).

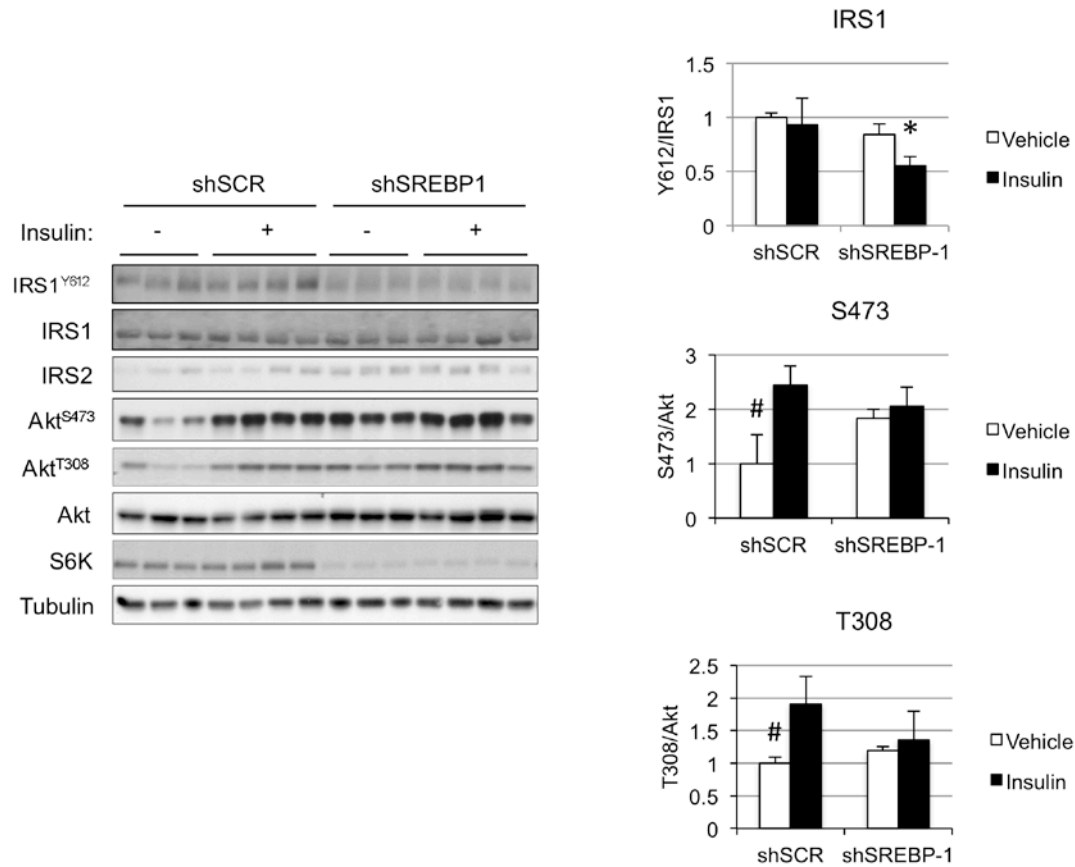


Figure 23. Insulin sensitivity analysis in *db/db* mice (liver). Groups of seven *db/db* mice were given 1×10^{11} vp gAd.shSCR or gAd.shSREBP1 through the tail vein. Three weeks later, mice were fasted for 5 hours and given vehicle or insulin via portal vein. Mice were euthanized 5 minutes later. Liver was flash frozen and protein lysates were generated. The densitometry analysis of IRS1^{Y612}, Akt^{Ser473} and Akt^{Thr308} relative to total Akt levels, is shown on the right. * $p < 0.05$ between shSCR and shSREBP groups; # $p < 0.05$ between vehicle and insulin-treated.

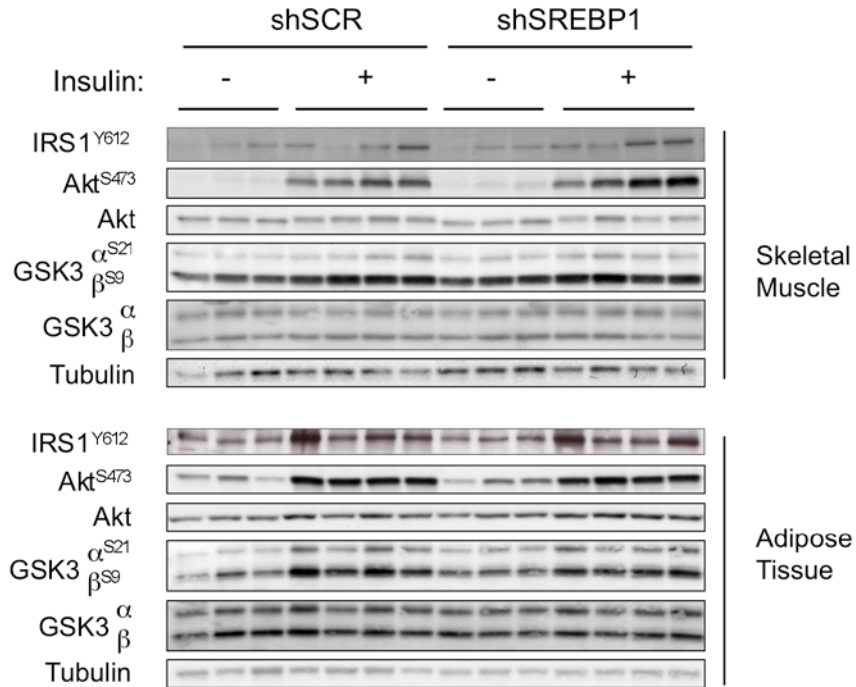


Figure 24. Insulin sensitivity analysis in *db/db* mice (skeletal muscle and adipose tissue). Groups of seven *db/db* mice were given 1×10^{11} vp gAd.shSCR or gAd.shSREBP1 through the tail vein. Three weeks later, mice were fasted for 5 hours and given vehicle or insulin via portal vein. Mice were euthanized 5 minutes later. Skeletal muscle and adipose tissue was flash-frozen and protein lysates were generated.

Insulin signaling kinetics

The increase in Akt^{S473} under hyperinsulinemic conditions (*db/db* mice), suggested a defect in inhibition of insulin signaling. To study this in more detail, we analyzed the response of hepatocytes to insulin in a time course experiment. Primary hepatocytes were cultured with 20 nM insulin and harvested 5, 15, 30, 46 and 60 minutes later. In SREBP-1 depleted cells, S6K1 and S6 phosphorylation were significantly lower, as expected from the lower levels of S6K1 (Fig. 25). However, Akt^{Ser473} phosphorylation was significantly higher at all time points. We then analyzed whether the response to insulin was shutdown with a similar kinetics. Primary hepatocytes were cultured with 20 nM insulin for 15 minutes; insulin was removed and cells were harvested at several time points. Although insulin signaling was shutdown with a similar kinetics, the signal remained higher in cells depleted of SREBP-1 relative to control cells (Fig. 25). These data suggest that insulin signaling may not be appropriately inhibited in the absence of SREBP-1, and lack of inhibition leads to increased Akt^{Ser473} phosphorylation.

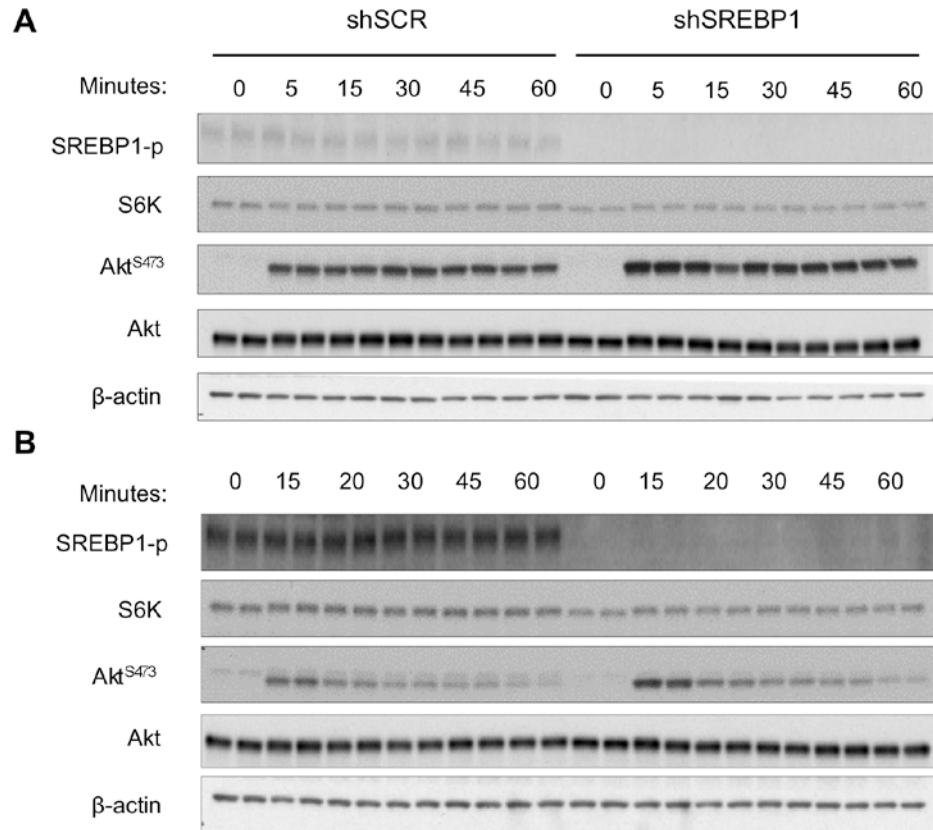


Figure 25. Insulin kinetics in primary hepatocytes. (A) Primary hepatocytes were transduced with gAd.shSCR or gAd.shSREBP-1 at MOI 3. Seventy-two hours post infection, cells were fasted for 1 hour and cultured with 20 nM insulin. Cells were harvested at the time points shown on the top. **(B)** Primary hepatocytes were transduced with gAd.shSCR or gAd.shSREBP-1 at MOI 3. Seventy-two hours post infection, cells were fasted for 1 hour and cultured with 20 nM insulin for 15 minutes. After removal of insulin, cells were harvested at the time points shown on the top.

Discussion

In the present study, we have shown that a positive feedback mechanism exists between SREBP-1 and mTORC1 through direct regulation of S6K1. The SREBP-1 binding sites are located in the first intron of the gene in a reverse orientation, in an area highly conserved between mouse and human, and this enhancer is likely to contribute to the overall level of expression of the gene. The SRE sites are located adjacent to SP1 binding sites, which are frequently found in promoters regulated by SREBP-1. There is a precedent for SREBP-1 binding to an enhancer located in an intron [77]. Caspase-2 has been shown to regulate lipid synthesis and its transcription is activated by SREBPs [187]. SREBP-1c, in particular, binds to an E-box in an enhancer located in the first intron [77]. Unlike the S6K1 gene, this site is not conserved across species, and is only present in human cells [77]. Thus, S6K1 exemplifies a unique case of an enhancer located in a highly conserved intronic region.

Functionally, the activation of S6K1 denotes a coordinated action between SREBP-1 and mTORC1 function. Several studies have demonstrated that mTORC1 mediates the response of insulin on activation of SREBP-1 and lipogenesis, as blocking mTORC1 activity with rapamycin inhibits SREBP-1 expression [172, 176, 177]. In addition, both mTORC1 and SREBP-1 activity are enhanced in liver of animal models of obesity and type 2 diabetes [171, 184, 188]. Furthermore, mice with liver-specific Raptor deficiency are protected against non-alcoholic fatty liver disease (NAFLD) [173]. However, it remains unclear whether S6K1 plays a role as regulator of SREBP-1. On one side,

inhibiting S6K activity with LYS6K2 does not affect the induction of SREBP-1 expression in response to insulin, suggesting another molecule downstream from mTORC1 induces this effect [172]. On the other, knocking down S6K1 in immortalized hepatocytes or in mouse liver, severely blunts SREBP-1 expression and triglyceride accumulation [177]. Regardless of whether S6K1 or a different mTORC1 downstream effector is implicated in the regulation of SREBP-1, our data suggest that SREBP-1 regulates S6K1 in a positive feedback mechanism to enhance protein synthesis. Thus, SREBP-1 facilitates multiple anabolic processes relevant to the fed state, including lipogenesis, protein synthesis, and glycogen synthesis.

The mTOR pathway functions as the hub for sensing nutrient abundance and changes in energy supplies [189]. Protein kinase B (Akt) is a critical signaling molecule that regulates a variety of cellular functions, including metabolism, survival, growth and proliferation [190]. The Akt/mTORC1 axis is deregulated in many cancers, and it has emerged as a critical regulatory point in the control of cell growth and cellular proliferation. Indeed, Akt is hyperactivated in many cancers [191-193], and mTORC1 has been used as a target to treat a variety of tumors [194, 195]. Enhanced mTORC1 activity in these tumors is associated with increased expression of SREBP-1, and emerging evidence is showing that lipogenesis is strongly associated with poor survival [115, 196]. Hence, SREBP-1 is receiving a lot of attention as a potential oncogene, as multiple studies have emphasized the association between its activity and cell growth and proliferation [196]. In fact, overexpression of SREBP-1 in HCC cell lines with low levels of

SREBP-1, accelerated their growth and reduced apoptosis. Remarkably, this was accompanied by increased levels of phosphorylated Akt^{Ser473} [115]. Conversely, in HCC cell lines expressing high SREBP-1 levels, knocking down SREBP-1 or any of its downstream effectors, reduced Akt^{Ser473} activity [115]. These data emphasize the connection between Akt/mTORC1/SREBP-1, to coordinate lipogenesis and cellular growth in liver. In agreement with these data, our results show that in normal mice, SREBP-1 deficiency is associated with lower IRS1^{Y612} and Akt^{Ser473, Thr308} (fasting conditions), concurring with previous reports in liver cell lines [115]. Although the mechanism leading to this reduction is yet to be elucidated, it is possible that is attributable to alterations in the composition of lipid rafts [181].

Previous studies have shown that mTORC1 is a negative regulator of mTORC2, and this effect is mediated by S6K1 [39, 186]. Thus, S6K1 negatively regulates the insulin signaling pathway by phosphorylating Rictor, thereby disrupting mTORC2 activity and precluding phosphorylation of Akt^{Ser473} [39, 186]. Our data indicates that *in vivo*, this negative regulation may be important when insulin levels are high, as occurs in *db/db* animals. In fact, the decrease in S6K1 resulting from silencing SREBP-1 in *db/db* mice, was associated with significantly higher Akt^{Ser473} under fed conditions. In an insulin sensitivity assay, fasting mice for 5 hours did not reduce Akt^{Ser473} to basal levels. Fasting for 24 hours was needed to reduce Akt^{Ser473} to basal levels, as seen in control *db/db* mice. This is consistent with the notion that insulin signaling is not appropriately shutdown, as IRS1^{Y612} remained lower relative to control animals, discarding the possibility of

increased insulin sensitivity. The observation that Rictor^{Thr1135} phosphorylation was lower in *db/db* mice lacking SREBP-1, suggests a possible mechanism for lack of inhibition of insulin signaling. Further studies are needed to investigate the detailed mechanisms through which this inhibitory activity takes place.

In conclusion, our data indicates that SREBP-1 binds to an enhancer in the first intron of the S6K1 gene, and is likely to contribute to the overall expression levels of this gene. *In vivo*, silencing SREBP-1 has significant implications for insulin signaling. In normal mice, reduced Akt^{Ser473} is observed, in particular under fasting conditions, and no effects are seen during the fed state. However, under hyperinsulinemic conditions, SREBP-1 depletion leads to significant changes in Akt^{Ser473}, and our data suggest that this is the result of lack of inhibition rather than enhanced sensitivity to insulin. Our data provides evidence that SREBP-1 coordinates multiple metabolic pathways by not only activating lipogenesis, but also promoting protein synthesis, and thus, it coordinates multiple aspects of the anabolic response in response to nutrient abundance.

CONCLUSIONS AND FUTURE DIRECTIONS

The non-alcoholic fatty liver disease (NAFLD) epidemic has been estimated to affect 75-100 million people in the United States, and continues to be the leading cause of liver diseases in the world [107]. In addition, non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC), affecting 5% and 0.006% of the US general population, respectively, are also becoming a concern [106, 107]. The link between the mTOR pathway and insulin signaling in relation to the pathophysiology of the aforementioned diseases has been broadly studied. The transcription factor SREBP-1, a downstream effector of insulin signaling through mTORC1, is up-regulated in these metabolic diseases, activating the *de novo* lipogenesis and glycolysis pathways. However, its role outside of being a contributing factor to hepatic lipid accumulation and insulin resistance, was not well known. Therefore we sought to explore its role in other pathways such as carbohydrate metabolism, insulin signaling, and mTOR.

We proposed to silence SREBP-1 in *db/db* mice, a diabetes mouse model, which exhibits hyperinsulinemia, hyperglycemia, and insulin resistance, as well as β -cell dysfunction [197], to examine the impact on carbohydrate metabolism. In normal animals, SREBP-1 depletion increased *Pck1* and reduced glycogen deposition under fed conditions, providing evidence that SREBP-1 is necessary to regulate carbohydrate metabolism during the fed state. As expected, knocking SREBP-1 down in *db/db* mice resulted in a significant reduction in *de novo* lipogenesis and triglyceride accumulation. However, mice remained

hyperglycemic, and no improvements in glucose or insulin tolerance were seen. As observed in normal mice, silencing SREBP-1 in *db/db* animals resulted in up-regulation of gluconeogenesis gene expression as well as decreased glycolysis and glycogen synthesis gene expression. Furthermore, glycogen synthase activity and glycogen accumulation were significantly reduced, suggesting SREBP-1 is needed under fed conditions to promote glycogen synthesis. Thus, SREBP-1 depletion leads to significant changes in carbohydrate metabolism and suggests that SREBP-1 plays additional roles in metabolism besides controlling lipogenesis. Future studies will be needed to further evaluate whether the alterations in mRNA/protein levels observed lead to increased hepatic glucose output, using pyruvate tolerance tests or metabolic tracers *in vitro* and/or *in vivo*.

Another outcome of these studies was the observation that FAO did not increase upon silencing SREBP-1, as we had predicted. This led to the notion that perhaps in this animal model mitochondria are not healthy to sustain additional FAO, or that CPT1A activity may be limiting. Individuals with obesity and type 2 diabetes exhibit hepatic lipid accumulation, which contributes to the induction of insulin resistance and impaired insulin signaling [24]. As mentioned previously, chronic exposure to insulin enhances the accumulation of lipids in the liver by stimulating *de novo* lipogenesis, thus inhibiting fatty acid oxidation (FAO). This inhibition is mediated by malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase 1A (CPT1A) [156]. Since CPT1A is a rate-limiting enzyme in the oxidation of fatty acids in mitochondria, increasing its activity has been devised as a therapeutic approach to decrease hepatic steatosis and improve

insulin sensitivity. In fact, increasing the levels of CPT1A has resulted in attenuated insulin resistance and intracellular lipid regulation in several studies [150-152, 198]. To determine if insulin resistance could be improved by overexpression of CPT1A in a controlled environment, we used adenoviral vectors expressing wild type or malonyl-CoA resistant CPT1A (gAd.CPT1A-WT, or gAd.CPT1A-Mu) in primary hepatocytes. Hepatocytes were treated under either hyperinsulinemic or hyperlipidemic environments, and the effects of overexpressing CPT1A as a mechanism to reduce steatosis and improve insulin sensitivity were evaluated. Our results showed that overexpressing CPT1A did not prevent the insulin resistance due to hyperinsulinemia or hyperlipidemia, regardless of when insulin resistance was established, i.e. before or after overexpression of CPT1A. These data indicate that the effects observed *in vivo* may require expression of CPT1A for longer time than what is possible in primary cultures. Despite the lack of efficacy at improving insulin resistance, these studies have enabled us to establish a system to induce insulin resistance using lipids and/or insulin for *in vitro* experiments in primary hepatocytes. This system allows more control of the cellular environment to perform studies that complement the data generated from *in vivo* experiments, and we used it for studying insulin signaling in relation to SREBP-1 deficiency.

Studies using genome-wide chromatin immunoprecipitation followed by sequencing (ChIP-seq) have shown that SREBP-1 binds to the promoter of S6K1 in mouse liver as well as in human hepatocellular carcinoma HepG2 cells [78, 179]. mTORC1 regulates SREBP-1 and S6K [101]. Here we have provided

evidence that SREBP-1 may regulate S6K1, in a positive feedback loop. We demonstrated that in *db/db* mice, depletion of SREBP-1 resulted in a significant decrease in S6K under fed and fasted conditions. In addition, protein and mRNA levels were decreased in primary hepatocytes treated with shSREBP-1 adenovirus compared to the control group. Thus, decreasing SREBP-1 had a significant effect on S6K expression. Furthermore, the drop of S6K was rescued by expression of constitutively active SREBP-1c. In addition, using luciferase reporter assays we demonstrated that the two SRE binding sites located in the first intron of the gene, are functional SREBP-1 binding motifs. Mutation of both SRE sites resulted in a significant decrease in luciferase expression. These two sites are conserved in mouse and human. Altogether, these data suggest that SREBP-1 directly regulates S6K. Future studies should include ChIP assays to confirm that SREBP-1 associates with the SRE binding sites in the S6K1 gene, as well as perform experiments in the HepG2 cell line to confirm that these conserved sites are also functional in humans.

S6K has also been shown to phosphorylate multiple serine residues on IRS1 [199]. Phosphorylation of IRS1 on these residues inhibits its interaction with the insulin receptor, leading to decreased phosphorylation of tyrosine's and reduced downstream signaling [200]. Our data showed that the SREBP-1 depleted *db/db* mice had lower levels of IRS1^{Y612}, suggesting other factors impede insulin signaling separate from S6K1 levels.

The mTOR pathway and the insulin signaling pathway are interconnected to decide the cellular fate (survival, growth, proliferation, apoptosis), based on

nutrient availability [101]. Our results have shown that knocking down SREBP-1, a key transcription factor during conditions of nutrient abundance, affects the mTOR and insulin signaling pathways. *Db/db* mice treated with shSREBP-1 adenovirus showed increased levels of basal Akt^{Ser473}, compared to the control group, shSCR. The absence of SREBP-1 and the subsequent drop in glycolysis and ATP synthesis during fed conditions, may have been sensed as a drop in energy availability, thus inhibiting mTORC1 and activating mTORC2, resulting in increased Akt^{Ser473}. S6K is one of the multiple molecules that phosphorylate Rictor, a subunit of the mTORC2 complex [201]. Phosphorylation at the Thr1135 residue leads to weakened activity of mTORC2. Given that mTORC2 is responsible for phosphorylating Akt at Ser473, it is conceivable that reduced S6K1 would lead to lack of Rictor phosphorylation, thereby increasing mTORC2 activity and Akt^{Ser473} phosphorylation. Our data showed that silencing SREBP-1 in *db/db* mice increased Rictor protein levels, and decreased phosphorylation of Rictor^{Thr1135}. This may provide a mechanism by which lower S6K1 may have caused upregulation in Akt^{Ser473} phosphorylation. Thus, by regulating S6K1, SREBP-1 may indirectly control the insulin signaling pathway (Fig. 26). Our model predicts that silencing SREBP-1 leads to decreased insulin signaling, and hence, lower Akt^{Ser473} phosphorylation. However in a hyperinsulinemic state, the depletion of SREBP-1 results in improper upregulation of Akt^{Ser473}, due to the lack of mTORC2 inhibition.

The glycolysis/lipogenesis pathway is enhanced and contributes 26% of triglycerides in liver of NAFLD patients, relative to only 5% in normal individuals

[36]. In *ob/ob* and *db/db* mice, it is also enhanced approximately 10-fold [39]. Thus, reducing this pathway in *db/db* mice activated AMPK, which in turn, inhibited mTORC1, and increased mTORC2 activity. Thus, the increase in Akt^{Ser473} is most likely not due to increased insulin signaling, but an inappropriate response to the inhibition of insulin action. Moreover, this would suggest that a certain amount of SREBP-1 may be needed in insulin resistant states, and complete knock down of SREBP-1 is only beneficial in reducing hepatic steatosis.

Studies have shown that SREBP-1 is needed for cancer cell survival. This is due to the need for lipid and protein homeostasis to sustain growth [110]. Decreasing SREBP-1 led to stress and cell death, as well as ROS production and inhibition of protein synthesis [110]. More specifically, human hepatocellular carcinoma has been shown to be associated with increased lipogenic gene expression as well as increased Akt levels, correlating with a worst prognosis [115]. Thus, SREBP-1 depletion would be a valuable therapeutic tool in treating HCC and other cancers.

Overall, this work highlights the notion that SREBP-1 regulates other pathways besides its known role in fatty acid synthesis (Fig. 27). The data generated from this thesis has provided evidence that SREBP-1 regulates carbohydrate metabolism, and most likely protein synthesis, by activating S6K1. Moreover, we show that SREBP-1 is needed for proper insulin signaling. Thus, its depletion does not result in improved glucose control in obese *db/db* animals. Nevertheless, it is possible that partial depletion has therapeutic value, and

results in lower lipid accumulation while preventing the negative effects on carbohydrate metabolism associated with its complete depletion. Our data also provide a mechanism by which reducing SREBP-1 in cancer cell lines reduces cell viability, as these cells require protein production for sustained growth and proliferation. Hence, SREBP-1 could be an appropriate target for treatment of a variety of tumors.

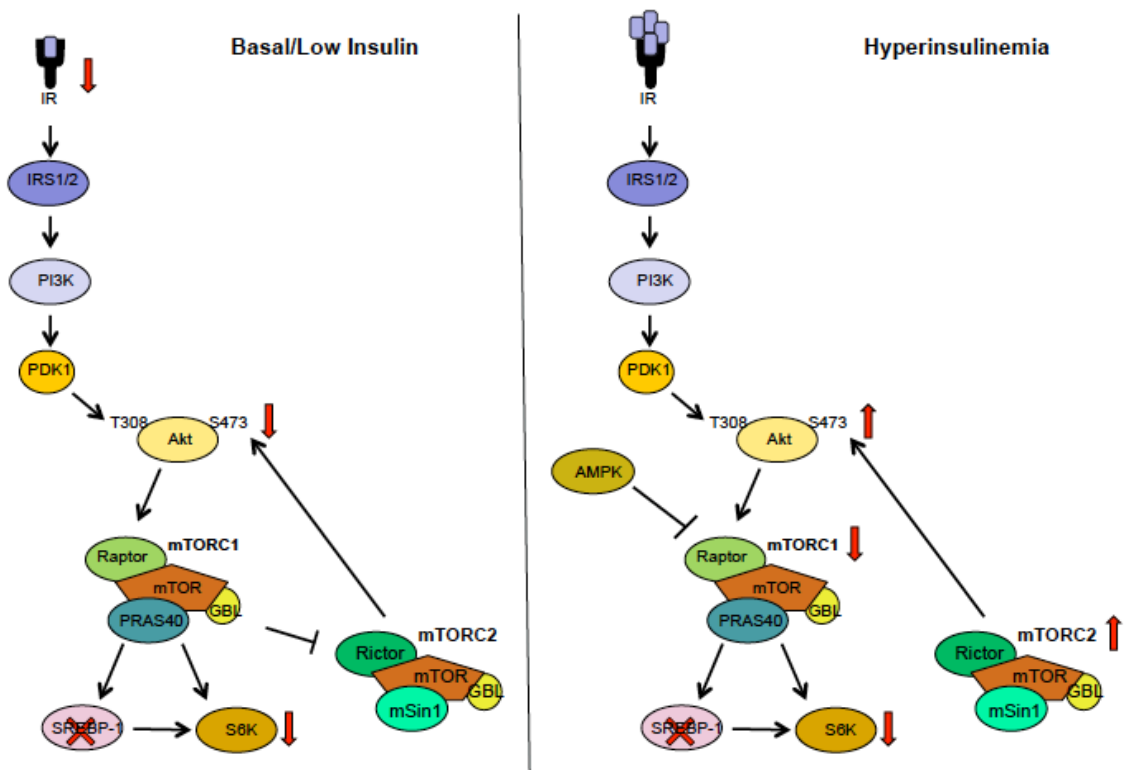


Figure 26. Effects of silencing SREBP-1 on the insulin and mTOR pathways. Under basal conditions, silencing SREBP-1 leads to a decrease in insulin signaling and Akt^{Ser473}. Under chronic insulin conditions, the lack of SREBP-1 decreases glycolysis and lipogenesis, activating AMPK, which negatively regulates mTORC1. The drop in S6K1 results in lack of mTORC2 inhibition, increasing Akt^{Ser473} phosphorylation.

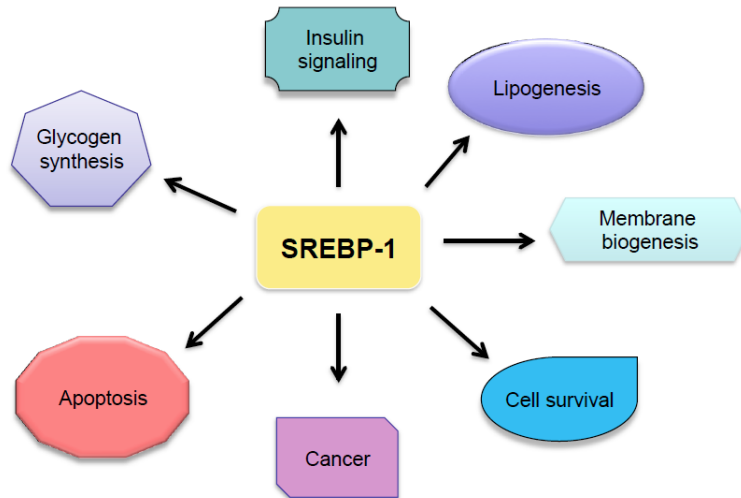


Figure 27. Central role of SREBP-1. This work, as well as other studies, have highlighted the various roles SREBP-1 plays in regulating metabolic processes as well as cell proliferation/growth and cancer stability.

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CURRICULUM VITAE

Victoria N. Jideonwo

EDUCATION

- 2010-2016 **Indiana University**, Indianapolis, IN
Ph.D. Medical and Molecular Genetics (2016)
Thesis: “Novel roles of sterol regulatory element-binding protein-1 in liver”
Advisor: Núria Morral, Ph.D.
Minor: Life Sciences
- 2003-2007 **Indiana University**, Bloomington, IN
Bachelor of Science in Biology, (2007)
Minors: Chemistry, African American Studies

HONORS, AWARDS, FELLOWSHIPS

- 2015-2016 President’s Diversity Dissertation Fellowship Award Recipient
- 2015 Award winner at Center for Diabetes and Metabolic Diseases Symposium
- 2012-2013 R01-DK 07859-05S1 Research supplements to promote Diversity in Health-Related Research
- 2006-2007 Louis-Stokes Alliance Minority (LSAMP) Scholars Award Recipient
- 2006 First Place Poster Presentation at Alliance for Graduate Education and the Professoriate (AGEP) Conference
- 2005-2006 NIH- Initiative for Maximizing Student Diversity (IMSD)

	Scholars Award Recipient
2003-2007	O'Bannon Honors Grant Recipient
2003-2007	Pell Grant Recipient
2003-2007	National Academic Scholarship Recipient

GRADUATE AND UNDERGRADUATE EXPERIENCE

2011-2016	<p>Ph.D. Candidate, Núria Morral Lab, Indiana University School of Medicine, Indianapolis, IN</p> <ul style="list-style-type: none"> • Studying the role of SREBP-1 and its effects on metabolic pathways in the liver • Studying the role of CPT1A and its effects on diabetes and obesity as well as liver metabolism • Skilled in performing transfections in primary hepatocytes • Responsible for primer design and sub-cloning procedures of constructs • Proficient in DNA purification, mitochondria isolation, and immunoblotting techniques • Performed luciferase assay and analysis • Performed RT-qpcr assay and analysis • Mentored undergraduates and medical students in summer research programs
2011	<p>Ph.D. Rotation Student, Kenneth White Lab, Indiana University School of Medicine, Indianapolis, IN</p> <ul style="list-style-type: none"> • Studied the role and effects of HIF1-alpha and FGF23 interaction in Autosomal dominant hypophosphatemia rickets • Performed western blotting procedures and analysis. • Performed immunohistochemistry procedures • Performed qRT-PCR procedures and analysis • Responsible for care of UMR-106 cells
2010	<p>Ph.D. Rotation Student, Edward Srour Lab, Indiana University School of Medicine, Indianapolis, IN</p> <ul style="list-style-type: none"> • Studied balancing effects of osteogenesis and adipogenesis on hematopoietic stem cells • Performed Quantitative-RT-PCR analysis • Performed Oil Red O staining procedure and analysis • Performed Flow cytometry phenotyping analysis

- Performed CFU Assays
- Responsible for harvesting and care of stromal cells

2008-2010

Biological Research Associate, Michael Wade Lab,
Indiana University, Bloomington, IN

- Studied complex genetic traits involved in speciation in *Tribolium Castaneum* as well as deleterious hybrid disadvantage phenotypes in offspring
- Skilled in performing microsatellite analysis and molecular diagnosis of parental and hybrid offspring amid inter-population crosses
- Familiar with genotyping and QTL-mapping of genes in whole organism
- Proficient in various molecular techniques such as gene amplification, DNA purification, RNA purification, sub-cloning, Restriction digests, Reverse Transcriptase PCR, genotyping, Q-PCR, and DNA sequencing
- Responsible for ordering laboratory reagents and equipment, making stock solutions and buffers, instructing and supervising new laboratory personnel

2007-2008

Biological Research Technician, Viola Ellison Lab,
Indiana University, Bloomington, IN

- Studied Eukaryotic genes involved in DNA replication and repair which may be associated with cancerous pathways and cancer development, particularly, human gene Ctf18
- Proficient with molecular biology and biochemistry techniques such as PCR, western blotting, immunofluorescence, antibody purification, protein purification, siRNA transfections, DNA transfections, gene expression assays, immuno-precipitation, mitotic shake off, DNA purification, and, transformations
- Experienced in maintaining human cell lines, and administering various drug treatments on cells
- Responsible for ordering laboratory reagents, making stock solutions, and instructing and supervising/mentoring new laboratory personnel

2006-2007

Undergraduate Biology Research Assistant, NIH-IMSD
Scholarship Viola Ellison Lab, Indiana University,
Bloomington, IN

- Work primarily focused on sub-cloning and making GFP variant construct of human gene Ctf4.
- Performed western blotting, transformations, and gene amplification (PCR)

Summer 2006	Undergraduate Researcher, Louis-Stokes Alliance Minority Participant (LSAMP) Scholarship Viola Ellison Lab, Indiana University, Bloomington, IN
	<ul style="list-style-type: none"> • Work primarily focused on sub-cloning, PCR amplification, immunoblotting, transformations, DNA transfections via lipofectamine into Hela cells
Summer 2005	Undergraduate Researcher, NIH-IMSD Scholarship Viola Ellison Lab, Indiana University, Bloomington, IN
	<ul style="list-style-type: none"> • Work primarily focused on sub-cloning and PCR • Performed in vitro transcription/translation assay • Performed protein induction for gene expression of A1U and purification of A1U
2005-2007	Secretary for University Coalitions for Global Health
2003-2007	Biology Club Member
2008	Middle Way House Volunteer

PUBLICATIONS

1. **Jideonwo, V.**, Ruiz, R., Surendran, S., Ahn, M., Morral, N. SREBP-1 regulates the mTOR pathway through transcriptional activation of S6K. In preparation.
2. Surendran S., **Jideonwo, V.**, Merchun, C., Ahn, M., Murray, J., Ryan, J., Dunn, K., Kota, J., Morral, N. Gene targets of mouse miR-709: regulation a distinct pools. Scientific Reports 6: 1-10 (2016)
3. Ruiz, R*, **Jideonwo, V***, Ahn, M., Surendran, S., Tagliabracci, VS., Hou, Y., Gamble, A., Kerner, J., Irimia-Dominguez, JM., Puchowicz, MA., DePaoli-Roach, A., Hoppel, C., Roach, P., Morral, N. Sterol Regulatory Element Binding Protein-1 (SREBP-1) is required to regulate glycogen synthesis and gluconeogenic gene expression in mouse liver. Journal of Biological Chemistry 289:5510-5517 (2014). *Equally contributed.

4. Drury DW, Ehmke RC, **Jideonwo VN**, Wade MJ. Developmental trajectories and breakdown in F1 interpopulation hybrids of *Tribolium castaneum*. *Ecology and evolution* 3(7):1992-2001 (2013).
5. Farrow EG, Yu X, Summers LJ, Davis SI, Fleet JC, Allen MR, Robling AG, Stayrook KR, **Jideonwo V**, Magers MJ, Garringer HJ, Vidal R, Chan RJ, Goodwin CB, Hui SL, Peacock M, White KE. Iron deficiency drives an autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (FGf23) knock-in mice. *Proceedings of the National Academy of Sciences of the USA* 108(46):E1146-1155 (2011).
6. Drury DW, **Jideonwo VN**, Ehmke RC, Wade MJ.. An unusual barrier to gene flow: perpetually immature larvae from inter-population crosses in the flour beetle, *Tribolium castaneum*. *Journal of evolutionary biology* 24 (12):2678-2686 (2011).

PRESENTATIONS

1. **Victoria Jideonwo**, Miwon Ahn, Sneha Surendran, Rafaela Ruiz, Yonyyong Hou, Núria Morral. Role of SREBP-1 as Regulator of S6k1 in the Liver. American Diabetes Association's 76th Scientific Sessions, New Orleans, LA, June 10-14, 2016
2. **Victoria Jideonwo**, Miwon Ahn, Sneha Surendran, Rafaela Ruiz, Yongyong Hou, Núria Morral. Regulation of insulin signaling and S6K by the transcription factor SREBP-1. 1st Annual Center for Diabetes and Metabolic Diseases Symposium, Indiana University School of Medicine Indianapolis, IN, August 7, 2015
3. **Victoria Jideonwo**, Yongyong Hou, Sneha Surendran, Aisha Gamble, Núria Morral. Investigation for the role of CPT1A over-expression for treatment of non-alcoholic fatty liver disease. 5th Annual Midwest Graduate Research Symposium (MGRS), University of Toledo, Toledo, OH, March 29, 2014
4. **Victoria Jideonwo**. A role for Carnitine Palmitoyltransferase 1A (CPT1A) overexpression in treatment of non-alcoholic fatty liver disease. Department of Medical and Molecular Genetics Research Club, Indiana University School of Medicine, Indianapolis, IN, March 25, 2014
5. **Victoria Jideonwo** and Viola Ellison. Analysis of Subcellular Localization and Colocalization of Sister Chromatid Cohesion Establishment Factor Ctf4. Poster presented at LSAMP Indiana and Midwest Crossroads AGEP Second Annual Joint Conference, West Lafayette, IN November 2006.