INSIG-MEDIATED REGULATION OF HEPATIC LIPID SYNTHESIS

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DEDICATION

To my dearest wife Stephanie and our family

INSIG-MEDIATED REGULATION OF HEPATIC LIPID SYNTHESIS

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

July, 2005

ACKNOWLEDGMENTS

This work would not have been possible without the help of many people. First, I thank my lovely wife Stephanie for her emotional, intellectual, financial, and patient support throughout the entirety of my medical and scientific training.

It has been an esteemed privilege of mine to have worked under Michael Brown and Joseph Goldstein. I can add little to the commendations and compliments that have already been bestowed on them, other than to whole-heartedly agree that they are well-deserved. I hope that some fraction of their scientific prowess, incredible energy, attention to detail, and appreciation for the big picture has rubbed off onto me and will see me through my future endeavors.

I would also like to thank the members of my dissertation committee – Andrew Zinn, Keith Parker, and Steven Kliewer. Their positive attitudes and insightful comments were a tremendous support during the past two years.

Much of this work is part of a larger collaboration between myself and Guosheng Liang. It has been pleasure to work with Guosheng. His incisive candor and good humor kept me motivated and focused on the problems worth studying. I am happy that Guosheng has returned to our department from his stint at Eli Lilly in a timely fashion so that he can continue with the emergent findings in our mice. Robert Hammer was invaluable in the production of the transgenic and knockout mice and was always willing to provide helpful suggestions or share his vast knowledge of animal research. Jay Horton readily gave relevant and useful comments, constructive criticisms, and essential support for all the animal studies presented here.

Hiroshi Kuriyama and Kiyosumi Takaishi provided helpful assistance during the Insig transgenic and knockout mice studies. It has been my delight to work with Bret Evers, who will have much success in continuing the MSTP legacy with the Insig mice. Wei-Ping Li and James Richardson were indispensable for the histology studies.

I would like to thank many people for their assistance with the studies presented here, especially Monica Mendoza. Also, I thank Isis DeSoto, Richard Gibson, and Liz Lummus for invaluable help with animal studies; Scott Clark, Emily Brown, Deborah Morgan, and Rachael Salas for excellent technical assistance; Jeff Cormier for DNA sequencing and help with RT-PCR; and Lisa Beatty for invaluable assistance with cell culture.

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Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2005

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Cholesterol synthesis in mammals is tightly regulated by end-product feedback inhibition. 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes a rate determining reaction that is highly regulated by transcriptional and post-transcriptional mechanisms. As cellular cholesterol accumulates, the transcription of HMGR mRNA is suppressed and the proteosomal degradation of HMGR protein is accelerated. The sterol-regulated transcription of HMGR and other lipogenic genes is controlled by sterol regulatory element binding proteins (SREBPs). These membrane-bound transcription factors are escorted by SREBP cleavage activating protein (SCAP) from the endoplasmic reticulum (ER) to the Golgi apparatus where SREBPs are proteolytically processed to their active forms. In cultured

cells, feedback inhibition of SREBP processing is mediated by Insigs. When sterols accumulate, Insigs block SREBP activation by retaining SCAP in the ER. Insigs also mediate rapid, sterol-dependent turnover of HMGR protein. When sterols accumulate, Insigs bind to HMGR and stimulate its ubiquitination and degradation. Although Insigs are key regulators of cholesterol homeostasis in cultured cells, their role in the intact mammal was undefined. To explore this question, gain-of-function and loss-of-function analyses were performed by studying the livers of genetically engineered mice. First, transgenic mice that overexpress Insig-1 in liver (TgInsig-1) were generated. In the livers of TgInsig-1 mice, nuclear SREBPs (nSREBPs) were reduced and SREBP processing was supersensitive to inhibition by feeding high-cholesterol diets. The block in SREBP processing reduced the mRNA levels of SREBP target genes. Levels of HMGR protein were reduced and declined further with cholesterol feeding. Next, knockout mice that lack Insig-1, Insig-2, or both Insigs were generated. In the livers of Insig double knockout mice, cholesterol and triglycerides accumulated to high levels, and despite their accumulation, nSREBPs and mRNAs of SREBP target genes were not suppressed. SREBP processing was insensitive to inhibition by feeding high-cholesterol diets. HMGR protein levels were increased and failed to decline with cholesterol feeding. As a consequence of Insig overexpression or deficiency and the respective effect on SREBPs and HMGR, hepatic cholesterol and fatty acid synthesis in living animals was decreased in TgInsig-1 mice and increased in Insig double knockout mice. These studies indicate that Insigs are essential regulators of hepatic lipid synthesis.

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LIST OF ABBREVIATIONS

bHLH-Zip – basic helix-loop-helix leucine zipper

CREB – cAMP-responsive element binding protein

dpc – days post coitum

FAS – Fatty acid synthase

FDP – farnesyl diphosphate

f/f – flox / flox genotype

G6PD – glucose-6-phosphate dehydrogenase

GPAT – glycerol-3-phosphate acyltransferase

HMG-CoA reductase, HMGR – 3-hydroxy-3-methylglutaryl-CoA reductase

INSIG – insulin-induced gene

L-Insig-1$^{-/-}$ – mice with inducible deletion of Insig-1 in liver, genotyped *Insig-1*$^{f/f}$*;MX1-Cre*

Insig-1$^{-/-}$*2*$^{-/-}$ – mice with homozygous germline deletions Insig-1 and Insig-2

LCE – long-chain fatty acyl CoA elongase

LDL – low density lipoprotein

LXR – liver X receptor

MX1 – myxovirus resistance-1

NPC1 – Niemann-Pick C1

nSREBP – nuclear form of sterol regulatory element binding protein

PEPCK – phosphoenolpyruvate caryboxykinase

pIpC – polyinosinic acid-polycytidylic acid

pSREBP – membrane-bound precursor form of sterol regulatory element binding protein

SCAP – SREBP cleavage activating protein

SREBP – sterol regulatory element binding protein

S1P – site 1 protease

S2P – site 2 protease

SCD-1 – stearoyl CoA desaturase-1

SSD – sterol-sensing domain

TgInsig-1 – mice carrying the apoE-human Insig-1 transgene

WT – wild-type control mice

CHAPTER ONE
Introduction

CHOLESTEROL HOMEOSTASIS IN THE MAMMAL

Sterol requirements in animals

Cholesterol is an essential component of all animal cell membranes. Levels of cholesterol are tightly regulated since states of sterol deficiency and excess are pathologic at the cellular and organismal levels: sterol insufficiency degrades the integrity of a cell's membranes whereas excess sterols can form toxic intracellular crystals. Genetic deficiencies in enzymes required for cholesterol synthesis lead to reduced cholesterol levels *in utero* and are associated with prototypical multiple malformation syndromes such as the Smith-Lemli-Opitz Syndrome (Porter 2002), while excess plasma cholesterol initiates the formation of atherosclerotic plaques, the pathology responsible for the majority of sudden cardiac deaths in western societies (Steinberg 2005).

Coordinate regulation of sterol uptake and synthesis by SREBPs

Mammalian cells have two major sources of cholesterol: *de novo* synthesis from acetyl carbons or receptor-mediated uptake from cholesterol-rich plasma lipoproteins. To maintain a constant level of membrane cholesterol, cells sense their sterol content and coordinately modulate the transcription of genes required for the uptake and *de novo* synthesis of cholesterol (Goldstein and Brown, 1990). This negative end-product feedback regulation is accomplished by the actions of a family of transcription factors known as Sterol Regulatory

Element Binding Proteins (SREBPs) (Brown and Goldstein, 1997). These transcription factors become activated when cells become depleted of sterols and upregulate the transcription of genes required for cholesterol uptake, such as the low density lipoprotein (LDL) receptor, and for cholesterol synthesis, such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (Horton et al., 2003). SREBPs are synthesized as transcriptionally inert precursor molecules (pSREBP) approximately 1150 amino acids in length that are composed of two major cytosolic domains separated by a membrane anchor made from 2 transmembrane spanning helices and a short loop facing the ER lumen. The N-terminal domain, of approximately 480 amino acids length, comprises a transcription factor of the basic helix loop helix leucine-zipper family (bHLH-Zip). The C-terminal domain, approximately 590 amino acids, is responsible for protein-protein interactions required for the activation of the bHLH-ZIP (Sato et al., 1994). Three SREBP proteins, denoted SREBP-1a, 1c, and -2 are encoded by two chromosomal genes (Hua et al., 1995). SREBP-1a and -1c are produced from a single SREBP-1 gene and differ in their use of alternate promoters and first exons (Yokoyama et al., 1993; Shimomura et al., 1997). The extreme N-termini of all three isoforms comprise acidic sequences which serve as transcriptional activating domains essential for the recruitment of coactivators such as CBP and Sp1 (Naar et al.,1998). In human SREBP-1a and SREBP-2, these acidic regions are 42 and 48 amino acids, respectively, whereas in SREBP-1c this sequence is much shorter and contains only 24 amino acids; in consequence SREBP-1c is a much weaker transcriptional activator than SREBP-1a or -2 (Yokoyama et al., 1993; Shimano et al., 1997A). SREBPs are unique among bHLH-Zip transcription factors in that a highly conserved arginine in the basic DNA-

binding region is replaced by a tyrosine. This replacement allows SREBPs to bind nonpalindromic SREs, such as ATCACCCCAC, the canonical binding site defined in the LDLR promoter, in addition to palindromic E-Boxes, namely CANNTG, which are the typical binding elements for bHLH transcription factors (Kim et al., 1995). Although all three SREBPs bind SREs with high affinity, SREBP-2 preferentially activates genes involved in cholesterol synthesis and uptake while SREBP-1c preferentially activates genes involved in fatty acid and triglyceride synthesis (Shimano et al., 1997A; Horton et al., 1998B). SREBP-1a potently activates both classes of genes (Shimano et al., 1996).

For the N-terminal bHLH-Zip region to reach the nucleus and activate the transcription of genes which contain SREs in their enhancer regions, it must be released from the ER membrane. This proteolytic release is accomplished by the actions of two membrane-bound proteases, termed the site-1 and site-2 proteases (S1P, S2P). The first cleavage event occurs in the lumen of the Golgi apparatus where the leucine-serine bond in the sequence RXXLS, contained in the luminal loop between the transmembrane spanning regions that separates the N and C terminal domains of SREBP, is cleaved by S1P, a subtilisin family serine protease (Sakai et al., 1998). The bHLH-Zip is then tethered to the membrane only by virtue of the first transmembrane (TM) spanning region. The second cleavage occurs within this transmembrane spanning region in the Golgi membrane, when S2P, a zinc metalloprotease, cleaves the leucine-cysteine bond in the sequence DRSRILLC (Rawson et al., 1997). Only three intramembrane residues, ILL, remain attached to the N-terminus such that it can be mobilized from the Golgi membrane and can enter the nucleus to modulate transcription of lipogenic genes.

Sterols inhibit the transcriptional activity of pSREBPs by blocking their proteolytic processing to form mature nSREBPs (Wang et al., 1994) by sequestering pSREBPs in the ER away from S1P and S2P, which reside in Golgi membranes. pSREBPs move to the Golgi in a sterol-regulated fashion via interactions between the C-terminus of SREBPs with SREBP cleavage activating protein (SCAP), a 1276 amino acid integral membrane protein with 8 membrane-spanning regions (Hua et al., 1996; Nohturfft et al., 2000). The SCAP-SREBP complex traffics to the Golgi via COPII-coated vesicles budding from the ER (Espenshade et al., 2002). The membranous regions of SCAP interact with proteins of the COPII vesicular machinery: Sar1p, a GTPase required for sorting of cargo proteins and vesicle budding, and Sec23/24, COPII coat proteins (Sun et al., 2005). Transmembrane helices 2 through 6 of SCAP form a pentahelical transmembrane bundle termed the sterol-sensing domain (SSD) that has sequence homology to pentahelical bundles present in several other proteins, which are all thought to be somehow related to cholesterol: HMG-CoA reductase, Niemann-Pick type C1 (NPC1), and Patched (Kuwabara and Labouesse 2002). Three mutations, termed sterol resistant mutants, in the SSD of SCAP abolish the ability of sterols to inhibit SREBP proteolysis: D443N, Y298C, and Y315F (Yabe et al., 2002).

Insigs and the Cholesterol-Sensing Mechanism

The key sterol-regulated step of SREBP activation is the exit of the SCAP-SREBP complex from the ER. Once the substrate pSREBPs are delivered to membranes containing S1P and S2P, cleavage and formation of nuclear SREBPs (nSREBPs) is constitutive (DeBose-Boyd et al., 1999). Key experiments by Yang suggested that the sterol-sensing mechanism involved a

sterol-dependent interaction between SCAP and a saturable ER retention factor (Yang et al., 2000). These studies lead to the purification of Insig-1 (insulin-induced gene-1), a factor which co-precipitated with affinity-tagged SCAP TM 1-6 and which restored sterol-dependent suppression of SREBP processing in cells that overexpress SCAP (Yang et al., 2002). Database searches revealed the existence of a closely-related isoform encoded by a different chromosomal locus, Insig-2 (Yabe et al., 2002).

Insigs are ER-residing integral membrane proteins that are predicted to contain 6 membrane-spanning regions (Feramisco et al., 2003). Mouse Insig-1 and Insig-2 are 259 and 225 amino acids, respectively (Yabe et al., 2002). When overexpressed in cultured cells, both Insig-1 and Insig-2 sensitize SREBP processing to inhibition by sterols. Insigs enhance the ability of sterols to induce a conformational change in SCAP that can be detected by the appearance of a new fragment in tryptic digests of microsomal SCAP protein produced by an alternative site of proteolytic cleavage in sequences surrounding TM 7 and 8 of SCAP (Brown et al., 2002; Adams et al., 2003). Sterol-resistant SCAP mutants that fail to bind Insigs have a reduced propensity to adopt the sterol/Insig-induced conformation change. Conversely, a mutant SCAP (D428A) that binds to Insigs in the absence of exogenously added sterols has an increased propensity to adopt the sterol/Insig-induced conformation, and has a reduced ability to support SREBP processing under sterol-depleted conditions (Feramisco et al., 2005). Inasmuch as Insigs are required for sterol-regulated binding between SCAP and COPII vesicle proteins (Sun et al., 2005), it is likely that the sterol/Insig-induced conformation change in SCAP disrupts this binding to prevent the vesicular budding of the SCAP/SREBP complex from the ER.

The effect of Insig deficiency on SREBP processing in cultured cells was demonstrated by RNAi-mediated knockdown of Insig-1 and Insig-2 (Adams et al., 2004) and by the generation of CHO cell lines lacking Insig-1 and Insig-2 (Lee et al., 2005), in which sterol treatment did not block SREBP processing. These studies establish a requirement for Insigs in mediating feedback regulation on SREBP proteolysis in cultured cells.

Multivalent Regulation of Sterol Synthesis: HMG-CoA reductase

HMG-CoA reductase catalyzes the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate, which is the rate-determining reaction of hepatic sterol biosynthesis (Dietschy and Brown, 1974). Resultant isoprenyl units derived from mevalonate are incorporated into a diverse array of final end products in addition to sterols, such as steroids, bile acids, isopentenyl adenine, haem A, ubiquinone, dolichol, farnesylated proteins, and other molecules (Goldstein and Brown, 1990). Treatment of cultured cells with statins, competitive inhibitors of HMG-CoA reductase, result in the rapid accumulation of HMG-CoA reductase protein to hundreds-fold its content in untreated cells (Nakanishi et al., 1998). This massive induction reflects the multiplicative interaction of three levels of HMG-CoA reductase regulation: the induction of HMG-CoA reductase transcription, mediated by the sterol-regulation activation of SREBPs as described above, accounts for an 8-fold change; nonsterol mevalonate-derived products can suppress HMG-CoA reductase translation by 5-fold; lastly both sterol and nonsterol mevalonate-derived products can accelerate HMG-CoA reductase protein degradation by 5-fold (Goldstein and Brown, 1990). The effect of sterols and mevalonate to accelerate HMG-CoA reductase degradation requires the transmembrane

spanning regions that constitute the SSD of HMG-CoA reductase; the half-life of the soluble catalytic domain of HMG-CoA reductase is very long and is not affected by sterol and/or mevalonate treatment (Gil et al., 1985).

Insigs and the Regulated Degradation of HMG-CoA reductase

The finding that Insigs bind the SSD of SCAP led to the hypothesis that Insig may bind and modulate the functions of other proteins which contain SSDs, such as HMG-CoA reductase. When overexpressed in cultured cells, Insigs accelerated the degradation of HMG-CoA reductase in the presence of sterols and mevalonate (Sever et al., 2003A). Coexpression of WT SCAP but not Y298C SCAP blocked this effect, which suggested that SCAP and HMG-CoA reductase were competing for the same binding site on Insig. While the Insig:SCAP interaction leads to the ER retention of SCAP, the Insig:HMG-CoA reductase interaction causes the ER-associated degradation (ERAD) of HMG-CoA reductase at the proteosome. Insig deficiency in cultured cells introduced by RNAi (Sever et al., 2003B) or by inactivating mutations in Insig-1 (Sever et al., 2004) or by mutations in both Insig-1 and Insig-2 (Lee et al., 2005) dramatically reduced the effect of sterols to induce the ubiquitination and degradation of HMG-CoA reductase, which established a requirement for Insigs in mediating sterol feedback regulation on HMG-CoA reductase degradation in cultured cells. Mutations in a tetrapeptide sequence, YIYF, located in the SSD of HMG-CoA reductase blunted the ability of Insigs to bind and stimulate the ubiquitination of HMG-CoA reductase and established the functional binding of Insig with the SSD of a second protein (Sever et al., 2003B).

REGULATION OF HEPATIC LIPID SYNTHESIS

SREBP Pathway and Insigs in Mammalian Liver

The studies detailed above have been carried out primarily in cultured cells. Of the three SREBP isoforms, SREBP-1c and SREBP-2 are predominant in liver; SREBP-1a is quantitatively minor (Horton et al., 2002). Hepatic SREBP expression is sensitive to the nutritional cues through the insulin / glucagon ratio: SREBP-1c expression in hepatocytes is potently upregulated by insulin and rapidly is downregulated when insulin is removed or antagonized with glucagon (Foretz et al., 1999; Shimomura et al., 1999). Three conserved DNA binding sites in the mouse SREBP-1c promoter, a pair of Liver-X-Receptor Response Elements (LXRE) sites and an SRE, cooperate to mediate the effect of insulin to induce SREBP-1c expression in primary hepatocytes (Chen et al., 2004). The nature of this insulin-mediated synergism between the SRE and LXREs remains unclear. Expression of SREBP-1a and -2 are much less sensitive than SREBP-1c to the effect of insulin. Independent of effects of insulin per se, the transcription of hepatic SREBP-1c is also potently regulated by the nuclear hormone receptors LXRα and β. Feeding of synthetic LXR agonists or high cholesterol diets, which generate the production of endogenous oxysterol ligands that activate LXRs, increases SREBP-1c mRNA (Repa et al., 2000).

Nutritional and hormonal regulation of the SREBP pathway has been studied in intact animals primarily by two paradigms: fasting and refeeding or streptozotocin treatment. During fasting and refeeding, mice are fasted until plasma insulin levels are very low and then refed a diet containing high amounts of carbohydrate that the mice overeat, inducing a

dramatic upswing in the plasma insulin levels. In the fasted state, pSREBP-1 disappears, and both nSREBP-1 and -2 disappear. HMG-CoA reductase protein also disappears in fasting. In the refed state, precursor and nuclear SREBP-1 are dramatically increased. HMG-CoA reductase protein and nSREBP-2 are restored to their pre-fasting levels, whereas levels of SCAP and pSREBP-2 are not affected by fasting and refeeding (Horton et al., 1998A). Streptozotocin treatment, which induces hypoinsulinemia by selectively killing pancreatic β cells, produces a similar phenotype to fasting in that pSREBP-1 and nSREBP levels are strongly reduced (Shimomura et al., 1999). Reintroduction of insulin into the strepotzotocin-treated animal rapidly restores SREBP1-c expression and SREBP processing, which suggests that the effect of streptozotocin on SREBP-1c is due to insulin loss and not a secondary effect of the drug treatment or the diabetic state.

Expression of Insig isoforms is also regulated by nutritional status. Insig-1 is an SREBP target itself; its mRNA level varies in concert with nSREBPs (Horton et al., 2003; Yabe et al., 2002). Thus, Insig-1 is highly expressed in the refed or the streptozotocin + insulin conditions when nSREBPs, especially nSREBP-1c, are abundant. Insig-2 expresses two isoforms, 2a and 2b, which result from the usage of alternate promoters and non-coding first exons. Whereas Insig-2b is ubiquitously expressed and not affected by feeding status, Insig-2a is expressed selectively in liver and is potently induced by fasting or streptozotocin treatment (Yabe et al., 2003). In cultured hepatocytes, insulin potently represses Insig-2a transcription. Consequently, in liver, the mRNAs for the predominant Insig isoforms are reciprocally regulated in relation to hormonal cues: Insig-1 mRNA is high in the fed, high-

insulin state and low in the fasted, low-insulin state whereas Insig-2a mRNA is low in the fed, high-insulin state and high in the fasted, low-insulin state.

Components of the SREBP pathway have been studied in intact animals using gain-of-function and loss-of-function analyses in the livers of genetically engineered mice. The aim of these studies was to validate whether components of the pathway, delineated for the most part by somatic cell genetics in cultured CHO cells, had a quantitatively significant impact on lipid synthesis *in vivo* (reviewed in Horton et al., 2002). Only a fraction of mice with homozygous germline deletions in SREBP-1 survive beyond the embryonic period; those that do compensate by increasing the expression of SREBP-2 and consequently have elevated cholesterol synthesis due to a large increase in the expression of genes in the cholesterol biosynthetic pathway. Mice selectively lacking SREBP-1c have normal viability and also show compensatory increases in SREBP-2. Refeeding or LXR-agonist-induced lipogenic responses are blunted in SREBP-1c$^{-/-}$ mice. Germline deletions in SREBP-2 or SCAP are lethal during the embryonic period, whereas mice with an inducible, liver-specific deletion in S1P or SCAP have dramatically reduced levels of hepatic nSREBPs and SREBP target mRNAs, reduced plasma and liver cholesterol and triglycerides, reduced insulin-stimulated lipogenesis, and reduced hepatic cholesterol and fatty acid synthesis as measured by of ^{3}H-water incorporation. The livers of mice which overexpress truncated SREBPs, which bypass processing and enter the nucleus constitutively, synthesize and accumulate large amounts of cholesterol (nSREBP-2; Horton et al., 1998B), large amounts of cholesterol and triglycerides (nSREBP-1a; Shimano et al., 1996), or modest amounts of triglycerides (nSREBP-1c; Shimano et al., 1997A), all with dramatically increased expression in subsets

of lipogenic SREBP target mRNAs. The livers of mice that express a sterol-resistant SCAP (D443N) accumulate large amounts of cholesterol and triglycerides and the processing of SREBPs is resistant to feedback suppression by diets containing high amounts of cholesterol and cholic acid (Korn et al., 1998). In summary, mice with a high hepatic content of nSREBPs develop fatty livers, increased transcription of lipogenic mRNAs, and increased *de novo* synthesis of lipids. Conversely, mice with a low hepatic content of nSREBPs have reduced transcription of lipogenic mRNAs, reduced levels of hepatic and plasma lipids, and decreased *de novo* synthesis of lipids, especially in the context of high-insulin, post-absorptive states such as refeeding.

The Schoenheimer Effect and regulation of hepatic cholesterol synthesis

The first evidence of end-product feedback regulation of cholesterol synthesis was provided by Rudolf Schoenheimer 72 years ago (Schoenheimer and Breush, 1933). In these metabolic balance studies, mice were kept in glass flasks and fed diets containing low or high amounts of cholesterol. At the completion of the month-long feeding period, the entire contents of the flask, including the mouse, bedding, feces, and any remaining food was hydrolyzed; resultant sterols were extracted with ether, precipitated with digitonin, and weighed. The result of the studies were that on a low cholesterol diet, more sterol was found in the flask than could have been accounted for by the starting sterol content of the mouse, its bedding, and its food. This implied that net synthesis of sterols had occurred. In contrast, when placed on a high cholesterol diet, less sterol was found in the flask than had been in the starting contents. This implied that net destruction of sterol, i.e. synthesis of bile acids, had

occurred. Feeding an intermediate dose of cholesterol resulted in a low net increase in sterol, which furthermore demonstrated the dose-dependent ability of dietary cholesterol to inhibit sterol synthesis in the intact mammal. These results, however, could not differentiate between a decrease in the absolute synthesis or an increase in absolute catabolism of sterols to account for the decrease in net sterol balance when mice were fed the high cholesterol diet.

Schoenheimer's pioneering work in the use of isotopic tracers for metabolic analysis (reviewed in Kennedy 2001) made possible the observations by R. Gordon Gould, who showed that ^{14}C-acetate incorporation into digitonin-precipitable sterols in dog and rabbit liver slices was dramatically decreased in animals fed high-cholesterol diets (Gould and Taylor 1950, Gould 1951). These studies established that the absolute rate of hepatic sterol synthesis was in fact inhibited by the feeding of dietary cholesterol. Studies by Chaikoff (Tomkins et al., 1952) and later by and Siperstein (Dietschy and Siperstein, 1967) demonstrated that sterol synthesis in liver but not extrahepatic sites was inhibited by dietary cholesterol feeding. Furthermore, liver was shown in these and other studies to be the major site of cholesterol synthesis (reviewed in Dietschy and Wilson, 1970). Hearkening back to the original observation by Schoenheimer, it follows that feedback inhibition of sterol synthesis in liver, the quantitatively major site of sterol synthesis, was critical in the regulation of whole-body sterol balance.

Work by several groups demonstrated that the hepatic conversion of HMG-CoA to mevalonate, catalyzed by HMG-CoA reductase, was the major regulated enzymatic reaction which controlled the rate of sterol synthesis (Siperstein and Fagan 1966, reviewed in Dietschy and Wilson, 1970). Brown observed that the rates of HMG-CoA reductase activity

and ^{14}C-octanoate incorporation into sterols measured from the same livers varied proportionally over 100-fold range in rats subjected to various conditions which either downregulated (cholesterol feeding, fasting) or upregulated (cholestyramine feeding) hepatic sterol synthesis (Dietschy and Brown 1974, Brown et al., 1979).

Brown and Goldstein, HMG-CoA reductase and LDL: The historical context of Insigs

The observations of Brown and Goldstein that LDL cholesterol potently suppressed HMG-CoA reductase activity in cultured cells (Brown et al., 1973) and the lack of such suppression in FH patients (Goldstein and Brown 1973) eventually led to the discovery of the LDL receptor, which then eventually led to the discovery the SREBP pathway, which then ultimately led to the discovery of Insigs – and takes us to the present. The aim of the current studies is to establish the role of Insig proteins in mediating sterol feedback suppression of HMG-CoA reductase in the livers of living mice at both transcriptional and posttranscriptional levels and the consequences of this regulation on the overall cholesterol homeostasis in the intact mammal as originally defined by Schoenheimer 72 years ago.

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CHAPTER TWO

OVEREXPRESSION OF INSIG-1 IN LIVERS OF TRANSGENIC MICE INHIBITS SREBP PROCESSING AND REDUCES INSULIN-STIMULATED LIPOGENESIS

ABSTRACT

In the current studies we generated transgenic mice that overexpress human Insig-1 in liver under a constitutive promoter. Insig-1 and Insig-2 have been shown in cultured cells to block lipid synthesis in a cholesterol-dependent fashion by inhibiting proteolytic processing of SREBPs, membrane-bound transcription factors that activate lipid synthesis. Insigs exert this action in the ER by binding SREBP cleavage-activating protein (SCAP) and preventing it from escorting SREBPs to the Golgi where the SREBPs are processed to their active forms. In livers of Insig-1 transgenic mice, the content of all nuclear SREBPs (nSREBPs) was reduced and declined further upon feeding dietary cholesterol. The nuclear content of the insulin-induced SREBP isoform, SREBP-1c, failed to increase to a normal extent upon refeeding a high carbohydrate diet. The nSREBP deficiency produced a marked reduction in the levels of mRNAs encoding enzymes required for synthesis of cholesterol, fatty acids, and triglycerides. Plasma cholesterol levels were strongly reduced, and plasma triglycerides did not exhibit their normal rise after refeeding. These results provide *in vivo* support for the hypothesis that nSREBPs are essential for high levels of lipid synthesis in liver and indicate that Insigs modulate nSREBP levels by binding and retaining SCAP in the ER.

INTRODUCTION

Insig-1 and Insig-2 are polytopic membrane proteins of the ER that play a central role in the feedback control of lipid synthesis in animal cells. Insigs regulate lipid synthesis by binding in a sterol-dependent fashion to two ER proteins: HMG CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis, and SREBP cleavage-activating protein (SCAP), an escort protein required for the cleavage and activation of the SREBP family of membrane-bound transcription factors. Sterol-stimulated binding of Insigs to HMG CoA reductase leads to its ubiquitination and proteosomal degradation (Sever et al., 2003A; Sever et al, 2003B). Sterol-stimulated binding of Insigs to SCAP leads to ER retention of the complexes between SCAP and SREBPs, thereby preventing the SREBPs from entering the Golgi for proteolytic processing (Yang et al., 2002; Yabe et al., 2002). ER retention prevents the proteolytic generation of the transcriptionally active nuclear forms of SREBPs (nSREBPs), thereby limiting transcription of SREBP target genes, which include all of the known genes required for synthesis of cholesterol, fatty acids, triglycerides, and phospholipids (Horton et al., 2003). In cultured cells, the net result of Insig action is to decrease lipid synthesis whenever sterols accumulate to high levels within cells.

The two known human Insig isoforms, Insig-1 and -2, are 59% identical over their lengths of 277 and 225 amino acids, respectively. Both are deeply embedded in ER membranes through the presence of six membrane-spanning segments (Feramisco et al., 2003). Studies in cultured cells indicate that the two Insigs have overlapping functions in binding both HMG CoA reductase and SCAP, but the two Insig genes are controlled

differently. Insig-1 is itself a target of nSREBPs, and its mRNA rises and falls coordinately with nSREBP levels (Yang et al., 2002; Janowski, 2002). The *Insig-2* gene has two promoters/enhancers that give rise to two transcripts with different noncoding first exons, but identical coding exons (Yabe et al., 2003). In cultured cells one of these transcripts, designated Insig-2b, is unvarying and is not influenced by nSREBP levels. The other transcript, Insig-2a, is expressed nearly exclusively in liver, and it reaches high levels during insulin deficiency, such as occurs in fasting. When insulin levels rise, the Insig-2a transcript is strongly suppressed (Yabe et al., 2003).

All detailed studies of Insig function have been carried out in cultured fibroblast-like cells, predominantly Chinese hamster ovary (CHO) cells. In these cells, overexpression of Insig-1 or -2 by transfection causes a marked leftward shift in the sterol-response curves for degradation of HMG CoA reductase (Sever et al., 2003A; Sever et al, 2003B) and inhibition of SREBP processing (Yang et al., 2002; Yabe et al., 2002), rendering both processes much more sensitive to sterols. When expressed at extremely high levels, Insig-1 can trap the SCAP/SREBP complex in the ER even without the addition of exogenous sterols. The sterol-sensitizing function of Insigs is also apparent in an *in vitro* assay that measures a conformational change in SCAP upon sterol addition (Adams et al., 2003). Expression of mammalian Insig-1 or -2 in Drosophila cells together with mammalian SCAP confers the ability of sterols to suppress SCAP transport and SREBP processing (Dobrosotskaya et al., 2003). Combined knockdown of Insig-1 (by 95%) and Insig-2 (by 50%) through RNA interference abolishes the ability of sterols to accelerate degradation of HMG CoA reductase

(Sever et al., 2003B), but it does not reduce the ability of sterols to block SREBP processing, perhaps owing to the failure to reduce Insig levels sufficiently.

In the present studies, we explore the role of Insig-1 in regulating synthesis of cholesterol, fatty acids, and triglycerides in the mammalian liver. For this purpose, we produced transgenic mice that overexpress human Insig-1 in liver. The data show that Insig-1 overexpression renders the liver much more sensitive to sterol-mediated feedback suppression of lipid synthesis, and it largely prevents the acute increase in lipid synthesis that accompanies refeeding of previously fasted mice. The latter finding suggests that insulin-mediated suppression of Insig-2a is required in order for insulin to increase lipid synthesis in liver and that increased lipid synthesis does not occur when Insig levels are kept high through overexpession of Insig-1.

RESULTS

Figure 2-1 shows the expression in mouse tissues of the human Insig-1 transgene driven by a version of the *apoE* promoter/enhancer that is believed to be liver specific (Simonet et al.,1993). By northern blotting, human Insig-1 mRNA was detectable only in liver and kidney (Figure 2-1a). Despite expression of the mRNA in kidney, the human protein was undetectable by immunoblotting in this organ (Figure 2-1b). In contrast, the liver expressed abundant amounts of human Insig-1 protein, which gives two bands, owing to the use of two start-sites for translation (Yang et al., 2002). Mouse Insig-1 gives only a single band, owing to a 16-amino acid deletion that removes the downstream initiator methionine, which is at

Table 2-1
Comparison of wild type and TgInsig-1 mice

Parameter	Wild type	TgInsig-1
Body weight (g)	29.0 ± 1.0	28.3 ± 0.9
Liver weight (g)	1.7 ± 0.04	1.7 ± 0.07
Epididymal fat weight (g)	0.25 ± 0.04	0.29 ± 0.05
Liver cholesterol concentration (mg/g)	1.9 ± 0.03	1.7 ± 0.03*
Liver triglyceride concentration (mg/g)	4.3 ± 0.5	2.9 ± 0.4
Total plasma cholesterol (mg/dl)	105 ± 5	61 ± 4*
Total plasma triglycerides (mg/dl)	119 ± 10	71 ± 9*
Plasma insulin (ng/ml)	0.90 ± 0.11	1.2 ± 0.19
Plasma glucose (mg/dl)	141 ± 11	153 ± 5
Plasma free fatty acids (mM)	0.38 ± 0.03	0.40 ± 0.03

16-week old male (5 per group) mice were fed a chow diet ad libitum prior to study. The wild type mice were littermates of the transgenic mice. Each value represents the mean ± SEM of 5 values. Asterisks denote the level of statistical significance (Student's *t* test) between wild type and TgInsig-1 mice. *, $p < 0.01$.

residue 37 in the human sequence. As a result, the single mouse Insig-1 protein is intermediate in size between the two human forms. In livers of these *ad lib*-fed mice, the level of human Insig-1 protein was many fold higher than the level of endogenous mouse Insig-1 (Figure 2-1b).

The transgenic mice appeared grossly normal. As shown in Table 2-1, at 16 weeks they had normal body weights, liver weights, and fat pad weights. The liver cholesterol content was significantly reduced. The triglyceride content was also reduced, but the data did not reach statistical significance. Plasma cholesterol and triglyceride levels were

significantly reduced by about 40%. Plasma glucose, insulin, and free fatty acids were unaffected.

In cultured cells, Insig overexpression increases the sensitivity of SREBP processing to inhibition by sterols. To test for this phenomenon *in vivo*, wild type and transgenic littermate mice were fed diets containing varying amounts of cholesterol for two days, after which the amounts of membrane-bound precursor and nSREBPs were measured by SDS-PAGE and immunoblotting (Figure 2-2a). When consuming the unsupplemented chow diet, which contains 0.02% cholesterol, the transgenic mice showed a lower content of nSREBP-1 and nSREBP-2 than did the wild type mice (Figure 2-2b) These values declined further when small amounts of cholesterol were added to the diet. As dietary cholesterol increased, the decline in both nSREBPs was much more pronounced in transgenic than in wild type mice. This decline occurred despite the fact that the cholesterol content of transgenic livers was lower than that in wild type mice at all levels of dietary cholesterol (Figure 2-2c).

To determine the effects of Insig-1 overexpression on SREBP target genes, we measured the relevant mRNAs by quantitative real-time RT-PCR in livers from cholesterol-fed mice (Figure 2-3). The liver produces two quantitatively major isoforms of SREBP, designated SREBP-1c and SREBP-2 (Horton et al., 2002). SREBP-1c activates predominantly the genes involved in fatty acid and triglyceride biosynthesis. SREBP-2 activates primarily the genes required for cholesterol synthesis. The mRNA for SREBP-1c is enhanced by nSREBPs in a feed-forward reaction. It is also induced by insulin and by the nuclear receptors LXRα and LXRβ, whose activities are stimulated by oxygenated sterols, some of which can be derived from the cholesterol biosynthetic pathway or from dietary

cholesterol (Horton et al., 2002; Repa et al., 2000; Debose-Boyd et al., 2001). The mRNA for SREBP-2 is positively regulated by nSREBPs, but not by insulin or LXRs (Horton et al.,2002). The liver also produces small and unvarying amounts of another isoform, SREBP-1a, which can activate both fatty acid and cholesterol synthesis.

The Insig-1 transgene had no effect on the small amount of SREBP-1a mRNA in liver, and there was no response to cholesterol feeding (Figure 2-3, first row). In contrast, the mRNA for SREBP-1c was severely reduced in the transgenic livers, even on the 0.02% cholesterol chow diet. Cholesterol feeding induced a biphasic response. In wild type littermates fed 0.05% cholesterol, the mRNA for SREBP-1c declined, perhaps owing to the reduction in nSREBPs. However, as dietary cholesterol increased, the amount of SREBP-1c mRNA rose, an effect that we attribute to the activation of LXRs (Debose-Boyd et al., 2001). The effects of dietary cholesterol on SREBP-1c mRNA in the transgenic mice paralleled those in wild type mice, but the absolute level in transgenic mice was always much lower than that in wild type mice. The response of SREBP-2 mRNA was much less complex. This mRNA was reduced in the transgenic livers, and it declined in parallel in wild type and transgenic animals as the dietary content of cholesterol rose. This decline is most likely caused by the loss of the feed-forward effect, owing to the cholesterol-mediated decline in nSREBPs (see Figure 2-2). SCAP mRNA was unaffected by the transgene or by cholesterol feeding.

All of the measured mRNAs in the fatty acid and triglyceride synthesis pathways were reduced in the Insig-1 transgenic mice fed the chow diet (0.02% cholesterol) (second row in Figure 2-3). In wild type mice cholesterol feeding partially reduced the mRNA for

acetyl CoA carboxylase, but this was not observed in the Insig-1 transgenics. The mRNA for fatty acid synthase (FAS) was reduced by cholesterol in both lines of mice. Cholesterol feeding had little effect on the mRNAs for stearoyl CoA desaturase (SCD-1) or glycerol-3-phosphate acyltransferase (GPAT). The mRNAs for three enzymes of cholesterol biosynthesis and the LDL receptor were reduced in the Insig-1 transgenics on the chow diet (third row in Figure 2-3), and they all declined further with cholesterol feeding. The mRNAs for LXRα and ABCA1 were not affected by the transgene or by cholesterol feeding. In contrast, the mRNAs for ABCG5 and ABCG8 were induced by cholesterol feeding, which reflects their known stimulation by LXRα and LXRβ. The Insig-1 transgene had little effect on these mRNAs, which encode transporters that secrete cholesterol and plant sterols into bile (Berge et al., 2000).

The data of Figures 2-2 and 2-3 indicate that overexpression of Insig-1 retards the processing of SREBP-1c and -2, and renders this process more sensitive to inhibition by cholesterol. The net effect is a reduction in the mRNAs for SREBP target genes. Increases in nSREBP-1c have also been invoked to explain the increase in hepatic fatty acid synthesis that occurs when fasted animals are refed with a high carbohydrate diet, which stimulates insulin release and thereby increases the mRNA for SREBP-1c (Horton et al., 2002). To test the effect of the Insig-1 transgene on this response, we subjected the transgenic mice to a 12-hour fast followed by 12 hours of refeeding.

As shown by the immunoblots of Figure 2-4, in wild type mice fasting reduced the amount of the precursor and nuclear forms of SREBP-1c, and refeeding induced a major "overshoot" in nSREBP-1c as well as in its precursor. In contrast, the transgenic mice

showed a reduction in nSREBP-1c on an *ad lib* diet, and there was no further reduction with fasting (although the precursor did fall in these mice). Refeeding restored the SREBP-1c precursor, but the amount of nSREBP-1c remained well below that in the wild type littermates. In wild type mice, fasting reduced nSREBP-2, and this was restored to control levels by refeeding without any "overshoot." The response was similar in Insig-1 transgenic mice, except that the level of nSREBP-2 was reduced when compared with wild type littermates. As previously reported (Yabe et al., 2003), in the wild type mice fasting caused the disappearance of Insig-1 (owing to the decrease in nSREBPs) and the appearance of Insig-2 (owing to the decrease in insulin). Refeeding reversed these changes. As expected, fasting and refeeding did not affect the elevated human Insig-1 in the transgenic mice. The induction of Insig-2 by fasting occurred normally in these animals. The modest increase of Insig-2 protein in the fasted transgenic mice compared to the wild type controls was not observed in other studies.

Figure 2-5 shows the levels of relevant mRNAs in livers of the fasted and refed mice. With regard to genes of the SREBP pathway, refeeding induced a marked rise in SREBP-1c mRNA in wild type but not transgenic mice (see Discussion for explanation). Refeeding also increased the mRNAs encoding three enzymes of cholesterol synthesis (HMG CoA synthase, HMG CoA reductase, and farnesyl diphosphate synthase). The response was blunted, but still detectable in the Insig-1 transgenics. Lesser increases were seen in squalene synthase and the LDL receptor. As expected, refeeding caused marked increases in all of the measured mRNAs encoding enzymes of fatty acid and triglyceride synthesis. The response was severely blunted in the Insig-1 transgenics. Refeeding suppressed the mRNAs for IRS-2

and PEPCK, two genes known to be repressed by insulin (Shimomura et al., 2000). As a control, we measured the mRNA for apoE, which was not affected by refeeding in wild type or transgenic mice.

As expected from the changes in mRNA levels, refeeding caused a marked 18-fold increase in hepatic fatty acid synthesis in wild type mice, as determined by measurement of the incorporation of intraperitoneally injected [^{3}H]water (Figure 2-6a). This response was severely blunted in the Insig-1 transgenics. Refeeding caused a much lesser stimulation of fatty acid synthesis in the kidney, and this was similar in the wild type and transgenic mice. Sterol synthesis was also markedly elevated in livers of the refed wild type mice, and this elevation was reduced 3-fold in the transgenic livers (Figure 2-6b). The kidneys of wild type and transgenic mice showed a much smaller increase in sterol synthesis upon refeeding.

Figure 2-7 shows the plasma cholesterol and triglyceride levels in the animals that were used in the current studies. In the cholesterol feeding experiment, plasma cholesterol levels were lower in the Insig-1 transgenic mice and remained lower as dietary cholesterol was increased to 0.5% (Figure 2-7a). Plasma triglyceride levels were not significantly lower in the transgenic mice as compared with their wild type littermates. In the fasting-refeeding experiments, plasma cholesterol was also significantly reduced in the Insig-1 transgenics (Figure 2-7b). Refeeding the high carbohydrate-low fat diet caused a marked increase in plasma triglycerides in wild type mice, reflecting the marked increase in hepatic fatty acid synthesis in these animals. This increase did not occur in the Insig-1 transgenics.

DISCUSSION

The current results indicate that overexpressed Insig-1 traps the SCAP/SREBP complex in the ER of liver cells in living mice, thereby reducing the ability of SREBPs to activate transcription of genes encoding enzymes of cholesterol and fatty acid biosynthesis. In addition to reducing basal levels of nSREBPs, the overexpressed Insig-1 enhanced the ability of dietary cholesterol to further reduce these levels. Moreover, the overexpressed Insig-1 prevented the increase in nSREBP-1c that normally accompanies refeeding of previously fasted mice and thereby markedly reduced fatty acid synthesis in these animals. This latter observation is similar to the observation previously made in gene knockout mice that lack SCAP or Site-1 Protease, both of which are required to process SREBPs (Horton et al., 2002, Matsuda et al., 2001; Yang et al., 2001). Considered together, these data establish the essential role of nSREBPs in mediating the insulin-stimulated increase in fatty acid synthesis that occurs upon the feeding of high carbohydrate diets to mice.

The immunoblot data of Figure 2-4 illustrate the dramatic shifts in Insig isoforms that occur after fasting and refeeding in wild type mice. Previous studies documented this shift at the mRNA level, but the data of Figure 4 are the first confirmation at the protein level, owing to the recent generation of antibodies capable of recognizing endogenous Insig-1 and -2. Upon fasting, Insig-1 disappears and is replaced by Insig-2. Upon refeeding, Insig-2 markedly decreases and Insig-1 reappears. Inasmuch as SREBP-1c processing is blocked during fasting and returns after refeeding, these data suggest that Insig-2 may be responsible for this regulation.

In the Insig-1 transgenic mice, SREBP-1c processing was not enhanced to a normal extent during refeeding, even though Insig-2 levels declined (see lower panel of Figure 2-4). Apparently, the unphysiologically high levels of Insig-1 in the livers of the transgenic animals prevented SREBP-1c processing during refeeding, an action that might not occur when Insig-1 is present at its usual low level. To distinguish between the roles of Insig-1 and -2 in liver, we are currently preparing transgenic mice that overexpress Insig-2. We are also creating gene knockout animals that lack expression of the genes encoding Insig-1, Insig-2, and both.

The refed transgenic mice failed to show the normal rise in SREBP-1c mRNA that results from insulin action (Figure 2-5). This failure occurred even though plasma insulin rose to similar levels in the refed transgenic mice as in the wild type mice (Appendix B). Insulin had its normal effect on transcription of other hepatic genes, as reflected in the normal repression of IRS-2 and PEPCK mRNAs in the livers of the transgenic mice upon refeeding (see Figure 2-5). In studies to be published elsewhere, we have found that the promoter/enhancer of the *SREBP-1c* gene contains a functional sterol regulatory element, and this element is essential for the insulin-mediated enhancement of SREBP-1c mRNA levels. We believe that insulin fails to activate the *SREBP-1c* gene in the Insig-1 transgenic mice because of the deficiency of nSREBPs that results from the block in SREBP processing.

The current studies, considered together with studies in mice deficient in SCAP or Site-1 Protease, establish the role of SREBPs in mediating both cholesterol-repressible transcription and insulin-induced transcription of lipogenic genes in mouse liver. Complex control mechanisms determine the nuclear content of SREBPs under any metabolic state.

These mechanisms are a net result of metabolic and hormonal effects on transcription of the SREBP genes and on proteolytic processing of the SREBP proteins. Insigs, along with SCAP, appear to play central roles in this regulation, which in turn is essential for normal homeostasis of carbohydrates and lipids in mice.

METHODS

Materials and general methods. Blood was drawn from the retro-orbital sinus; the plasma was separated immediately and stored at -70^{0}C. Plasma glucose was measured using a glucose kit from Wako Chemicals USA Inc. (Richmond, Virginia, USA; Cat. No. 994-90902). Plasma free fatty acids were measured using a NEFA kit from Wako (Cat. No. 994-75409). Plasma insulin was measured using a rat insulin RIA kit from Linco Research Inc. (St. Charles, Missouri, USA; Cat. No. SRI-13K). Cholesterol and triglycerides in plasma and liver were measured as previously described (Yokode et al., 1990). Immunoblot analyses of mouse SREBP-1 and -2 were carried out as previously described (Shimano et al., 1997B).

Generation of transgenic mice overexpressing human Insig-1. To generate transgenic mice overexpressing human Insig-1 (TgInsig-1) in the liver, we used a pLiv-11 vector that contains the constitutive human *apoE* gene promoter and its hepatic control region (a gift from Dr. John Taylor) (Simonet et al.,1993; Allan and Taylor., 1996). The transgenic plasmid (pLiv-11-hInsig-1) was generated by cloning a cDNA fragment encoding the open reading frame of human Insig-1 into MluI-ClaI sites of pLiv-11. The 11-kb SalI-SpeI fragment of pLiv-11-Insig-1 was then isolated and injected into fertilized eggs to generate transgenic mice as previously described (Shimano et al., 1996). Tail DNA dot blot analyses

were performed to identify founder mice that harbored the integrated transgene. Positive founders were then subjected to partial hepatectomy and expression levels of the human Insig-1 transgene were determined by northern blot analysis. Mice with high levels of transgene expression in liver were bred to C57BL/6J X SJL/J F1 mice and four lines of TgInsig-1 mice were established.

The transgenic mice were maintained as hemizygotes by breeding with wild type C57BL/6J X SJL/J F1 mice. To genotype transgenic mice, tail DNA was prepared using a direct lysis kit from Viagen Biotech Inc. (Los Angeles, California, USA; Cat. No. 102-T) and used for PCR with the following primers:
5' primer, 5'-ATGGAGAGGAGGGGGCTGAGAATTGTGTGG-3'; and
3' primer, 5'-CTAAATTGGATTTTGCCAATAATGTCCAGC-3'. Tail DNA of TgInsig-1 mice produced a PCR product of 530-bp. All mice were housed in colony cages with a 12-hour light/12-hour dark cycle and fed Teklad Mouse/Rat Diet # 7002 from Harlan Teklad Premier Laboratory Diets (Madison, Wisconsin, USA). For animal experiments, nontransgenic littermates were used as controls for transgenic mice. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center at Dallas.

Diet studies. For the cholesterol feeding study, mice were fed a chow diet (0.02% cholesterol) or chow diet supplemented with 0.05%, 0.15%, or 0.5% of cholesterol for 2 days prior to study. For the fasting and refeeding experiments, mice were divided into three groups: nonfasted, fasted, and refed. The nonfasted group was fed ad libitum, the fasted group was fasted for 12 hours, and the refed group was fasted for 12 hours and then refed a

high carbohydrate/low fat diet (Cat. No. TD 88122; Harlan Teklad Diets) for 12 hours prior to study. The starting times for the feeding regimens were staggered so that all mice were sacrificed at the same time, which was at the end of the dark cycle.

Real-time RT-PCR. Total RNA was prepared from mouse tissues using an RNA STAT-60 kit from TEL-TEST "B" (Friendswood, Texas, USA). Equal amounts of RNA from 4 or 5 mice were then pooled and treated with DNase I (DNA-*free*™, Ambion Inc., Austin, Texas, USA). First strand cDNA was synthesized from 2 μg of DNase I-treated total RNA with random hexamer primers using the ABI cDNA synthesis kit (Cat. No. N808-0234, PE Biosystems, Foster City, California, USA). Specific primers for each gene were designed using Primer Express software (PE Biosystems). The real-time RT-PCR reaction was set up in a final volume of 20 μl containing 20 ng of reverse transcribed total RNA, 167 nM of the forward and reverse primers, and 10 μl of 2X SYBR Green PCR Master Mix (Cat. No. 4312704, PE Biosystems). PCR reactions were carried out in 384-well plates using the ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems). All reactions were done in triplicate. The relative amount of all mRNAs was calculated using the Comparative C_T method. Mouse apoB or cyclophilin mRNAs were used as the invariant controls. The primers for real-time PCR were described previously (Liang et al., 2002).

Antibodies against mouse Insig-1 and Insig-2. Polyclonal antibodies against mouse Insig-1 and Insig-2 were prepared by immunizing rabbits with $(His)_6$-tagged mouse Insig-1 and Insig-2 proteins produced from Sf9 cells using the BAC-to-BAC™ Baculovirus Expression System from Invitrogen (Carlsbad, California, USA). Briefly, cDNA fragments encoding the Insig-1 and Insig-2 open reading frames were generated by PCR using mouse

liver RNA as templates with a One-Step RT-PCR Kit from Clontech (Palo Alto, California, USA; Cat. No. K1403-1). The PCR products were subcloned into the pFastBac^{TM}HTA vector to generate pFastBac™HTA-Insig-1 and pFastBac™HTA-Insig-2, which were used to transform *E. coli* strain DH10Bac^{TM} to produce recombinant Bacmids. The recombinant Bacmids were then used to transfect Sf9 insect cells to produce recombinant baculoviruses. The recombinant baculoviruses were amplified and used to infect Sf9 insect cells to produce recombinant Insig-1 and Insig-2 proteins. The resultant $(His)_6$-tagged proteins were solubilized with Fos-Choline-13 from Anatrace Inc. (Maumee, Ohio, USA), purified by Ni-NTA affinity columns, and injected into rabbits.

Immunoblot analyses of Insig-1 and Insig-2. To prepare membrane fractions for immunoblot analyses, ~ 50 mg of frozen liver was homogenized in 1 ml buffer (20 mM Tris-HCl at pH 7.4, 2 mM $MgCl_2$, 0.25 M sucrose, 10 mM sodium EDTA, and 10 mM sodium EGTA) supplemented with protease inhibitor cocktail consisting of 5 mM DTT, 0.1 mM leupeptin, 1 mM PMSF, 0.5 mM Pefabloc, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 25 μg/ml ALLN, and 10 μg/ml aprotinin. After centrifugation at 8000 rpm for 5 min at 4°C in a microcentrifuge, the supernatant was centrifuged at $10^5 g$ for 30 min at 4°C in a Beckman TLA 100.2 rotor. The resulting membrane pellet was resuspended in 0.2 ml SDS-lysis buffer (10 mM Tris-HCl at pH 6.8, 1% (w/v) SDS, 100 mM NaCl, 1 mM sodium EDTA, and 1 mM sodium EGTA). After removal of an aliquot of the membrane fraction for measurement of protein concentration (BCA Kit, Pierce Biotechnology, Rockford, Illinois, USA), 150 μl of the remaining fraction was mixed with an equal volume of buffer containing 62.5 mM Tris-HCl at pH 6.8, 15% SDS, 8 M urea, 10% (v/v) glycerol, and 100 mM DTT. To this mixture

was added 100 µl of 4X SDS loading buffer (12 % SDS, 0.02% bromophenol blue, 30% glycerol, 0.15 M Tris.HCl at pH 6.8, and 6% β-mercaptoethanol). Equal amounts of protein from 4 to 5 mice per group were then pooled, and aliquots (45 µg) of the pooled membrane fraction were incubated at 37°C for 20 min and subjected to SDS-PAGE on 12% gels. After electrophoresis, the proteins were transferred to Hybond-C extra nitrocellulose filters from Amersham Biosciences (Piscataway, New Jersey, USA), and the filters were pre-incubated for 1 hour with blocking buffer (1X PBS with Tween 20 (Sigma-Aldrich Corp., St. Louis, Missouri, USA; Cat. No. P3563) containing 5% (v/v) newborn calf serum (BioWhitaker, Walkersville, Maryland, USA; Cat. No. 14-416Q) and 5% (w/v) milk), followed by incubation for 3 hours at room temperature with blocking buffer containing a 1:1000 dilution of either anti-Insig-1 or anti-Insig-2 antiserum. Bound antibodies were visualized with peroxidase-conjugated, affinity-purified donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, Pennsylvania, USA) using the SuperSignal CL-HRP substrate system (Pierce Biotechnology). Filters were exposed to Kodak X-Omat™ Blue XB-1 film at room temperature for 10-30 sec.

Cholesterol and fatty acid synthesis in vivo. Rates of cholesterol and fatty acid synthesis were measured in TgInsig-1 mice and their nontransgenic littermates using ^{3}H-labeled water as previously described (Shimano et al., 1996). The mice were either fasted for 12 hours or fasted for 12 hours and then refed a high carbohydrate/low fat diet (Cat. No. TD 88122; Harlan Teklad Diets) for 12 hours prior to study. The rates of cholesterol and fatty acid synthesis were calculated as µmoles of ^{3}H-radioactivity incorporated into fatty acids or digitonin-precipitable sterols per hour per gram of tissue.

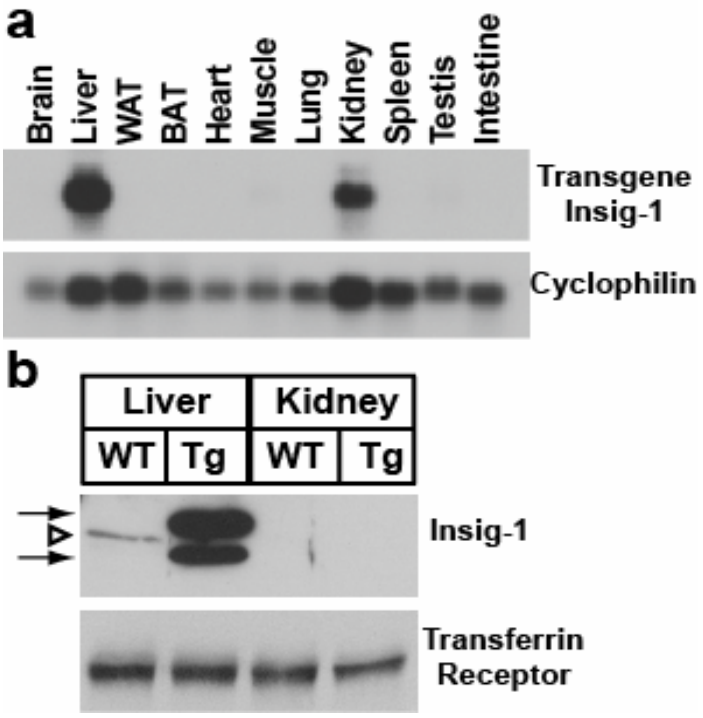


Figure 2-1
Transgene expression in TgInsig-1 mice. (**a**) Tissue distribution of human Insig-1 transgene mRNA. Total RNA was extracted from tissues of transgenic mice consuming a chow diet. A total of 20 μg of RNA was subjected to electrophoresis and blot hybridization with [α-^{32}P]-labeled cDNA probes for human Insig-1 and mouse cyclophilin. After stringent washing, the membranes were exposed to Kodak X-Omat™ Blue XB-1 films for 4-12 hours at –80°C. (**b**) Immunoblot analysis of endogenous mouse Insig-1 and transgenic human Insig-1 from livers and kidneys of wild type and TgInsig-1 mice. Membrane fractions from 5 wild type (WT) and 5 TgInsig-1 (Tg) mice (same as those described in Table 2-1) were prepared and pooled as described in Methods. Aliquots of the pooled membrane fraction (45 μg protein) were subjected to SDS-PAGE and immunoblot analysis as described in Methods. Filters were exposed to Kodak X-Omat™ Blue XB-1 film for 15 sec at room temperature. Open triangle denotes endogenous mouse Insig-1 (28 kDa); arrows denote transgenic human Insig-1 (doublet of 30 kDa and 26 kDa). Immunoblot of transferrin receptor (antibody from Zymed Laboratories Inc., San Francisco, California, USA; Cat. No. 13-6800) served as a loading control. WAT, white adipose tissue; BAT, brown adipose tissue.

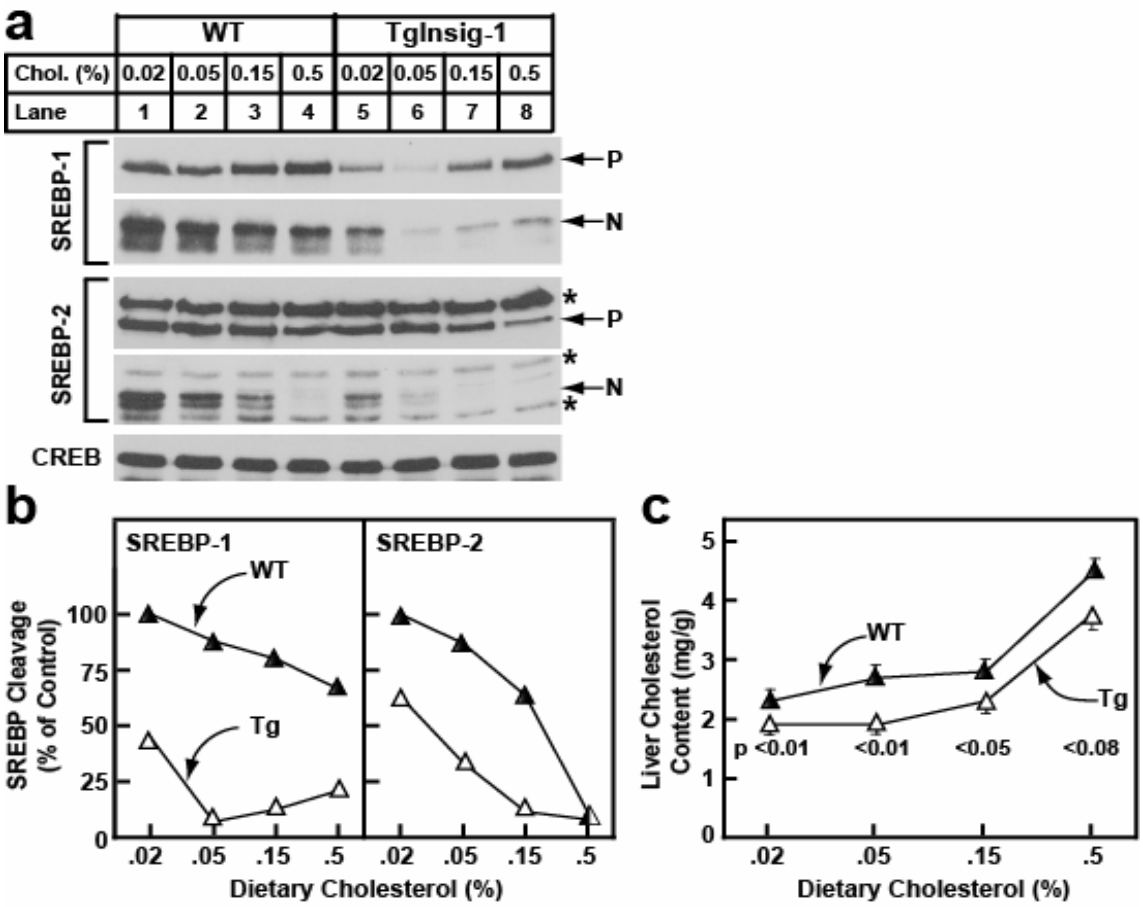


Figure 2-2

Overexpression of Insig-1 increases sensitivity of SREBP processing to inhibition by dietary cholesterol. 8-week old female mice (4 per group) were fed an ad libitum chow diet supplemented with the indicated amount of cholesterol for 2 days prior to study. The wild type mice were littermates of the transgenic mice. A detailed description of these mice is provided in Appendix A. (**a**) Immunoblot analysis. Livers from each group (4 mice per group) were pooled, and 30-μg aliquots of the membrane and nuclear extract fractions were subjected to SDS-PAGE and immunoblotted with antibodies against SREBP-1 and SREBP-2. P and N denote precursor and nuclear forms of SREBPs, respectively. Asterisks denote non-specific bands. CREB (cAMP-responsive element binding) protein was used as a loading control for the nuclear extract fractions (antibody from Cell Signaling Technology, Beverly, Massachusetts, USA; Cat. No. 9192). (**b**) The gels of nuclear extract fractions in (**a**) were scanned and quantified by densitometry. Intensities of the cleaved nuclear forms of SREBP-1 and SREBP-2 in lane 1 (wild type mice fed with 0.02% cholesterol) were arbitrarily set at 100%. (**c**) Cholesterol content of livers from wild type and TgInsig-1 mice fed for 2 days with the indicated amount of cholesterol. Each value represents the mean ± SEM of data from 4 mice. The levels of statistical significance (Student's *t* test) between the wild type and TgInsig-1 groups are shown as p values.

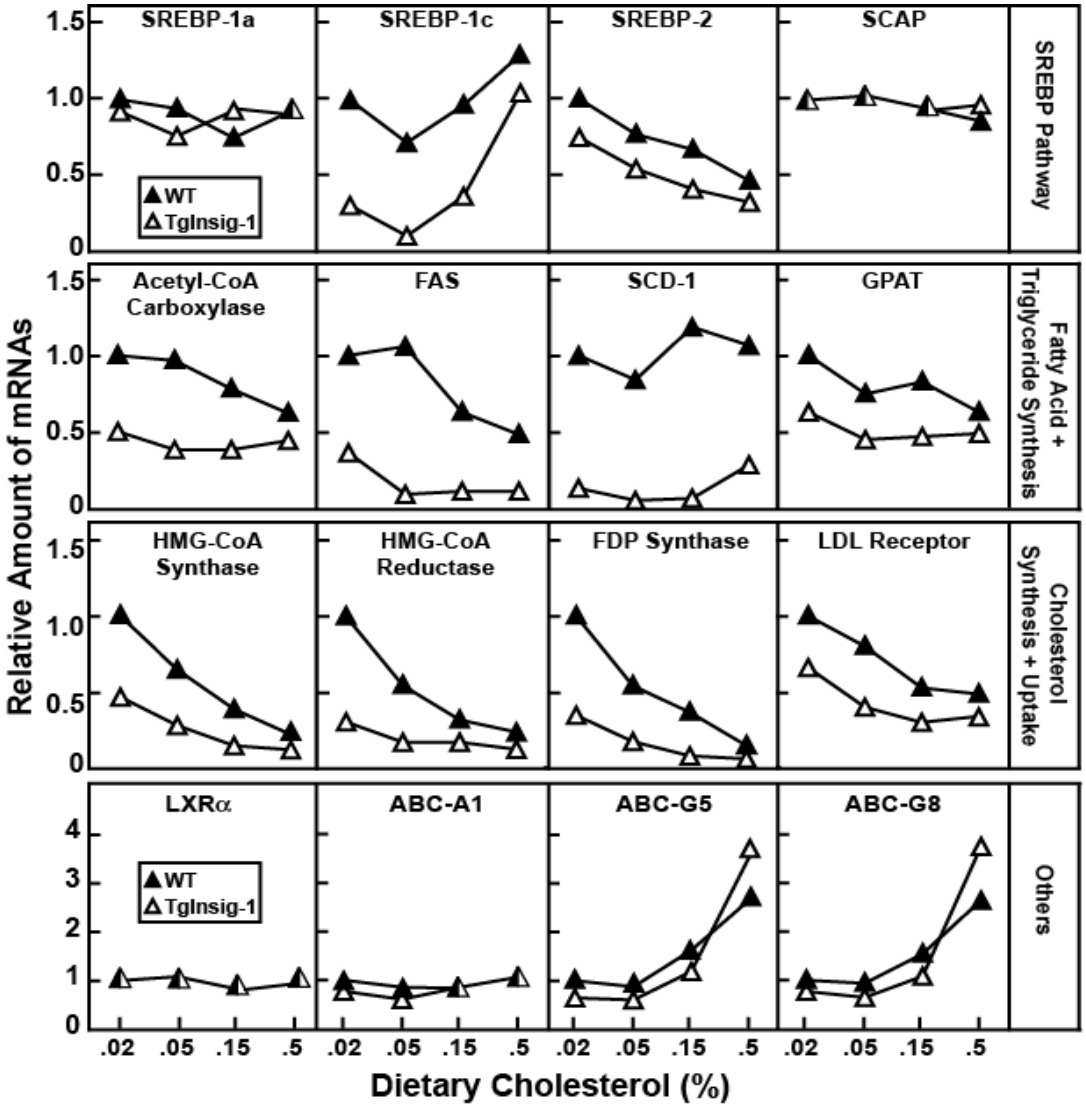


Figure 2-3
Relative amount of various mRNAs in livers from wild type and TgInsig-1 mice fed with increasing amounts of cholesterol (0.02 to 0.5%). The mice used here are the same as those used in Figure 2-2 and Appendix A. Total RNA from 4 mouse livers was pooled and subjected to real time PCR quantification as described in Methods. Each value represents the amount of mRNA relative to that in wild type mice fed a chow diet (0.02% cholesterol), which is arbitrarily defined as 1. FDP, farnesyl diphosphate; FAS, fatty acid synthase; SCD-1, stearoyl CoA desaturase-1; GPAT, glycerol-3-phosphate acyltransferase; LXR, liver X receptor.

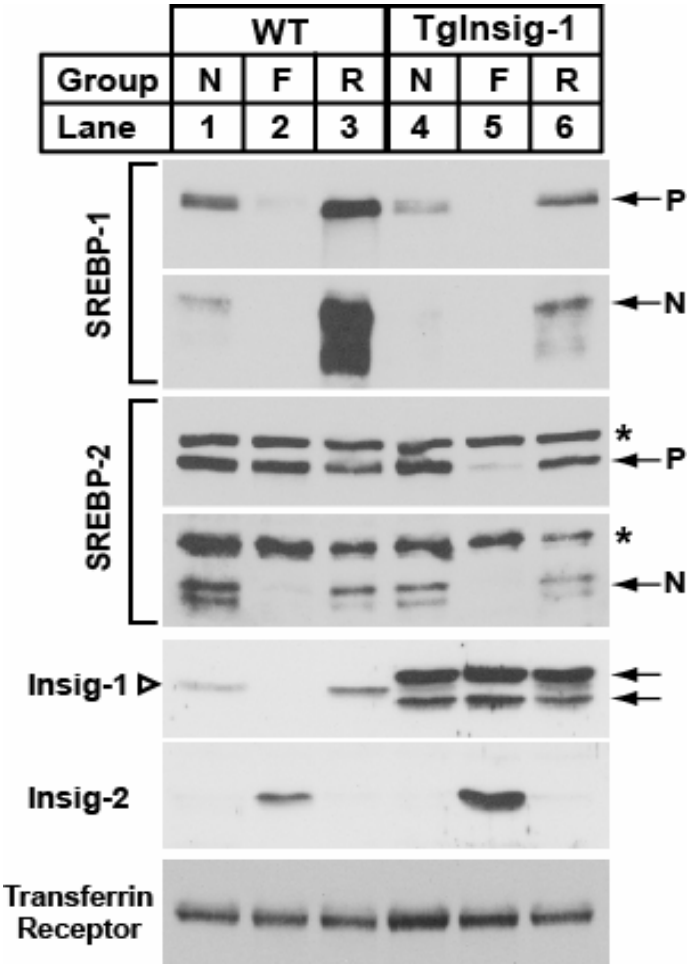


Figure 2-4

Effect of fasting and refeeding on SREBP and Insig proteins in livers of wild type and TgInsig-1 mice. 16-week old male mice (5 per group) were subjected to fasting and refeeding as described in Methods. The wild type mice were littermates of the transgenic mice. A detailed description of these mice is provided in Appendix B. The nonfasted group (N) was fed a chow diet ad libitum, the fasted group (F) was fasted for 12 hours, and the refed group (R) was fasted for 12 hours and then refed a high carbohydrate/low fat diet for 12 hours prior to study. Nuclear extract fractions were prepared from pooled livers (5 mice per group); membrane fractions were prepared individually and pooled as described in Methods. Aliquots of membrane and nuclear extract fractions (45 μg) were subjected to SDS-PAGE and immunoblot analysis as described in Methods and Figures 1 and 2. P and N denote precursor and nuclear forms of SREBPs, respectively. Asterisks denote non-specific bands. Open triangle denotes endogenous mouse Insig-1 (28 kDa) (lanes 1-3); arrows denote transgenic human Insig-1 (doublet of 30 kDa and 26 kDa) (lanes 4-6).

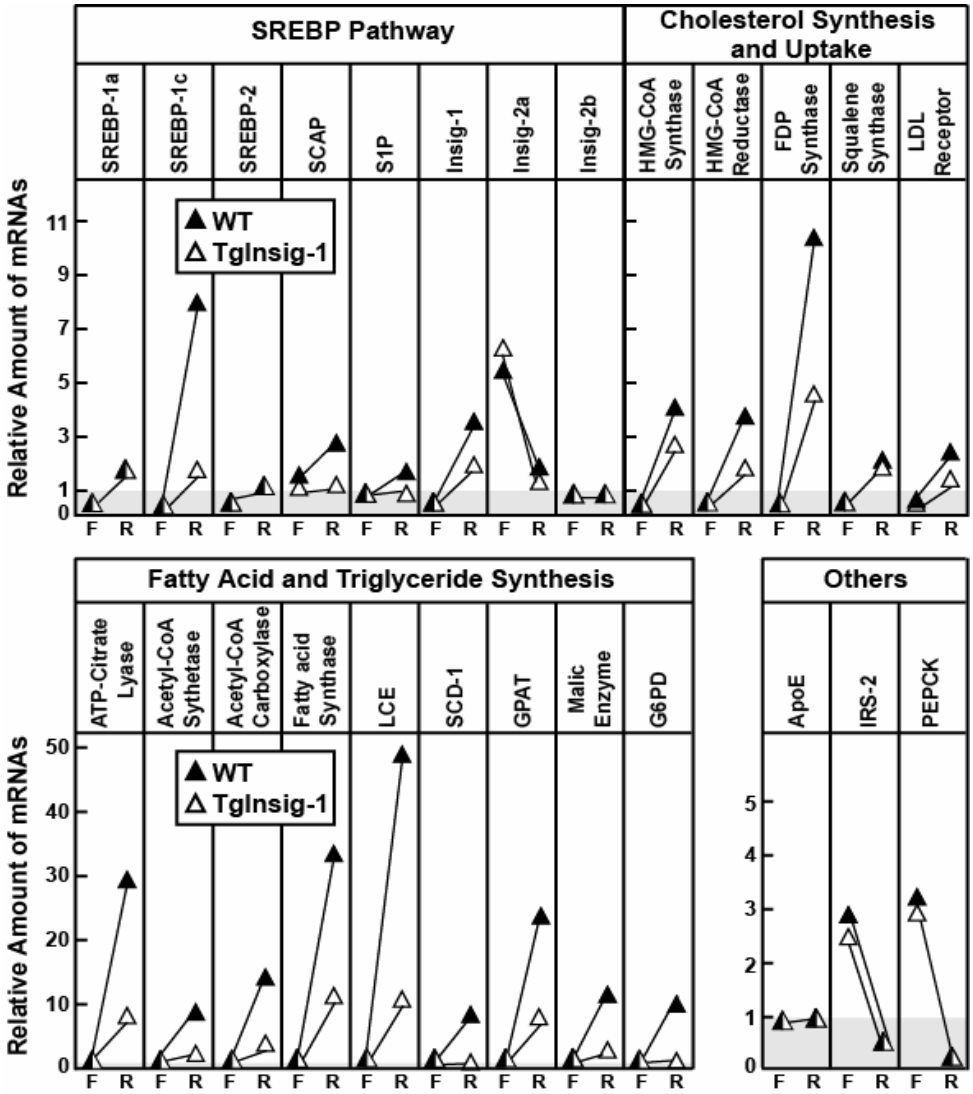


Figure 2-5
Relative amount of various mRNAs in livers from wild type and TgInsig-1 mice subjected to fasting and refeeding. The mice used here are the same as those used in Figure 2-4 and Appendix B. Total RNA from livers of mice was pooled (5 mice per group) and subjected to real time PCR quantification as described in Methods. Each value represents the amount of mRNA relative to that in the nonfasted wild type mice, which is arbitrarily defined as 1. For purposes of clarity, only the fasted (F) and refed (R) values are shown. LCE, long-chain fatty acyl elongase; G6PD, glucose-6-phosphate dehydrogenase; IRS-2, insulin receptor substrate-2; PEPCK, phosphoenol pyruvate carboxykinase; S1P, site-1 protease.

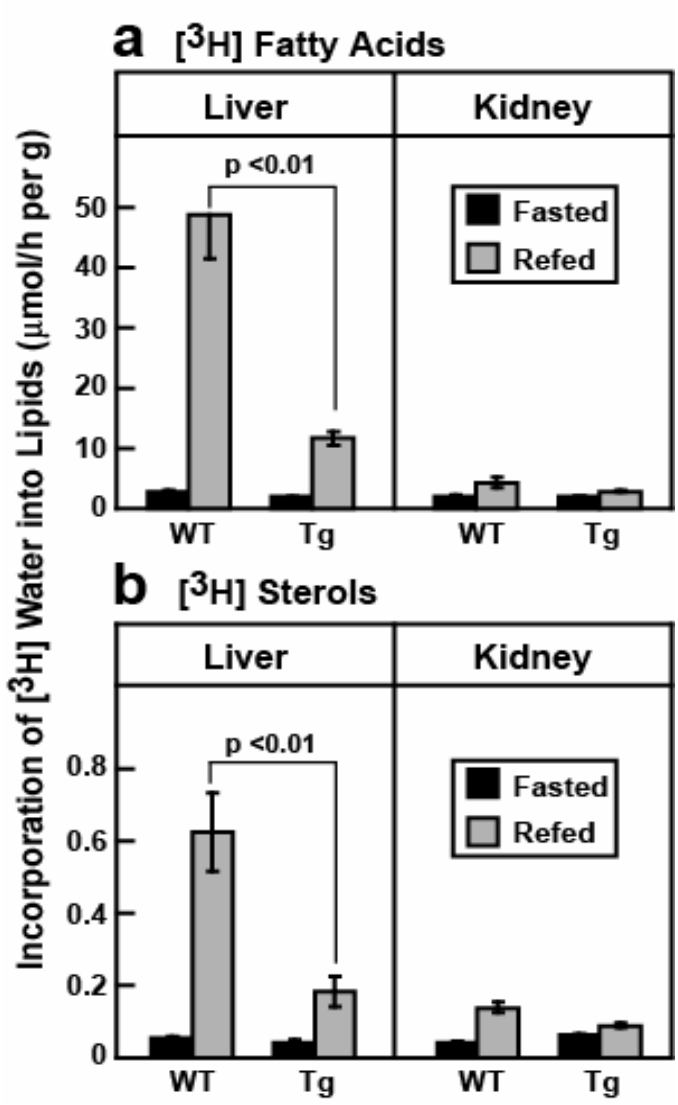


Figure 2-6
In vivo synthesis rates of fatty acids (**a**) and sterols (**b**) in liver and kidney from wild type and TgInsig-1 mice subjected to fasting and refeeding. Mice (16-week old male, 5 per group) were either fasted for 12 hours or fasted for 12 hours and then refed a high carbohydrate/low fat diet for 12 hours prior to study. Each mouse was then injected intraperitoneally with ^{3}H-labeled water (50 mCi in 0.25 ml of isotonic saline), and 1 hour later the tissues were removed for measurement of ^{3}H-labeled fatty acids and digitonin-precipitable sterols. Each bar represents the mean ± SEM of values from 5 mice. The levels of statistical significance (Student's *t* test) between the wild type and TgInsig-1 groups are shown as p values.

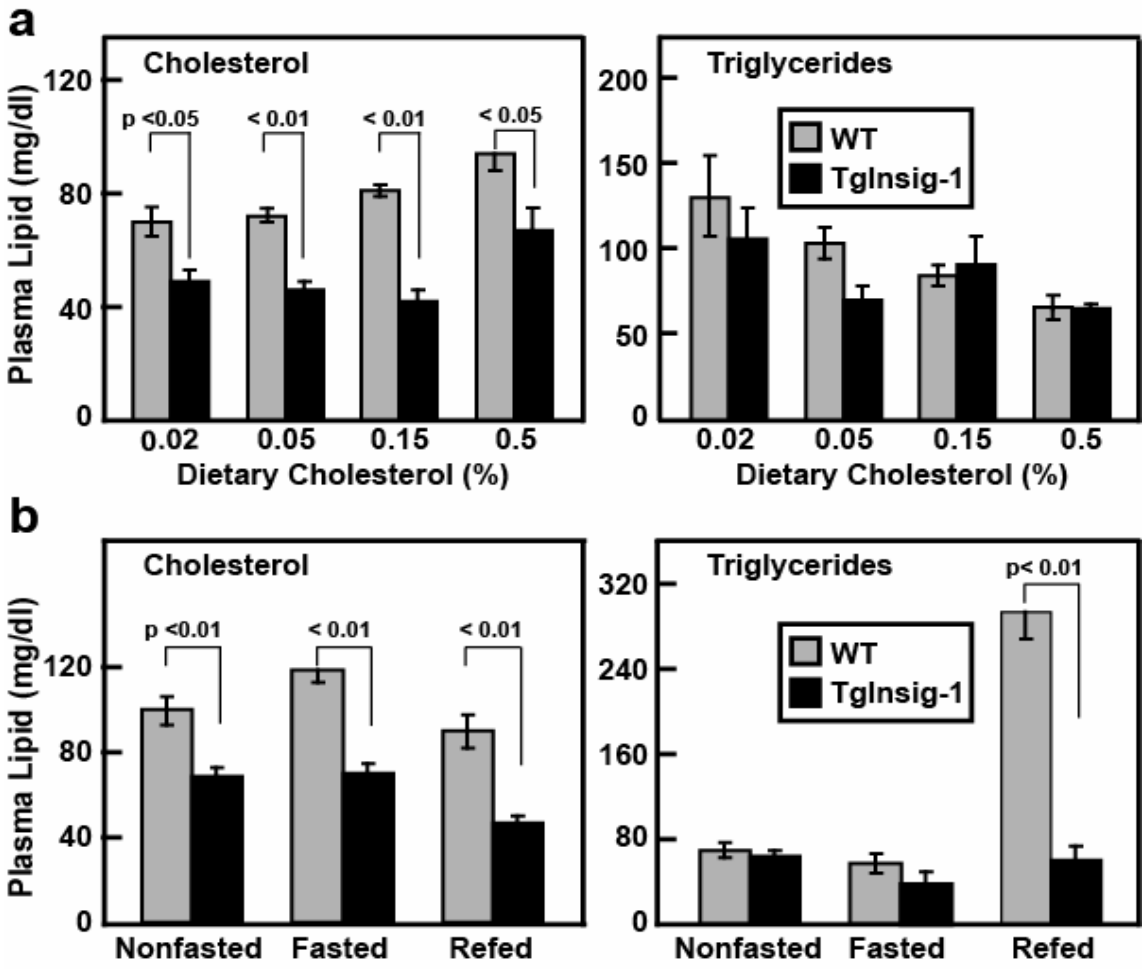


Figure 2-7
Plasma lipid levels in wild type and TgInsig-1 mice fed different amounts of cholesterol (**a**) or subjected to fasting and refeeding (**b**). Mice used in (**a**) are described in Figure 2-2; mice used in (**b**) are described in Figure 2-4. Each value is the mean ± SEM of data from 4 or 5 mice. The levels of statistical significance (Student's *t* test) between the wild type and TgInsig-1 groups are shown as p values.

CHAPTER THREE

SCHOENHEIMER EFFECT EXPLAINED – FEEDBACK REGULATION OF CHOLESTEROL SYNTHESIS IN MICE MEDIATED BY INSIG PROTEINS

ABSTRACT

End-product feedback inhibition of cholesterol synthesis was first demonstrated in living animals by Schoenheimer 72 years ago. The current studies define Insig proteins as essential elements of this feedback system in mouse liver. In cultured cells, Insig proteins are required for sterol-mediated inhibition of the processing of sterol regulatory element-binding proteins (SREBPs) to their nuclear forms. Here, we produced mice with germline disruption of the *Insig-2* gene and Cre-mediated disruption of the *Insig-1* gene in liver. On a chow diet, these double knockout mice over accumulated cholesterol and triglycerides in liver. Despite this accumulation, levels of nuclear SREBPs and mRNAs for SREBP target genes in lipogenic pathways were not reduced. Whereas cholesterol feeding reduced nuclear SREBPs and lipogenic mRNAs in wild type mice, this feedback response was severely blunted in the double knockout mice, and synthesis of cholesterol and fatty acids was not repressed. The amount of HMG-CoA reductase protein was elevated out of proportion to the mRNA in the double knockout mice, apparently owing to the failure of cholesterol to accelerate degradation of the enzyme. These studies indicate that the essential elements of the regulatory pathway for lipid synthesis function in liver as they do in cultured cells.

INTRODUCTION

In 1933, Rudolph Schoenheimer, a physician then working in Germany, performed a metabolic balance study in mice and observed that the animals synthesized large amounts of cholesterol when fed a low-cholesterol diet and that synthesis stopped when the mice were fed cholesterol (Schoenheimer and Breusch, 1933). This experiment provided the first evidence for end-product feedback regulation of a biosynthetic pathway, a discovery that predated by two decades the demonstration of end-product feedback inhibition in bacteria and prefigured much of modern molecular biology and metabolic science. With the advent of radioisotopes, Gould showed in the early 1950s that the cholesterol feedback system operated in the liver of living animals (Gould 1951; Gould et al., 1953).

In subsequent years many details of cholesterol feedback were elucidated, including the identification of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) as a highly regulated rate-controlling step (reviewed in Brown and Goldstein, 1980). However, the fundamental molecular mechanism by which liver cells sense cholesterol and use that information to regulate cholesterol production remained obscure until recent studies were conducted in cultured mammalian cells.

The cultured cell studies showed that transcription of HMG-CoA reductase and other genes in the cholesterol biosynthetic pathway is dependent upon sterol regulatory element-binding proteins (SREBPs), a family of sterol-regulated, membrane-bound transcription factors (Brown and Goldstein, 1997). The SREBPs begin life as integral proteins of the endoplasmic reticulum (ER). To gain access to nuclear DNA, the SREBPs must move in vesicles to the Golgi complex where they are processed proteolytically (Norturfft et al., 1999;

Norturfft et al., 2000). ER-to-Golgi transport is mediated by SCAP, a polytopic ER membrane protein that forms complexes with newly synthesized SREBPs. SCAP acts as a nucleation site for COPII proteins, which mediate the incorporation of the SCAP-SREBP complex into COPII coated vesicles that move to the Golgi (Espenshade et al., 2002; Sun et al., 2005).

In cultured cells feedback inhibition of cholesterol synthesis is mediated by Insigs, polytopic ER membrane proteins that serve as anchors (Yang et al., 2002; Yabe et al., 2002). When the cholesterol content of ER membranes rises, cholesterol binds to a membrane-embedded region of SCAP designated the sterol-sensing domain (Rahakrishnan et al., 2004), thereby causing a conformational change (Brown et al., 2002; Adams et al., 2004). The altered SCAP binds to Insigs, thus preventing exit of the SCAP-SREBP complex from the ER. As a result, the nuclear content of processed SREBPs declines, the cholesterol biosynthetic genes are not transcribed actively, and cholesterol synthesis ceases. When cultured cells are depleted of cholesterol, SCAP undocks from Insigs and the SCAP-SREBP complex then moves to the Golgi for processing and activation of cholesterol synthesis.

Insigs have a second action in the feedback regulation of cholesterol synthesis, namely, they mediate the rapid proteolytic degradation of HMG-CoA reductase (Sever et al., 2003A; Sever et al., 2003B). Like SCAP, HMG-CoA reductase is synthesized on ER membranes. The catalytic domain of the enzyme is attached to the membrane by virtue of an NH_2-terminal segment that contains 8 membrane-spanning helices (Gil et al., 1985; Roitelman et al., 1992). Like SCAP, the membrane-embedded portion of HMG-CoA reductase contains a sterol-sensing domain (Brown and Goldstein, 1999). When sterols

accumulate in ER membranes, they trigger the binding of HMG-CoA reductase to Insigs, initiating a process by which HMG-CoA reductase is ubiquitinated and degraded (Sever et al., 2003A; Sever et al., 2003B). When cells are depleted of sterols, HMG-CoA reductase has an extended lifetime, the amount of active enzyme increases, and this contributes to an increase in cholesterol synthesis in sterol-depleted cells (Nakanishi et al., 1988).

Two mammalian *Insig* genes have been identified, *Insig-1* and *Insig-2* (Dabe et al., 2002). They encode highly similar proteins (59% identity). In cultured cells the two *Insig* genes have redundant functions. Reduction of both Insig mRNAs, as achieved with RNA interference (Sever et al., 2003B) or by mutational inactivation in hamster cells (Lee et al., 2005) leads to an increase in nuclear SREBPs. More important, the nuclear SREBPs fail to decline after sterol treatment. The reduction in both Insig-1 and Insig-2 causes the level of HMG-CoA reductase protein to be elevated to an even greater extent than the level of reductase mRNA, owing to a failure of sterols to stimulate reductase degradation. Mutant hamster cells that are deficient in Insig-1, but not Insig-2, show partial defects in regulation of reductase degradation and SREBP processing (Sever et al., 2004).

The regulatory system in mouse liver is more complicated because the two *Insig* genes are under reciprocal regulation (Yabe et al., 2002). Expression of *Insig-1* in liver is dependent upon nuclear SREBPs, especially one isoform, designated SREBP-1c. Transcription of the *SREBP-1c* gene is induced by insulin (Chen et al., 2004). Thus, Insig-1 mRNA disappears during fasting when insulin levels are low and *SREBP-1c* is repressed. Conversely, Insig-1 mRNA rises to high levels upon refeeding when insulin rises and nuclear SREBP-1c increases (Yabe et al., 2003). In direct contrast, the *Insig-2* gene in liver is

transcribed by a hepatocyte-specific promoter (Insig-2a) that is repressed by insulin (Yabe et al., 2003). Under fasting conditions, when Insig-1 falls, Insig-2 rises. Upon refeeding, the two proteins are switched: Insig-2 disappears and is replaced by Insig-1.

A regulatory role for Insig proteins in liver was inferred from experiments in transgenic mice that overexpress Insig-1 (Engelking et al., 2004). In these mice the content of nuclear SREBPs was reduced, and the target lipogenic mRNAs were all decreased. Nuclear SREBPs and target mRNAs declined further in a supersensitive fashion in response to cholesterol feeding. These experiments demonstrated that Insig-1 could act in liver when it was overexpressed, but they did not indicate whether Insigs play a role under normal conditions. In the current paper, we have addressed these questions by disrupting the genes for *Insig-1* and *Insig-2*, together or separately, in livers of mice. The results indicate that Insigs are essential components of the cholesterol feedback system that was defined by Schoenheimer and Gould.

RESULTS

Figure 3-1A shows the gene targeting strategy for mouse *Insig-1*. A neomycin resistance cassette (*neo*) flanked by *loxP* and *frt* sites was inserted into the 5′-flanking region of the *Insig-1* gene. An additional *loxP* site was inserted into the first intron. Cre-mediated recombination removes exon 1 of *Insig-1*. Mice carrying the floxed *Insig-1* allele were bred to transgenic mice that express Cre recombinase driven from the interferon-responsive *MX1* promoter (*MX1*-Cre) to derive mice homozygous for the floxed *Insig-1* allele and hemizygous for the *MX1*-Cre transgene (*Insig-1*$^{f/f}$*;MX1-Cre*). When injected four times with

a synthetic double-stranded ribonucleotide polyinosinic acid-polycytidylic acid (pIpC), these mice produce interferon, which activates the *MX1* promoter, thereby producing the Cre recombinase in liver and other tissues (Kuhn et al., 1995). Figure 3-1B shows Southern blot analysis of *NcoI* -digested genomic DNA extracted from the livers of pIpC-treated mice of the indicated genotypes and probed with a 299-bp genomic fragment at the 5′ end of the *Insig-1* gene. In wild type mice and mice hemizygous for the *MX1*-Cre transgene, a 9-kb band is observed that is unaffected by the expression of the Cre transgene. In *Insig-1*$^{f/f}$ mice, a 3-kb band is observed which is markedly decreased in *Insig-1*$^{f/f}$*;MX1-Cre* mice and is replaced by a 6-kb band that results from recombination between *loxP* sites. This recombination eliminates sequences encoding the first 119 of the 259 amino acids of Insig-1.

Figures 3-1C and 3-1D show the expression of Insig-1 by northern blot analysis of total RNA and by immunoblotting of membrane proteins prepared from the same livers as those used in Figure 3-1B. Levels of hepatic Insig-1 mRNA and protein were identical in wild-type and *MX1*-Cre mice and were reduced by 20% in *Insig-1*$^{f/f}$ mice, perhaps owing to transcriptional interference from the *neo* cassette. Insig-1 mRNA and protein declined by more than 90% in *Insig-1*$^{f/f}$*;MX1-Cre* mice injected with pIpC as compared with wild type mice. In other studies using animals that were not injected with pIpC, the expression of Insig-1 mRNA in *Insig-1*$^{f/f}$*;MX1-Cre* was the same as in *Insig-1*$^{f/f}$ mice (data not shown).

Figure 3-1E shows the strategy for germline targeting of mouse *Insig-2*. Exons 2 and 3 of *Insig-2* were replaced by a *neo* cassette, which eliminates sequences encoding the first 123 of 225 amino acids of Insig-2. Mating between *Insig-2*$^{+/-}$ mice yielded +/+, +/-, and -/- mice at the expected 1:2:1 ratio. Figure 3-1F shows representative Southern blots of *NcoI*-

digested genomic DNA extracted from the tails of *Insig-2* +/+, +/-, and -/- littermates probed with a 505-bp genomic fragment containing exon 6 of the *Insig-2* gene. The expression of Insig-2 mRNA and protein in these mice is shown in Figure 3-1G and 3-1H, respectively. When probed with a cDNA containing part of the 3´-untranslated region of Insig-2, an aberrant transcript is observed in *Insig-2* +/- and -/- mice, perhaps owing to a transcript initiating in exons 1a or 1b and splicing directly into exons 4, 5, or 6 (Figure 3-1G). In such a transcript, the first available inframe methionine is located in exon 5. If translated, this transcript would yield a peptide of only 23 amino acids. Levels of hepatic Insig-2 protein, as detected by immunoblotting with antiserum raised against full-length mouse Insig-2, were reduced by approximately 50% in *Insig-2* +/- livers and were undetectable in -/- livers (Figure 3-1H).

Mice carrying the floxed *Insig-1* allele were also bred to transgenic mice that express Cre recombinase driven from the adenovirus *EIIA* promoter (*EIIA-Cre*). *EIIA-Cre* mice express Cre in germ cells which results in germline deletion of the floxed *Insig-1* allele. *Insig-1*$^{+/-}$*;Insig-2*$^{+/-}$ (+-/+-) mice were mated to each other in an attempt to produce *Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ (--/--) mice. When the surviving adults were genotyped, 0 of 6 expected --/-- mice were observed, and only 3 of 12 expected --/+- mice were observed (Table 3-1).

Further breeding experiments were conducted in which +-/-- mice were interbred, and pregnant females were killed at 12.5 and 18.5 days post coitum (dpc). At 12.5 dpc, the observed ratio of ++/-- , +-/--, and --/-- was 46:81:41, which is consistent with the expected 1:2:1 ratio (Table 3-1). At 18.5 dpc, 82% of the expected --/-- embryos were

Table 3-1
Segregation of disrupted *Insig-1* and *Insig-2* alleles in mice

Mating	Age of offspring	Number of litters	Number of offspring	Genotype of offspring, Insig-1 / Insig-2 Observed number (expected number)				
Insig-1/Insig-2 Genotype				++/++	+-/--	--/+-	--/--	‡ All other genotypes
+-/--M X +-/--F	12.5 dpc	20	168	*	81 (84)	*	41 (42)	46 (42)
+-/--M X +-/--F	18.5 dpc	14	112	*	58 (56)	*	23 (28)	31 (28)
+-/+-M X +-/+-F	Adult	11	95	7 (6)	11 (12)	3 (12)	0 (6)¶	74 (59)

Genotype was determined by PCR analysis of DNA prepared from embryonic tissues at the indicated day post coitum (dpc) or from tails of adult mice.

‡ Other genotypes include: +-/++, ++/+-, +-/+-, --/++, and ++/--.

* Not applicable.

¶ $p < 0.01$ by Fisher Exact test

observed. Further studies into the cause of the neonatal lethality in -/- mice are ongoing and are beyond the scope of this work.

In order to study the metabolic effects of total Insig deficiency in adult livers, *Insig-1*$^{f/f}$*;MX1-Cre* mice were bred with *Insig-2*$^{-/-}$ mice. Mice of 4 genotypes were produced: *Insig-1*$^{f/f}$ (designated control), *Insig-1*$^{f/f}$*;MX1-Cre* (designated *L-Insig-1*$^{-/-}$ to denote the conditional deficiency of Insig-1 in Liver when induced with pIpC), *Insig-1*$^{f/f}$; *Insig-2*$^{-/-}$ (hereafter designated *Insig-2*$^{-/-}$), and *Insig-1*$^{f/f}$*;Insig-2*$^{-/-}$;*MX1-Cre* (designated *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$). For the studies described in Figures 3-2 to 3-6, all of the mice in each group were injected four times with pIpC and then studied between 7 and 14 days after the last injection.

When fed a chow diet *ad libitum* and observed 14 days after the last pIpC injection, mice from all genotypes were indistinguishable by external appearance and body weight (Table 3-2). Plasma cholesterol was slightly elevated and plasma triglycerides were slightly reduced in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice ($p<0.05$, *Student's t* test). Plasma insulin, glucose, and free fatty acids were similar among the 4 groups.

Figure 3-2A shows immunoblots of pooled hepatic membrane fractions and nuclear extracts from pIpC treated mice of the 4 genotypes described in Table 3-2. Mice with single deficiencies of hepatic Insig-1 or Insig-2 did not compensate by increasing the expression of Insig-2 or Insig-1, respectively. Levels of hepatic Insig-1 protein were dramatically reduced in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ and *L-Insig-1*$^{-/-}$ mice. Hepatic Insig-2 protein was undetectable in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ and *Insig-2*$^{-/-}$ mice. Levels of the membrane-bound precursors of

Table 3-2

Comparison of control, *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$, *L-Insig-1*$^{-/-}$, and *Insig-2*$^{-/-}$ mice

Parameter	**Control**	***L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$**	***L-Insig-1*$^{-/-}$**	***Insig-2*$^{-/-}$**
Number of mice	6	6	6	6
Body weight (g)	26.6 ± 1.0	24.5 ± 0.9	27.6 ± 1.2	25.4 ± 0.5
Liver weight (g)	1.39 ± 0.05	1.42 ± 0.07	1.43 ± 0.09	1.37 ± 0.05
Plasma cholesterol (mg/dl)	81 ± 3	110 ± 7*	88 ± 4	75 ± 6
Plasma triglycerides (mg/dl)	76 ± 8	55 ± 5*	68 ± 5	65 ± 6
Plasma insulin (ng/ml)	1.04 ± 0.13	0.85 ± 0.08	1.02 ± 0.10	0.98 ± 0.16
Plasma glucose (mg/dl)	149 ± 4	147 ± 8	145 ± 6	138 ± 7
Plasma free fatty acids (mM)	0.36 ± 0.02	0.45 ± 0.06	0.40 ± 0.02	0.34 ± 0.02

10-week old male mice were fed a chow diet prior to study. Each mouse was treated with 4 intraperitoneal injections of pIpC (300 μg/injection), and tissues were obtained 14 d after the final injection. Mice had free access to food and were killed at the end of the dark cycle. Each value represents the mean +/- SEM of six values. *Asterisk* denotes the level of statistical significance between the control and *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$, *L-Insig-1*$^{-/-}$, or *Insig-2*$^{-/-}$ groups (Student's *t test*).

*****, $p < 0.05$

SREBP-1 and SREBP-2 (pSREBP-1 and -2) were largely unaffected by deficiency of one or both Insig proteins. A small increase in SCAP protein was consistently observed in the livers of mice deficient in Insig-1. Nuclear SREBP-1 (nSREBP-1) levels were increased by 2-fold in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice compared to control mice, while nuclear SREBP-2 (nSREBP-2) levels were unchanged. Hepatic HMG-CoA reductase protein levels were slightly elevated in *Insig-2*$^{-/-}$ mice and were dramatically elevated in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice.

Figure 3-2B shows the liver cholesterol and triglyceride content in the mice that were described in Table 3-2. The hepatic content of total cholesterol and triglycerides were increased by 4- and 6-fold, respectively in the *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice compared to control mice on the same diet. In the double knockout livers, the free cholesterol content rose by 1.4-fold, and the cholesteryl ester content rose by 15-fold (data not shown). Hepatic lipid levels were normal in mice with the other genotypes. These data indicate that loss of both Insigs is necessary in order to perturb lipid metabolism grossly in mouse liver. For this reason, in all subsequent studies we used *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice.

Table 3-3 shows the relative expression of various mRNAs in livers from *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice studied in three different experiments. The values in control mice for each experiment were assigned as 1. The mean value for Insig-1 mRNA was reduced by 95% compared to control mice, which reflects the direct action of the Cre recombinase. The *Insig-2* gene has two promoter/enhancer regions that give rise to two transcripts, denoted Insig-2a and Insig-2b (Yabe et al., 2003). The expression of both transcripts was undetectable in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ livers. The mean mRNA values for SREBP-1a,

Table 3-3

mRNAs in liver of *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice as compared with values in control mice

mRNA	Relative amount of mRNAs in liver of *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice			
	Exp. A*	Exp. B†	Exp. C¶	**Mean**
Insigs				
Insig-1	0.05	0.09	0.00	**0.05**
Insig-2a	0.00	0.00	0.00	**0.00**
Insig-2b	0.00	0.00	0.00	**0.00**
SREBP pathway				
SREBP-1a	1.1	1.5	1.3	**1.3**
SREBP-1c	1.1	1.8	2.5	**1.8**
SREBP-2	1.0	1.2	1.0	**1.1**
SCAP	1.2	1.3	1.4	**1.3**
Fatty acid synthesis				
ATP citrate lyase	1.8	1.9	4.2	**2.6**
Acetyl-CoA carboxylase	1.9	1.8	4.5	**2.7**
Fatty acid synthase	2.9	2.6	7.9	**4.5**
Stearoyl-CoA desaturase-1	2.9	3.1	10.2	**5.4**
Glucose-6-phosphate dehydrogenase	4.4	2.9	15.9	**7.7**
Cholesterol synthesis and uptake				
HMG-CoA synthase	0.6	1.5	1.5	**1.2**
HMG-CoA Reductase	1.4	2.4	2.1	**2.0**
FDP synthase	0.9	1.6	nd	**1.3**
LDL receptor	0.9	1.6	1.4	**1.2**
Others				
PEPCK	0.8	0.9	0.6	**0.8**
LXRα	0.9	1.4	1.2	**1.2**
ABCG5	2.2	3.1	4.7	**3.4**
ABCG8	2.0	2.9	4.0	**3.0**
Lipoprotein lipase	3.5	3.3	9.0	**5.3**

The mice used in these experiments (see below) were fed a chow diet *ad libitum* prior to the study. For each group, equal amounts of total RNA from the livers of 4 to 6 mice were pooled and subjected to real-time PCR quantification as described in Methods. ApoB was used as the invariant control. Values represent the amount of mRNA relative to that in control mice in each experiment, which is arbitrarily defined as 1. FDP, farnesyl diphosphate. PEPCK, phosphoenolpyruvate carboxykinase; nd, not measured.

*10-week-old male mice; 14 days after final pIpC injection (same mice as in Table 3-2)

†23 to 27-week-old female mice; 9 days after final pIpC injection

¶23 to 27-week-old male mice; 13 days after final pIpC injection (same mice as those in Table 3-5 fed the chow diet containing 0.02% cholesterol)

SREBP-2, and SCAP were unaffected by Insig deficiency whereas a small increase in SREBP-1c expression (mean of 1.8-fold) was observed. The mRNAs of 5 SREBP target genes involved in fatty acid synthesis were increased by 2.6- to 7.7-fold, consistent with the increase in nSREBP-1 in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ livers (see Figure 3-2A). mRNAs of 4 SREBP targets involved in cholesterol synthesis were generally the same as those in the control mice except for a 2-fold increase in HMG-CoA reductase mRNA. When considered in light of the large increase in the cholesterol content of the Insig-deficient livers, these 4 mRNAs were all inappropriately elevated (see below). mRNAs for ABCG5, ABCG8, and lipoprotein lipase - three genes known to be stimulated by LXRα and LXRβ (Repa and Mangelsdorf, 2002) - were increased in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ livers by 3.0- to 5.3-fold. We attribute their increase to the activation of LXRs by the sterols (Repa and Mangelsdorf, 2002) that accumulate in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ livers. The mRNA for LXRα itself was unaltered.

Figure 3-3A shows the gross appearance of the livers of control and *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ female mice fed a chow diet *ad libitum* 12 days after injection with pIpC. The liver of the *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mouse is pale, owing to the accumulation of lipids. The livers from mice of the other 3 genotypes were normal in appearance (data not shown). When liver sections were stained with Oil Red O, a fat-specific dye, the increase in lipid was dramatic in the *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice (Figure 3-3B).

In cultured cells, the reduction of Insig expression creates a relative resistance to the effects of sterols in blocking SREBP processing (Adams et al., 2004) and accelerating HMG-CoA reductase degradation (Sever et al., 2003B, Lee et al., 2005). A similar resistance was

apparent in the livers of *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. This resistance can be inferred from the observation that nuclear SREBPs were still present, and HMG-CoA reductase protein was increased in these livers despite the accumulation of cholesterol to levels that would have reduced these proteins in wild type mice.

To compare directly cholesterol regulation in livers of control and *L-Insig-1*$^{-/-}$; *Insig-2*$^{-/-}$ mice, the mice were fed diets containing varying amounts of cholesterol for 2.5 d, after which precursor and nuclear SREBPs and HMG-CoA reductase were measured by immunoblotting (Figure 3-4A). Metabolic measurements in these mice are provided in Table 4. When consuming the unsupplemented chow diet, the levels of nSREBP-1 were elevated by 2-fold, and levels of nSREBP-2 were unaltered in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. In control mice fed 2% cholesterol, nSREBP-2 was reduced by 93% (Figures 3-4A and 3-4B). This decline was severely blunted in *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice, in whom nSREBP-2 decreased only 17%. When fed high cholesterol diets, nSREBP-1 decreased slightly in control mice, and even less in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. nSREBP-1 remained elevated in the knockout animals at all levels of dietary cholesterol.

Whereas HMG-CoA reductase protein levels were markedly reduced in control mice fed the 0.2% and 2.0% cholesterol diets, the elevated levels in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice were not affected (Figure 3-4A). Insig-1 and Insig-2 protein levels were unaffected by cholesterol feeding in control mice and were undetectable in the knockout animals (Figure 3-4A). Hepatic cholesterol content was markedly elevated in the knockout mice, and it did not

Table 3-4

Effects of dietary cholesterol feeding in control and *L-Insig-1$^{-/-}$;Insig-2$^{-/-}$* mice

	Control			*L-Insig-1$^{-/-}$;Insig-2$^{-/-}$*		
Dietary cholesterol (%)	**0.02%**	**0.2%**	**2.0%**	**0.02%**	**0.2%**	**2.0%**
Number of mice	6	6	6	4[§]	6	6
Body weight (g)	24.8 ± 1.1	24.7 ± 0.4	25.2 ± 0.4	25.7 ± 0.8	25.8 ± 0.9	25.4 ± 1.1
Liver weight (g)	1.25 ± 0.04	1.26 ± 0.02	1.35 ± 0.06	1.82 ± 0.07*	1.72 ± 0.12[‡]	1.93 ± 0.15[‡]
Liver cholesterol content (mg/g)	3.2 ± 0.2	4.3 ± 0.4	11.3 ± 0.6	22.4 ± 3.1*	25.4 ± 2.5*	26.0 ± 1.5*
Liver triglyceride content (mg/g)	15.5 ± 3.0	22.8 ± 6.9	28.8 ± 3.8	75.5 ± 11.7*	84.2 ± 9.1*	106.3 ± 5.0*
Plasma cholesterol (mg/dl)	85 ± 11	91 ± 3	91 ± 12	102 ± 4	95 ± 9	113 ± 12
Plasma triglycerides (mg/dl)	64 ± 10	46 ± 6	39 ± 5	41 ± 9	36 ± 4	34 ± 5
Plasma insulin (ng/ml)	0.46 ± 0.18	0.53 ± 0.11	0.56 ± 0.19	0.97 ± 0.16	0.63 ± 0.21	0.59 ± 0.14
Plasma glucose (mg/dl)	100 ± 5	108 ± 8	112 ± 7	126 ± 11	108 ± 6	123 ± 6
Plasma free fatty acids (mM)	0.47 ± 0.06	0.35 ± 0.05	0.32 ± 0.04	0.35 ± 0.04	0.45 ± 0.07	0.44 ± 0.09

Female mice (20-24 weeks of age) were fed *ad libitum* a chow diet containing the indicated amount of cholesterol for 2.5 d prior to study. Each mouse was treated with 4 intraperitoneal injections of pIpC (300 μg/injection), and tissues were obtained 11 d after the final injection. Each value represents the mean ± SEM of 4 or 6 values. *Asterisks* denote the level of statistical significance (Student's *t* test) between the control and *L-Insig-1$^{-/-}$;Insig-2$^{-/-}$* groups.

[§]Two of the 6 mice in this group did not respond to treatment with pIpC as assessed by quantitative real time PCR. The percent reduction in Insig-1 mRNA in these 2 mice was < 40% as compared to > 90% in the other *L-Insig-1$^{-/-}$;Insig-2$^{-/-}$* mice.

[‡], $p < 0.01$

*, $p < 0.001$

increase further with cholesterol feeding (Figure 3-4C). Hepatic cholesterol increased in the control mice, but it did not reach the high levels seen in the knockout animals.

To confirm that the elevated levels of HMG-CoA reductase protein in the *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ livers were associated with increases in HMG-CoA reductase activity, we assayed the enzyme activity in liver homogenates. Inasmuch as most HMG-CoA reductase molecules are phosphorylated and inactive (Brown et al., 1975; Sato et al., 1993), we homogenized the livers in two different buffers. One of these contains sodium fluoride, which stops dephosphorylation during homogenization and therefore is believed to reveal true endogenous HMG-CoA reductase activity. The other contains sodium chloride, which permits dephosphorylation and therefore represents the maximal activity from the enzyme (Brown et al., 1975). In control mice, cholesterol feeding markedly reduced HMG-CoA reductase activity regardless of the homogenization buffer (Table 3-5). In the *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice on a chow diet, reductase activity was markedly elevated, and it failed to decline after cholesterol feeding. The relative results were the same when the livers were homogenized in fluoride or chloride.

Relevant hepatic mRNAs were quantified by real-time PCR in the livers of mice fed low-cholesterol chow (0.02%, L) or the high cholesterol (2.0%, H) diet (Figure 3-5). Liver expresses two major isoforms of SREBPs: SREBP-1c and SREBP-2 (Shimomura et al., 1997). Although their functions overlap, SREBP-1c preferentially activates the genes involved in fatty acid and triglyceride synthesis while SREBP-2 preferentially activates the genes required for cholesterol synthesis (Horton et al., 2002). mRNAs for both SREBP-1c and -2 are enhanced by nSREBPs themselves in a feed-forward activation loop and, in

Table 3-5
HMG-CoA reductase activity in livers of cholesterol-fed mice

Genotype of Mice	Cholesterol in Diet	HMG-CoA reductase Activity (pmol / min per mg protein)	
		+ NaF	+ NaCl
Control	0.02%	161 ± 10	998 ± 19*
	1.5%	39 ± 5	59 ± 13
L-Insig-1$^{-/-}$;*Insig-2*$^{-/-}$	0.02%	2169 ± 120	5423 ± 203
	1.5%	2436 ± 45	5024 ± 157

Male mice (23 to 27 weeks of age, 5 per group) were fed *ad libitum* a chow diet containing the indicated amount of cholesterol for 2.5 d prior to study. Each mouse was treated with 4 intraperitoneal injections of pIpC (300 µg/injection), and tissues were obtained 13 days after the final injection. Livers from 5 mice in each group were individually homogenized in buffer containing either NaF or NaCl, after which the homogenates were pooled and the resulting microsomes were assayed for HMG-CoA reductase activity in the presence of either of NaF or NaCl as indicated.
*Mean ± SEM of triplicate assays.

addition, SREBP-1c mRNA is induced by insulin and by agonists of LXRs (Horton et al., 2002; Repa et al., 2000). In control mice, SREBP-1c mRNA was induced 2-fold by high-cholesterol feeding, which we attribute to the activation of LXRs by oxysterols generated from dietary sterols (Debose-Boyd et al., 2001). In the sterol-overloaded livers of *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice, SREBP-1c mRNA was elevated by 2-fold and did not rise further after cholesterol feeding. SREBP-2 mRNA was suppressed by 46% in control mice fed the high cholesterol diet, and this decline was blunted in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. The decline of SREBP-2 mRNA in control mice is likely due to the loss of feed-forward activation as

SREBP processing is reduced by the increase in hepatic cholesterol. In the double knockout mice, the loss of Insigs prevents cholesterol from blocking SREBP processing, and thus the SREBP-2 mRNA does not decline. Consistent with this hypothesis, SREBP-2 target mRNAs for genes involved in cholesterol synthesis (HMG-CoA reductase, HMG-CoA synthase, farnesyl diphosphate synthase, and squalene synthase) declined by more than 75% when control mice were fed the high cholesterol diet. These mRNAs were elevated by 1.7- to 2.6-fold in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$. They declined partially when the animals were fed the high cholesterol diet, but remained higher than or equal to levels in control mice fed the low cholesterol diet.

mRNAs for genes involved in fatty acid and triglyceride synthesis were elevated 2.5- to 5.5-fold in the *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. These mRNAs declined only slightly with cholesterol feeding in both the control and the knockout animals (Figure 3-5). mRNAs for the LXR-responsive genes ABCG5 and ABCG8 were induced by high cholesterol feeding in control mice. These mRNAs were elevated in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice, and they did not increase with cholesterol feeding, consistent with the hypothesis that hepatic LXRs were already activated by sterols in the livers of the *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice.

To test the effect of Insig deficiency on hepatic lipid synthesis, male control and *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice were fed diets containing 0.02% (low) or 1.5% (high) cholesterol for 2.5 d, after which in vivo lipid synthesis was determined by measurement of the incorporation of intraperitoneally-injected ^{3}H-labeled water into digitonin-precipitable sterols and fatty acids (Figure 3-6). High-cholesterol feeding reduced hepatic sterol synthesis by 93% in control mice. Hepatic sterol synthesis in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice was elevated

by 5-fold, and it did not decline with the high cholesterol diet. Hepatic fatty acid synthesis was 3-fold higher in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice fed the low cholesterol diet compared to control mice. The synthesis of sterols and fatty acids in brain, a tissue with a very low level of interferon-induced *MX1*-Cre recombination (Kuhn et al., 1995), was normal in *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice.

DISCUSSION

The current studies define Insigs as essential components of the cholesterol feedback response that Schoenheimer discovered in living mice more than 70 years ago (Schoenheimer and Breusch 1933). Considered together with previous experiments in mouse liver, the current data indicate that the entire pathway for SREBP processing functions in this organ and that this pathway is responsible for the synthesis of cholesterol as well as the feedback suppression of synthesis when cholesterol is consumed in the diet.

The first indication that SREBPs control cholesterol synthesis in liver came from the production of transgenic mice expressing truncated versions of SREBPs that lack a membrane-spanning segment and enter the nucleus without a requirement for processing (Horton et al., 2002). Overexpression of the nuclear forms of SREBP-1a or SREBP-2 markedly increased cholesterol synthesis in liver and led to massive intrahepatic accumulation of cholesterol (Shimano et al., 1996; Horton et al., 1998). Overexpression of the nuclear form of SREBP-1c was much less potent in this regard (Shimano et al., 1997A). To show that endogenous levels of SREBPs are sufficient for cholesterol overproduction, transgenic mice were created that express a mutant form of SCAP that escorts SREBPs

normally, but cannot bind to Insigs (Yang et al., 2002; Korn et al., 1998). These livers also overproduced cholesterol and were resistant to feedback inhibition by dietary cholesterol. Cre-mediated elimination of SCAP in the liver (Matsuda et al., 2001), or of one of the SREBP-processing proteases (Yang et al., 2001), reduced the amount of nuclear SREBPs and led to a severe reduction in cholesterol synthesis, indicating that nuclear SREBPs are required for transcription of the genes encoding enzymes of the cholesterol biosynthetic pathway in liver.

Although elimination of Insig-1 alone had a minor effect on nuclear SREBPs and its target mRNAs, full derepression of cholesterol synthesis required the elimination of both Insig-1 and Insig-2. These data indicate that Insig-2 plays an important role in liver. Although Insigs are required in sterol-mediated feedback inhibition of the processing of SREBP-1 and SREBP-2, the two SREBPs show a differential sensitivity to cholesterol. As shown in Figures 3-4A and 3-4B, cholesterol feeding to wild-type mice nearly eliminated nSREBP-2, but it reduced nSREBP-1 by only about 50%. Both of these responses were mediated by Insigs since they did not occur in the *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice. In wild-type mice, the partial resistance of SREBP-1 may be due in part to an increase in the SREBP-1c precursor, owing to an increase in SREBP-1c mRNA (Figure 3-5), which is produced by sterol-mediated activation of LXRs (Repa at al., 2000). However, it seems unlikely that the increase in SREBP-1c precursor is solely responsible for this resistance. Inhibition of SREBP processing is mediated by the binding of the SCAP-SREBP complex to Insigs. The possibility therefore exists that the SCAP-SREBP-1 complex in liver may have a lower affinity for Insigs then does the SCAP-SREBP-2 complex. These possibilities, and others,

are open to study now that the role of Insigs in cholesterol feedback has been demonstrated in liver.

METHODS

Materials and general methods. Blood was drawn from the retro-orbital sinus; the plasma was separated immediately and stored at -70°C. Plasma glucose was measured with a Glucometer from Bayer (Elkhard, IN, Cat. No. 994-90902). Concentrations of plasma and liver cholesterol and triglycerides and of plasma free fatty acids and insulin were measured as described (Engelking et al., 2004). Polyinosinic-polycytidylic acid (pIpC) was obtained from Sigma-Aldrich (Cat. No. P1530) and Amersham Biosciences (Cat. No. 27-4729-01).

Generation of Insig knockout mice. The *Insig-1* gene was conditionally disrupted by the insertion of a *loxP, frt*-flanked *pgkneopA* cassette (Soriano et al., 1991) 2.3-kb upstream of the initiator ATG codon of exon 1 of *Insig-1* and the insertion of a downstream *loxP* site within intron 1. Regions of homology were generated by PCR using SM-1 ES cell genomic DNA as template and were then inserted into a targeting vector, pJB1 (kindly provided by Joachim Herz, University of Texas Southwestern Medical Center). Cre-mediated recombination removes exon 1, which encodes amino acids 1-119.

The *Insig-2* gene was disrupted by replacement of exons 2 and 3 with a *polIIsneopA* cassette (Ishibashi et al., 1993) to remove sequences that encode amino acids 1-123. Regions of homology were generated by PCR using SM-1 ES cell genomic DNA as template and inserted into the targeting vector pGEM3Zf(+) containing a *polIIsneopA* cassette. The integrity of all constructs was confirmed by restriction analysis and DNA sequencing.

SM-1 ES cells, derived from 129S6/SvEv blastocysts, were cultured on leukemia inhibitory factor-producing STO feeder cells and transfected with linearized targeting vectors as described previously (Ishibashi et al., 1993; Liang et al., 2002). ES cell clones that had undergone homologous recombination as determined by PCR and Southern blotting were isolated and injected into C57BL/6J blastocysts. Chimeric males with >50% *agouti* coat color were crossed to C57BL/6J females. From single ES cell lines, 7 chimeric males gave offspring that carried a single copy of the targeted *Insig-1* allele (referred to as *Insig-1*$^{+/f}$), and 6 chimeric males gave offspring that carried a single copy of the disrupted *Insig-2* allele (referred to as *Insig-2*$^{+/-}$).

To generate mice with inducible disruption of the floxed *Insig-1* allele, *Insig-1*$^{+/f}$ mice were bred to *MX1*-Cre transgenic mice to generate *Insig-1*$^{f/f}$;*MX1-Cre* mice (referred to as *L-Insig-1*$^{-/-}$ mice to denote the deletion of *Insig-1* in Liver) (Rohlmann et al., 1998). Littermates of *L-Insig-1*$^{-/-}$ mice lacking the Cre transgene were used as control mice. *L-Insig-1*$^{-/-}$ mice were bred to *Insig-2*$^{-/-}$ mice to generate *Insig-1*$^{f/f}$; *Insig-2*$^{-/-}$;*MX1-Cre* mice (referred to as *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$). Littermates of *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice lacking the Cre transgene are referred to as *Insig-2*$^{-/-}$ mice. Disruption of *Insig-1* in the liver was induced by intraperitoneal injection of pIpC, which induces interferon, which in turn induces the Cre recombinase driven by the *MX1* promoter (Kuhn et al., 1995). For the studies described here, each mouse (control, *L-Insig-1*$^{-/-}$, *Insig-2*$^{-/-}$, or *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$) received four intraperitoneal injections of 300 μl of a 1 mg/ml solution of pIpC in water or PBS administered every 48 h. Mice were analyzed between 7 and 14 days after the final

pIpC injection. Disruption of the floxed *Insig-1* allele in all mice was confirmed by either Southern blot analysis of *Nco*I-digested genomic DNA or by measurement of Insig-1 mRNA by quantitative real-time PCR of total RNA from livers of pIpC-treated mice. *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice demonstrating low levels of disruption of the floxed *Insig-1* allele were excluded from further analysis (see legend to Table 3-4). During the course of these studies, we observed batch-to-batch variation in the potency of pIpC, and in general higher disruption was seen when the pIpC was administered in PBS rather than water.

To generate mice with disruption of the floxed *Insig-1* allele in the whole animal, *Insig-1*$^{+/f}$ mice were bred to *EIIa-Cre* transgenic mice (Jackson Laboratory, strain FVB/N/Tg(EIIa-cre)C5379Lmgd/J, stock #003314). Cre recombinase driven by the adenovirus *EIIA* promoter is active in the tissues of the developing animal, including the germ cells (Lakso et al., 1996). Mice carrying a single copy of the disrupted *Insig-1* allele in all tissues (referred to as *Insig-1*$^{+/-}$) were bred to *Insig-2*$^{+/-}$ mice and the genotypes of the resultant offspring are shown in Table 1.

To genotype knockout animals, genomic DNA was prepared from adult tail or embryonic tissue using a direct lysis kit from Viagen Biotech (Cat. No. 102-T) and used for PCR with the following primers. Insig-1: 5' primer-1, 5'-CTCAAGAGGCAGTTGCCAGGGGACAGACTT-3', 5' primer-2, 5'-GCCACATAGGCTTCTGCATAGAAACACACT-3', and 3' primer (common), 5'–CACTTGACAAATGGTTATCAACGCTGTACA-3'. Insig-1 genotyping yielded PCR products of 296, 393, and 567 bp from the wild type, floxed, and disrupted alleles,

respectively. Insig-2: 5' primer-1, 5'-CCTGCATTGACAGGCATCTAGGAGAACCTC-3', 5' primer-2,

5'-GATTGGGAAGACAATAGCAGGCATGC-3', and 3' primer (common),

5'–AGGGTACTTCTTGGTGCTGACTATAACCAA-3'. Insig-2 genotyping yielded PCR products of 477 and 357 bp from the wild type and disrupted alleles, respectively.

All mice were housed in colony cages with a 12-h light/12-h dark cycle and fed Teklad Mouse/Rat Diet #7002 from Harlan Teklad Premier Laboratory Diets (Madison, WI). Unless otherwise stated, mice were fed a chow diet *ad libitum*, and tissues were collected at the end of the dark phase. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center at Dallas.

Diet studies. For the cholesterol feeding studies, mice were fed for 2.5 days with a chow diet (0.02% cholesterol) or chow diet containing cholesterol at a final concentration (w/w) of 0.2%, 1.5%, or 2.0%. High cholesterol diets were prepared by grinding solid cholesterol (MP Biomedicals, Aurora, OH, Cat. No. 101380) directly into the powdered chow diet (Mouse/Rat Diet #7001, Harlan Teklad Premier Laboratory Diets) with a mortar and pestle.

Quantitative real-time PCR and northern blot analysis. Total RNA was prepared from mouse tissues using an RNA STAT-60 kit (TEL-TEST “B”, Friendswood, TX). Equal amounts of RNA from 4 to 6 mice were pooled and subjected to quantitative real-time PCR as previously described (Engelking et al., 2004)). ApoB mRNA was used as the invariant

control. The primers for real-time PCR were described previously (Yabe et al., 2003; Yang et al., 2001; Liang et al., 2002).

For northern blot analysis, pooled total RNA was subjected to electrophoresis in a 1% agarose gel and transferred to Hybond XL membranes (Amersham Biosciences, Cat. No. RPN303S). cDNA probe preparation and hybridization were carried out as described (Shimomura et al., 1998). The Insig-1 probe consisted of the full-length mouse Insig-1 open reading frame (Engelking et al., 2004). Probes for Insig-2 and cyclophilin were described previously (Yabe et al., 2002; Luong et al., 2000).

Immunoblot analyses of liver nuclear extracts and membrane fractions. To prepare nuclear and membrane fractions for immunoblot analyses, aliquots of frozen liver (~100 mg) were homogenized in 1 ml buffer (20 mM Tris-chloride at pH 7.4, 2 mM $MgCl_2$, 0.25 M sucrose, 10 mM sodium EDTA, and 10 mM sodium EGTA) supplemented with protease inhibitor cocktail consisting of 5 mM dithiothreitol, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Pefabloc, 5 μg/ml pepstatin A, 25 μg/ml *N*-acetyl-leu-leu-norleucinal, 10 μg/ml aprotinin, and the equivalent of 0.1 tablet of Complete Mini Inhibitor Cocktail (Roche Diagnostics, Cat. No. 11836170001). The liver homogenate was centrifuged at 1000*g* for 5 min at 4°C. The 1000*g* supernatant was removed and used to prepare a membrane fraction as previously described (Engelking et al., 2004). To prepare nuclear extracts, the 1000*g* pellet was washed in 2 ml of homogenization buffer and collected by centrifugation at 1000*g* for 5 min at 4°C. The nuclear pellet was resuspended in 0.3 ml of buffer (20 mM Hepes-NaOH at pH7.6, 2.5% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, and the equivalent of 0.03 protease inhibitor tablet).

The suspension was rotated at 4°C for 1 h and centrifuged at $10^5 g$ for 30 min at 4°C. The supernatant from this spin is designated the nuclear extract. After aliquots of the nuclear extract and membrane fraction were removed for measuring protein concentration with the BCA Kit (Pierce Biotechnology), the remainder of each sample was mixed with 4X SDS loading buffer (12% (w/v) SDS, 0.02% bromophenol blue, 30% glycerol, 0.15 M Tris-chloride at pH 6.8, and 6% β-mercaptoethanol). Equal amounts of protein from the livers of 4 to 6 mice were then pooled, and an aliquot of the pooled sample (45 μg) was subjected to SDS-PAGE and immunoblot analysis (Engelking et al., 2004; Shimomura et al.,1997).

Rabbit polyclonal antibodies that detect mouse SREBP-1, SREBP-2, Insig-1, Insig-2, HMG-CoA reductase, and SCAP were described previously (Engelking et al., 2004; Sato et al., 1993; Shimano et al., 1997; Sakai et al., 1997). These antibodies were diluted in 5% bovine serum albumin (Sigma-Aldrich, Cat. No. A7906) in PBS-0.05% Tween-20 (Sigma, Cat. No. P3563) and used at the indicated concentrations: SREBP-1 (IgG fraction, 5 μg/ml), SREBP-2 (IgG, 2.9 μg/ml), Insig-1 (1:1000 dilution of serum), Insig-2 (1:1000 dilution of serum), HMG-CoA reductase (IgG, 3.4 μg/ml), and SCAP (IgG, 3.5 μg/ml). Immunoblots of CREB (cAMP-responsive element-binding protein) and transferrin receptor were used as loading controls for the nuclear extract and membrane fractions, respectively. Anti-CREB (Cat. No. 13-6800) and anti-transferrin receptor antibodies were each used at 0.5 μg/ml (Zymed, South San Francisco, CA, Cat. No. 13-6800 and 35-0900, respectively).

Cholesterol and fatty acid synthesis in vivo. Rates of cholesterol and fatty acid synthesis were measured in control and *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice using ^{3}H-labeled water as previously described (34). The mice were fed *ad libitum* with chow diets containing 0.02%

(low) or 1.5% (high) cholesterol for 2.5 day prior to study. The rates of cholesterol and fatty acid synthesis were calculated as μmoles of ^{3}H-labeled water incorporated into fatty acids or digitonin-precipitable sterols per hour per gram of tissue.

Assay of HMG-CoA reductase activity. Mice were sacrificed at the end of the dark phase, the livers were immediately removed, and 2 equal pieces of liver (~250 mg/piece) from each mouse were individually homogenized on ice with 10 stokes of a loose Dounce pestle followed by 5 strokes of a tight pestle in 1.5 ml of solution containing 0.3 M sucrose, 10 mM sodium EDTA at pH 7.4, 10 mM β-mercaptoethanol, and either 50 mM NaF or 50 mM NaCl. Homogenates from 5 mice in each group were then pooled and centrifuged for 15 min at 12,000*g* at 4°C, and the supernatant fraction was again centrifuged for 15 min at 12,000*g*. The resulting supernatant was centrifuged for 60 min at $10^{5}g$, and the resulting microsomal pellet was frozen at -80°C. Before assay of HMG-CoA reductase activity, each pellet was resuspended in 2 ml of assay buffer (20 mM imidazole at pH 7.4, 5 mM dithiothreitol, and either 50 mM NaF or 50 mM NaCl), and aliquots were removed for protein quantification and enzyme assay as previously described (Kita et al., 1980). Each assay was carried out for 30 min at 37°C in the presence of 87 μM DL[3-^{14}C]HMG-CoA (22,051 dpm/nmol). The amount of [^{14}C]mevalonate formed was quantified by thin layer chromatography. Reductase activity is expressed as the picomoles of [^{14}C]mevalonate formed per min per milligram of microsomal protein.

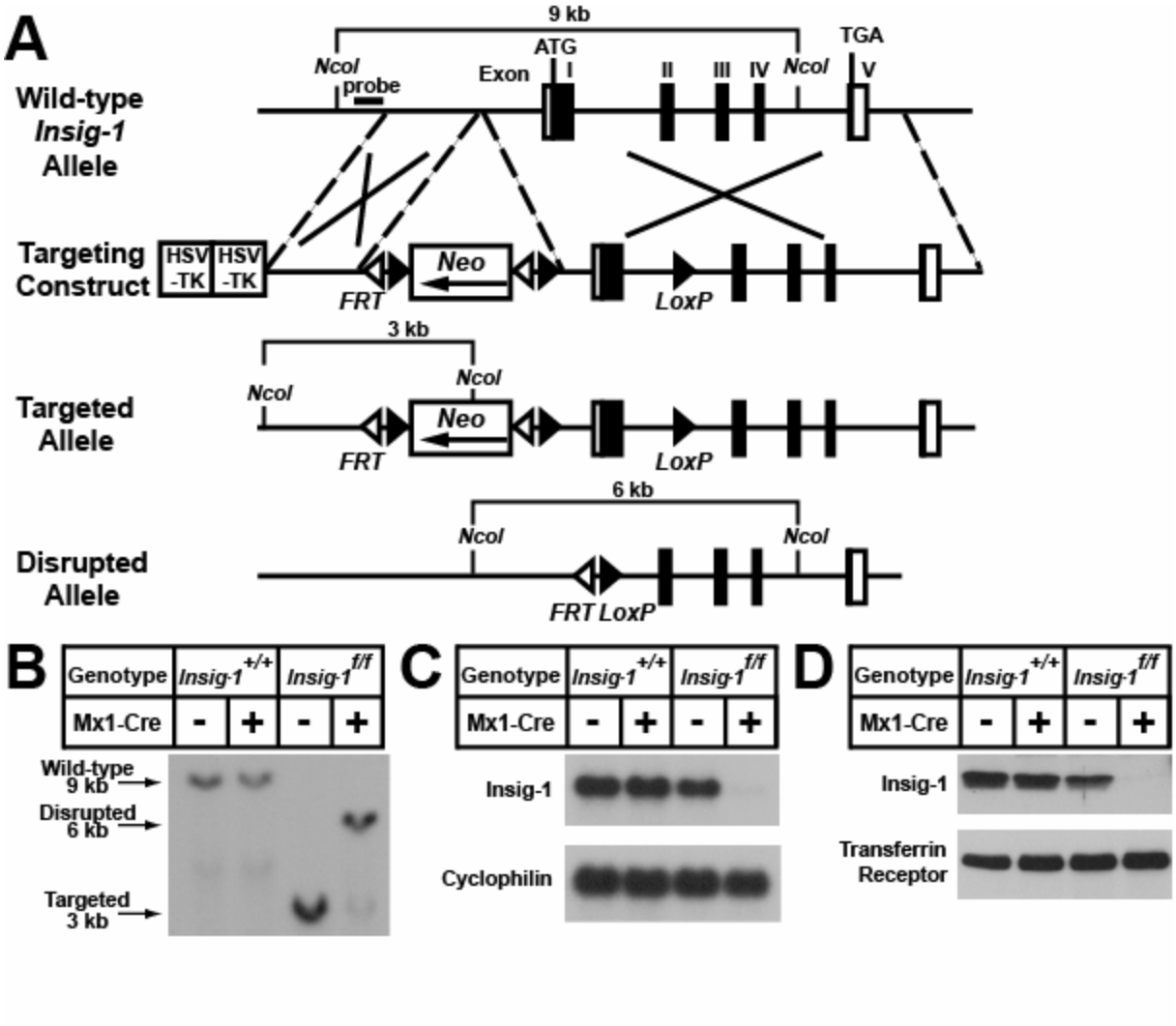


Figure 3-1, part A

Targeted disruption of *Insig-1* and *Insig-2* genes in mice**.** (**A**) Schematic of *Insig-1* gene targeting strategy. Cre-mediated excision of the sequence of between *loxP* sites deletes exon 1. The location of the probe used for Southern analysis is denoted by a solid box. (**B**) Representative Southern blot analysis of *Nco*I-digested DNA from livers of mice with the indicated genotypes that were treated with 4 intraperitoneal injections of pIpC (300 μg/injection) (**C**) Northern blot analysis of hepatic RNA of mice described in (**B**). Total RNA from liver was pooled, and 20 μg aliquots were subjected to electrophoresis and blot hybridization with [^{32}P]-labeled cDNA probes for mouse Insig-1 and mouse cyclophilin. (**D**) Immunoblot analysis of livers of mice described in (**B**). Liver membrane fractions were prepared as described in Methods, and aliquots (45 μg) were subjected to SDS-PAGE and immunoblot analysis. The Insig-1 protein (28.2 kDa) is indicated. *f/f = flox/flox.*

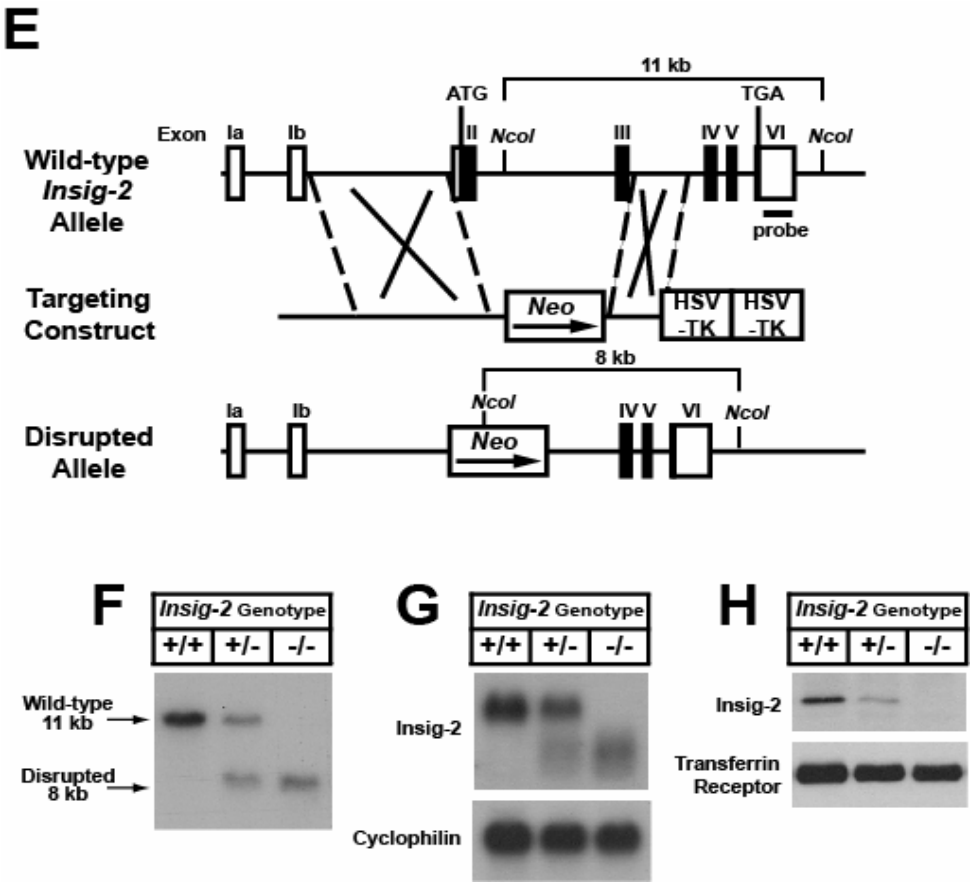


Figure 3-1, part B

(**E**) Schematic of *Insig-2* gene targeting strategy. The *Insig-2* allele was disrupted by replacement of exons II and III of the *Insig-2* gene with a *polIIsneopA* expression cassette. The DNA probe used for Southern analysis is denoted by a solid box. (**F**) Representative Southern blot analysis of *NcoI*-digested tail DNA of the offspring from mating of *Insig-2*$^{+/-}$ mice. (**G**) Northern blot analysis of hepatic RNA of mice described in (**F**). Total RNA from livers of mice was subjected to electrophoresis and blot hybridization with ^{32}P-labeled cDNA probes for mouse Insig-2 and mouse cyclophilin. (**H**) Immunoblot analysis of liver membranes from mice with the indicated Insig-2 genotype. The Insig-2 protein (24.9 kDa) is indicated.

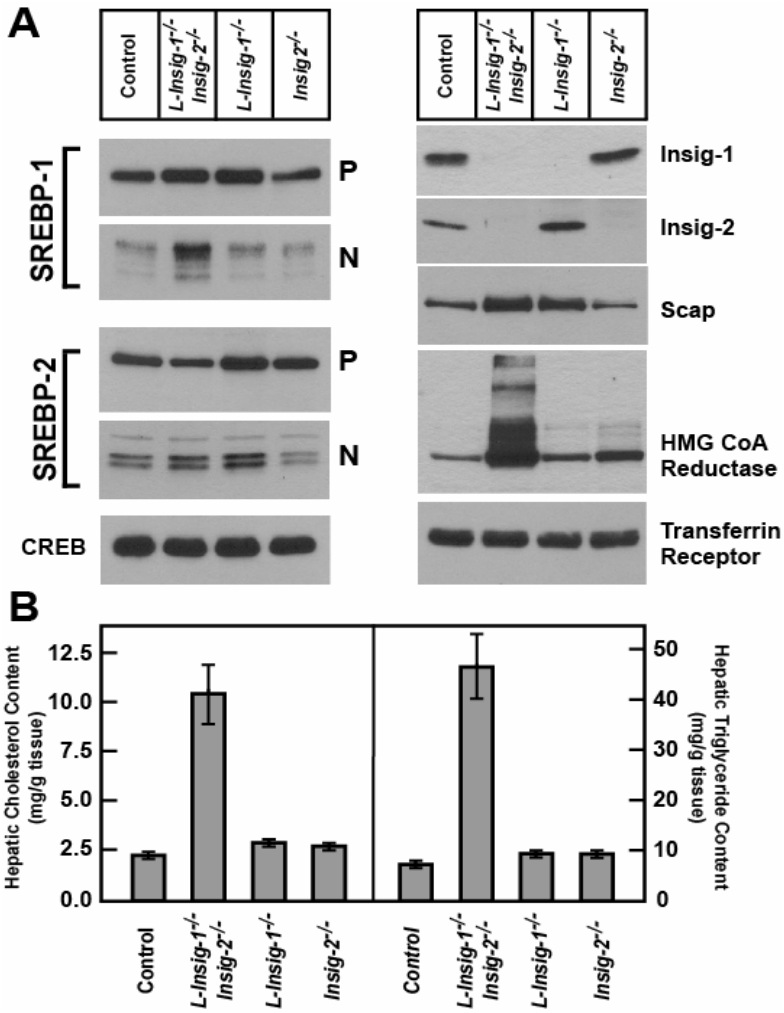


Figure 3-2
Immunoblot (**A**) and lipid analysis (**B**) of livers from control and Insig-deficient mice. The mice used in this figure are the same as those described in Table 2. (**A**) Immunoblot analysis from nuclear extract and membrane fractions obtained from with the following four genotypes: 1) *Insig-1*$^{f/f}$ (designated Control); 2) *Insig-1*$^{f/f}$; *Insig-2*$^{-/-}$;*MX1-Cre* (designated *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$); 3) *Insig-1*$^{f/f}$;*MX1-Cre* (designated *L-Insig-1*$^{-/-}$); and 4) *Insig-1*$^{f/f}$;*Insig-2*$^{-/-}$ (designated *Insig-2*$^{-/-}$). Each mouse was treated with 4 intraperitoneal injections of pIpC (300 μg/injection), and tissues were obtained 14 days after the final injection. Livers (6 mice per group) were separately pooled, and 45 μg aliquots of the pooled membrane and nuclear extract fractions were subjected to SDS-PAGE and immunoblot analysis. The precursor and nuclear forms of SREBPs are denoted as P and N, respectively. CREB protein (cAMP-responsive element binding protein) and the transferrin receptor were used in the immunoblots as loading controls for the nuclear extract and membrane fractions, respectively. (**B**) Hepatic cholesterol and triglyceride content of control and Insig-deficient livers. Each bar represents the mean ± SEM of data from 6 mice.

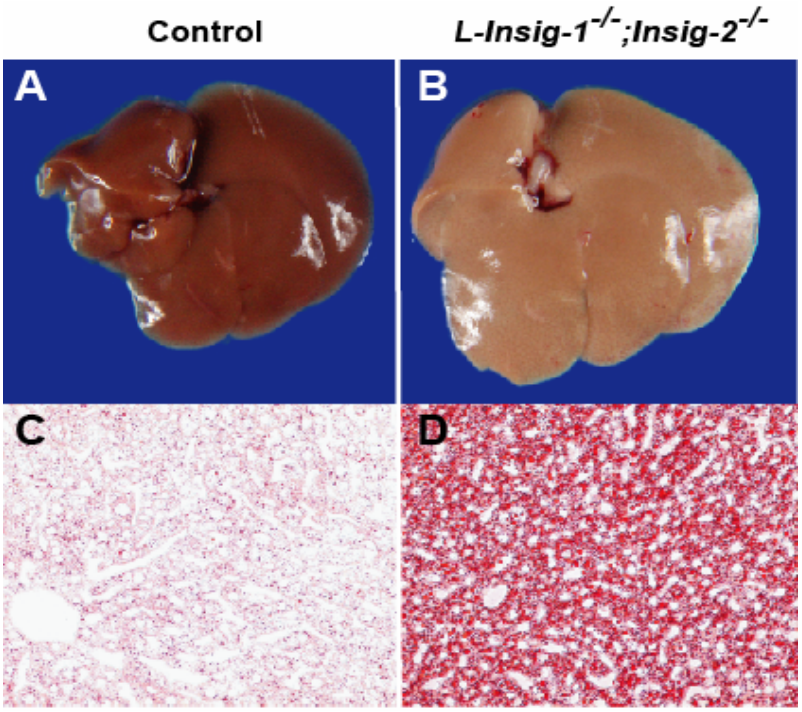


Figure 3-3

Lipid accumulation in livers of *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. Adult female mice were treated with 4 intraperitoneal injections of pIpC (300 µg/injection), and the livers were removed either 12 days (**A, B**) or 7 days (**C, D**) after the final injection. (**A, B**) Photographs of livers from chow-fed control and *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. (**C, D**) Oil Red O-stained histologic sections of the livers from chow-fed control (left) and *L-Insig-1*$^{-/-}$;*Insig-2*$^{-/-}$ mice. Mice were perfused through the heart with HBSS, fixed with 10% (v/v) formalin in PBS, and frozen sections of livers were stained with Oil Red O. Magnification: 20 X.

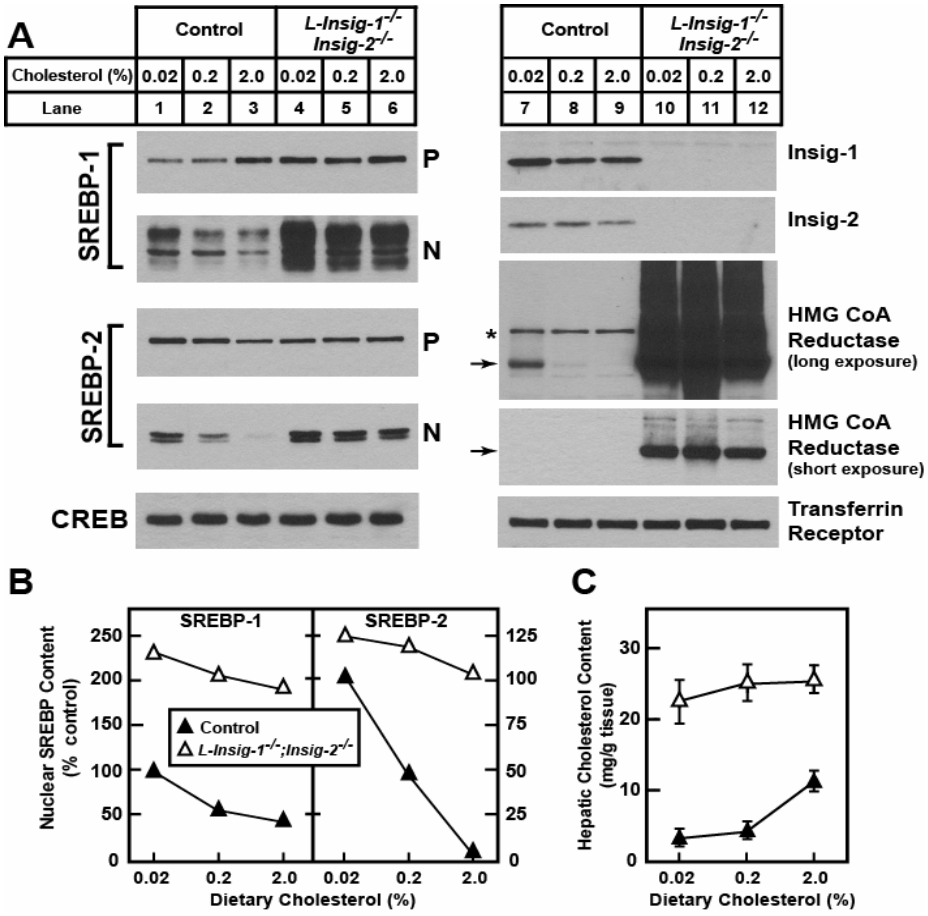


Figure 3-4

Markedly elevated levels of nuclear SREBPs and HMG-CoA reductase in the livers of Insig-deficient mice fed high cholesterol diets. The mice used in this figure are the same as those described in Table 3-4. Each mouse was treated with 4 intraperitoneal injections of pIpC (300 μg/injection), and 8.5 day after the final injection, mice were fed *ad libitum* a chow diet containing the indicated amount of cholesterol for 2.5 days prior to study. (**A**) Immunoblot analysis of SREBP-1 and SREBP-2 from livers of control and *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice fed with the indicated amount of cholesterol. Livers (4 or 6 mice per group) were separately pooled, and 45-μg aliquots of the membrane and nuclear extract fractions were subjected to SDS-PAGE and immunoblot analysis. Arrows indicate the position of migration on SDS gels of monomeric HMG-CoA reductase (97 kDa). Immunoblots of CREB and transferrin receptor were used as loading controls for the nuclear extract and membrane fractions, respectively. **(B**) The gels of nuclear extract fractions in (**A**) were scanned and quantified by densitometry. The intensities of the cleaved nuclear forms of SREBP-1 and SREBP-2 in lane 1 (control mice fed with 0.02% cholesterol) were arbitrarily set as 100%. (**C**) Hepatic cholesterol content of control and Insig-deficient mice. Each value represents the mean ± SEM of data from 4 or 6 mice.

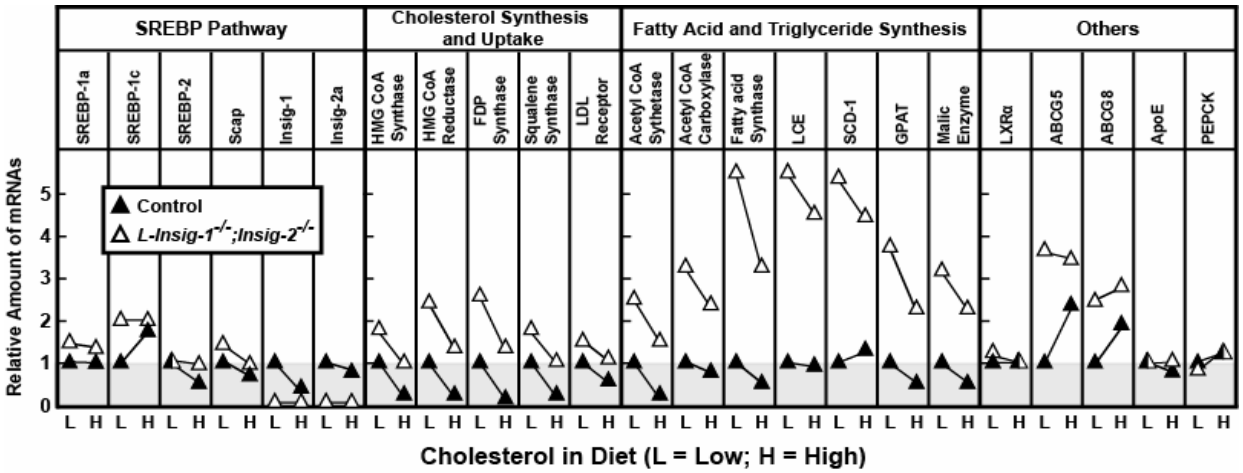


Figure 3-5

Relative amounts of various mRNAs in livers from control and *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice fed with diets containing a low (L) or high (H) amount of cholesterol (0.02 or 2.0% cholesterol, respectively). The mice used here are the same as those used in Figure 3-5 and Table 3-4. Total RNA from livers of mice was pooled and subjected to real time PCR quantification as described in Methods. Each value represents the amount of mRNA relative to that in the control mice fed with a chow diet (0.02% cholesterol), which is arbitrarily defined as 1. FDP, farnesyl diphosphate; LCE, long-chain fatty acyl-CoA elongase; SCD-1, stearoyl-CoA desaturase-1; GPAT, glycerol-3-phosphate acyltransferase; PEPCK, phosphoenolpyruvate carboxykinase.

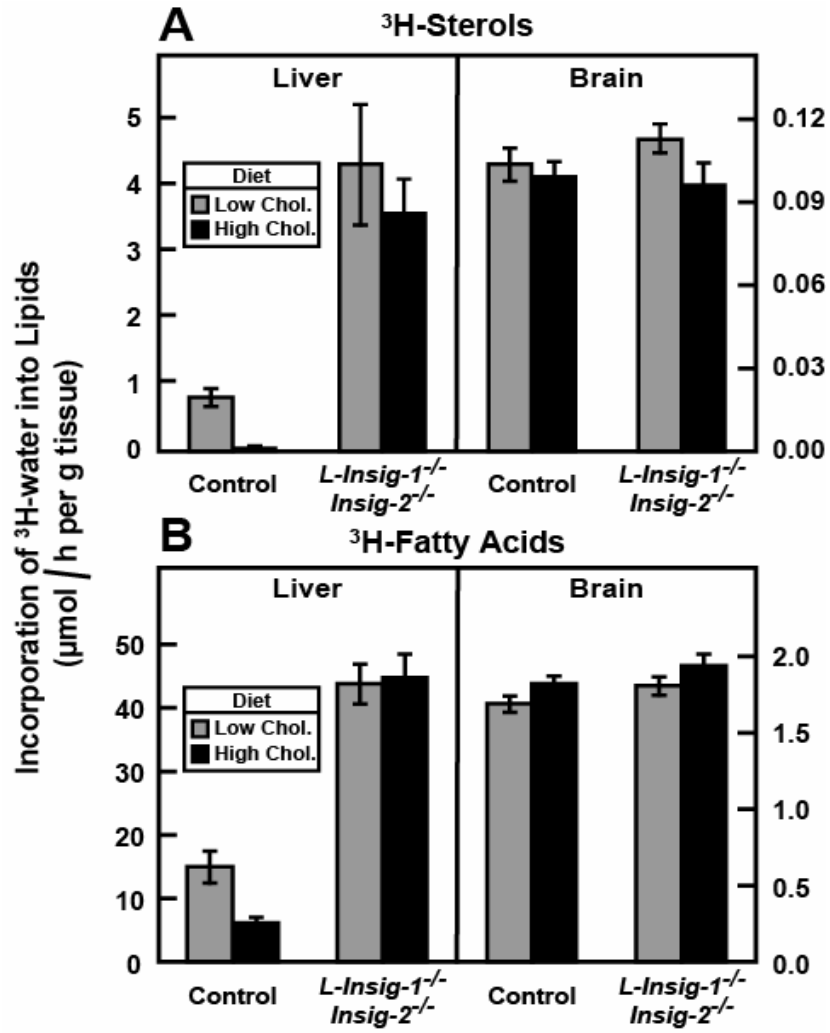


Figure 3-6

In vivo synthesis rates of sterols (**A**) and fatty acids (**B**) in livers and brains from control and *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$ mice. Mice (20 to 24-week-old male, 5 or 6 per group) were treated with 4 intraperitoneal injections of pIpC (300 µg/injection). Five and a half days after the final injection, mice were fed *ad libitum*, a chow diet containing 0.02% (low) or 1.5% cholesterol (high) for 2.5 day prior to study, at which time the mice were injected intraperitoneally with ^{3}H-labeled water (50 mCi in 0.20 ml of isotonic saline). One hour later the tissues were removed for measurement of ^{3}H-labeled fatty acids and digitonin-precipitable sterols. Each bar represents the mean ± SEM of the values from 5 or 6 mice.

CHAPTER FOUR
Conclusions and Recommendations

FEEDBACK REGULATION OF HEPATIC LIPID SYNTHESIS REQUIRES INSIG PROTEINS

Insigs Regulate Hepatic Lipid Homeostasis through SCAP and HMG-CoA reductase

Insigs are required for end-product feedback regulation of hepatic lipid synthesis

The results presented in Chapters two and three extend the previous studies performed in cultured cells by Yang, Dabe, and Sever which demonstrated that Insigs are key regulators of two arms of the cholesterol feedback response through SCAP/SREBP and HMG-CoA reductase. Importantly, the current studies demonstrate that Insigs can modulate cellular lipid contents and lipid biosynthetic rates, which had not been shown in previous studies in cultured cells. The current studies demonstrate that Insigs are essential elements of the regulatory mechanism controlling the rate of cholesterol synthesis in living animals first defined by Schoenheimer and Breusch in the 1930s.

When overexpressed in the livers of transgenic mice, Insig-1 sensitizes SREBP processing to inhibition by sterols. Livers of transgenic mice have a low hepatic nSREBP content, as the high levels of Insig-1 trap the SREBP/SCAP complex in the ER. These low levels of hepatic nSREBPs are supersensitive to the inhibitory effects of dietary cholesterol. The blockage of SREBP processing resulted in a low level of SREBP target mRNA levels. Furthermore, the overexpressed Insig-1 reduced the levels of HMG-CoA reductase protein, especially when dietary cholesterol was fed (see Appendix C). Since the mRNA level of

HMG-CoA reductase was reduced, it is likely the decline of reductase protein was due to a combinatorial effect of Insigs to inhibit HMG-CoA reductase transcription through decreasing nSREBPs and to accelerate its ubiquitination and degradation.

When inactivated in the liver by conditional disruption of Insig-1 and germline disruption of Insig-2, SREBP processing was resistant to inhibition by sterols, as the SCAP/SREBP complex could traffic from the ER regardless of the sterol content in the double knockout livers. This sterol resistance was further demonstrated by the markedly elevated HMG-CoA reductase levels in the livers of double knockout mice despite the accumulation of cholesterol to levels which would have eliminated it in mice with a normal complement of Insigs. The elevated levels of hepatic nSREBPs and HMG-CoA reductase were insensitive to the effects of dietary cholesterol to reduce them, even though the cholesterol content of the knockout livers remained much higher than that of the control mice when fed the high cholesterol diets. The constitutive SREBP processing resulted in elevated levels of SREBP target gene mRNAs. The data in Figure 3-6, which show that hepatic cholesterol synthesis is not suppressed by cholesterol feeding, define Insigs as essential mediators of the Schoenheimer effect, and reiterate that feedback regulation of the SREBP pathway and HMG-CoA reductase are key determinants of cholesterol homeostasis in living animals.

The observation that the steady-state level of HMG-CoA reductase protein was increased by approximately 30-fold whereas HMG-CoA reductase mRNA was increased by only 2-fold in Insig double knockout livers suggests that posttranscriptional mechanisms, likely decreased protein turnover, is a major effect of Insig deficiency. The analyses of

hepatic HMG-CoA reductase were limited to its steady-state protein levels, its mRNA, and its *ex vivo* activity level. For technical reasons I was not able to determine whether Insig deficiency affects the half-life of hepatic HMG-CoA reductase protein , such as by attempting an *in vivo* pulse-chase study using ^{35}S. Previous literature suggested against attempting an *in vivo* cycloheximide dosing experiment. The intent would be to show that, after cycloheximide dosing to block the synthesis of new HMG-CoA reductase, already synthesized HMG-CoA reductase protein would disappear at a slower rate in Insig-deficient livers compared to controls, especially in the cholesterol-fed state. However, several groups have shown that cycloheximide treatment actually blocks the effect of cholesterol treatment to accelerate reductase turnover, which implies that sterol-mediated acceleration of HMG-CoA reductase requires a short-lived regulatory protein (Chang et al., 1981; Chen et al., 1982). The recent observation of Ye that Insig-1 turns over rapidly in cultured cells (Lee and Ye, 2004) makes Insigs themselves attractive candidates as these putative short-lived regulatory factors, and the study of Insig protein turnover a key issue.

One paradox of Insig function is how Insig binding to the SSDs of two proteins results in two very different effects: ER retention of SCAP versus ER-associated degradation of HMG-CoA reductase. Curiously, levels of SCAP protein were consistently elevated by 2-3 fold without changes in levels of SCAP mRNA in livers of mice deficient in Insig-1 but were not altered in Insig-2 deficient mice (Figure 3-2). It is possible that the end results of retention versus degradation are not mutually exclusive: Insigs may also cause a slight increase in SCAP degradation, though the effect is not as dramatic as its effect on HMG-CoA reductase.

Insig double knockout mice share some similarities with transgenic mice that express a sterol-resistant (D443N) SCAP transgene (Korn et al., 1998). In the SCAP transgenic mice, nSREBPs are dramatically elevated and resistant to suppression by feeding of cholesterol and cholic acid. The increase in nSREBPs leads to a large increase in the transcription of lipogenic mRNAs, increased hepatic cholesterol and triglyceride levels, and increased *in vivo* lipid synthesis rates. Unlike Insig double knockout mice, however, the turnover of HMG-CoA reductase protein would not be predicted to be affected in SCAP transgenic mice. However, reductase mRNA levels were 18-fold increased in SCAP transgenic mice whereas they were elevated by only 2-fold in Insig double knockout livers. Ultimately, the dramatic increases in HMG-CoA reductase transcription seen in the SCAP transgenic mice or combinatorial effects on reductase transcription and degradation seen in the Insig double knockout mice both resulted in high levels of sterol synthesis and cholesterol accumulation in the livers of both mouse strains.

Insigs modulate insulin-stimulated lipogenesis through SREBP-1c

Similar to other mouse models with deficiencies in nSREBPs, such as mice that lack SCAP or Site-1 Protease (Matsuda et al., 2001; Yang et al., 2001), Insig-1 transgenic mice manifested a blunted increase in nSREBP-1c that normally accompanies the refeeding of previously fasted mice, which results in a blunted upshot in lipogenic mRNAs and fatty acid synthesis during refeeding. The increase in plasma triglycerides normally seen in refeeding of wild-type mice was absent in Insig-1 transgenic mice, which suggests that the ability the livers of these mice to convert excess carbohydrate energy into triglycerides and export it as VLDL for storage in adipose tissue was reduced.

The refed TgInsig-1 mice had a normal increase in plasma insulin, appropriate decreases in the mRNAs of insulin-responsive genes such as PEPCK and IRS-2, and a normal decline in Insig-2 protein. These data indicate that the blunted increase in SREBP-1c expression and nuclear protein were not due to generalized resistance to insulin action in the livers of TgInsig-1 mice. The loss of nSREBP-1c in refed TgInsig-1 mice is likely due to the combinatorial effects of the insig-mediated block in SREBP processing and the loss of feedforward activation of SREBP-1c transcription as fewer nSREBPs are available to bind the SRE in the SREBP-1c enhancer that is required for insulin's inducing effect (Chen et al., 2004). The observation that mice with conditional disruption of SREBP-2 in liver have dramatically reduced SREBP-1c mRNAs levels (J.D. Horton, unpublished observation) supports such a hypothesis.

Conversely, it might be predicted that Insig-deficient mice would hyperactivate SREBP-1c processing during high-insulin states such as refeeding and manifest an overexaggerated lipogenic response. Refeeding studies of mice deficient in Insig-1 or Insig-2 singly showed normal SREBP responses during refeeding; Insig double knockout mice fed an insulin-inducing, high-sucrose diet also failed to show an overexaggerated lipogenic response (data not shown). These negative results may be due to any number of factors. First, early in refeeding, a net deficiency in total Insig protein is observed. Insig-2 disappears within three hours of refeeding previously fasted mice while the reemergence of Insig-1, which lags the restoration of nSREBPs since it is an SREBP target, requires more than six hours (Appendix D). Since the total Insig protein levels are low early in refeeding, Insig knockout mice might be predicted to have a normal refeeding response, as is observed.

The observation that total Insig protein is low in early refeeding is consistent with the hypothesis proposed by Yabe that the fall in Insig-2a is required for the increase in nuclear SREBPs during refeeding. To test this hypothesis directly, I studied transgenic mice that constitutively express Insig-2 driven by a fragment of the human *apoE* promoter. The intent was to generate a transgenic line with same level of expression of Insig-2 as was present in fasted animals. Although I tried several transgenic constructs, the levels of transgene expression were always low, never reaching the level of endogenous Insig-2 expression in fasting mice. No dramatic alterations in terms of SREBP processing or HMG-CoA reductase were observed in these TgInsig-2 mice. The studies in chapter 2, using a line of TgInsig-1 mice in which the expression of transgenic human Insig-1 is approximately 30-fold higher than endogenous mouse Insig-1 as estimated by real-time PCR, demonstrate only that saturating levels of Insig expression are sufficient to block refeeding-induced lipogenesis.

Redundancy and Reciprocal Regulation of Hepatic Insig-1 and Insig-2

It is attractive to speculate that the *in vivo* roles of Insig-1 and Insig-2 not be solely redundant because of their reciprocal transcriptional regulation: in the fed state, Insig-1 is abundant; in the fasted state, Insig-1 disappears and is replaced by Insig-2. The immunoblot data of Figure 2-4 illustrate the dramatic shifts in the protein levels of Insig isoforms that occur after fasting and refeeding in wild type mice. If Insig-1 and Insig-2 are redundant, would it not be simpler to have a single Insig isoform that was constitutively expressed? Studies in the Insig double knockout mice indicate that Insig-1 and Insig-2 are largely redundant in liver, since dramatic loss of sterol feedback suppression on hepatic lipid synthesis required the loss of

both Insigs. Studies on mice deficient in single Insig isoforms were disappointing because they displayed only mild phenotypes in the ad libitum chow-fed state, during cholesterol feeding, and in the fasting/refeeding experiments. I had expected that a fed Insig-1 knockout mouse or a fasted Insig-2 knockout mouse would be functionally deficient in both Insigs unless a compensatory increase in the expression of the intact Insig isoform had occurred. Such compensatory increases were not observed (Appendix E), and still the phenotypes of fasted and fed Insig single knockout mice were mild. Likely, the small quantity of Insig-2 left in the fed Insig-1 knockout mouse was sufficient to maintain sterol-dependent regulation of SREBP processing and HMG-CoA reductase degradation. Although a deficit in the regulation of HMG-CoA reductase was noted in Insig-2 knockout mice during fasting (Appendix E), large increases in hepatic lipid contents or alterations in SREBP processing were not observed, which indicated that Insig-1 itself is sufficient to maintain gross lipid homeostasis.

These observations lead to several possible explanations for lack of dramatic changes in the livers of Insig single knockout mice. First, a trivial explanation: the small quantity of Insig-1 in fasted animals or Insig-2 in fed animals is not really small; the apparent disappearance Insig-1 in fasting or Insig-2 in feeding is due to of a lack of sensitivity in the polyclonal antibodies used to detect them. Second, small but statistically significant increases in esterified and total cholesterol were observed in the livers of Insig-1 and Insig-2 single knockout mice (same mice as those used in Figure 3-2). Since the interaction of Insigs and SCAP or HMG-CoA reductase likely are tertiary complexes that contain and are enhanced by sterols, these small increases in the hepatic cholesterol in Insig single knockout

mice could prime the remaining Insig protein so that it is capable of exerting control on SCAP:SREBP and HMG-CoA reductase. Finally, two examples of nonredundant functions of Insigs *in vivo*, one in liver and one in extrahepatic tissues, are described in the *Perspectives* below.

Perspectives for the future

Insigs and Regulation of Hepatic Cholesterol Synthesis during Fasting, Diurnal Rhythms, and High-Carbohydrate Feeding

Early studies by Brown and Dietschy demonstrated that HMG-CoA reductase activity is potently regulated by several influences other than maneuvers that alter the tissue cholesterol content, i.e. cholesterol feeding to increase hepatic cholesterol and inhibit HMG-CoA reductase activity or cholestyramine feeding to decrease hepatic cholesterol and induce it. These maneuvers include fasting and diurnal cycling (Dietschy and Brown, 1974; Brown et al., 1979). Furthermore, these maneuvers seem to regulate the abundance of the enzyme.

As noted above, HMG-CoA reductase protein did not decline with fasting in Insig-2 knockout or Insig double knockout livers, even though the suppression of nSREBPs was for the most part intact (Appendix E). These data suggest that the upregulation of Insig-2a in fasting serves to regulate HMG-CoA reductase levels, and provides one example of a regulatory function specific to a single Insig isoform. This functional specificity may be due to the sole expression of Insig-2 in fasting or may reflect intrinsic differences between Insig-1 and Insig-2, namely a higher affinity for Insig-2 and HMG-CoA reductase compared to Insig-1. To test the latter hypothesis, selective Insig-1 expression in fasting could be

achieved by breeding transgenic mice with a low, constitutive level of expression of Insig-1 with Insig-2 knockout mice and measuring the fasted HMG-CoA reductase content in the Insig-$2^{-/-}$;TgInsig-1 livers.

Although HMG-CoA reductase enzyme levels are stabilized in the fasted Insig-2 knockout livers, its activity and the *in vivo* sterol synthesis rates should be measured to verify whether or not the fasting-stabilized enzyme levels retains activity. The consequence of high levels of sterol synthesis during prolonged fasting and potential competition for substrate with ketogenesis is difficult to predict but should be investigated.

Preliminary studies also indicate that Insig-2 expression varies in reciprocal fashion with HMG-CoA reductase protein levels the diurnal cycle (Appendix F); the peak expression of HMG-CoA reductase coincides with the nadir of Insig-2 expression. To test the hypothesis that the diurnal variations in reductase abundance are due to reciprocal changes in Insig-2 expression, Insig-2 knockout mice should be subjected to diurnal cycling and HMG-CoA reductase protein levels measured.

Recent studies by Kuriyama also demonstrated that feeding of a fat-free, high carbohydrate diet potently decreased hepatic cholesterol synthesis and HMG-CoA reductase protein levels, albeit without large changes in the levels of Insig mRNAs (Kuriyama et al., 2005). Whether Insigs mediate the decline in HMG-CoA reductase caused by fat-free diet feeding is unknown but readily testable in Insig double knockout mice.

The aim of these studies is to define the role of Insigs in the regulation of HMG-CoA abundance in four established experimental manipulations that alter HMG-CoA reductase

abundance, activity, and in consequence cholesterol synthesis: cholesterol feeding (as shown in the current studies), fasting, diurnal variations, and high-carbohydrate feeding.

Insigs and lipid homeostasis in Gonads

The current studies focused on liver, because liver is responsible for the majority of total body sterol synthesis and it is the major site of sterol synthesis sensitive to inhibition by cholesterol feeding. However, Insig-1 is highly expressed in white adipose tissue in addition to liver and is expressed at lower levels in many tissues. The testes and ovaries of mice with a germline deficiency of Insig-1 (*Insig-1*$^{-/-}$) accumulate high levels of lipid (Appendix G) Histologic sections show that the lipid accumulation detected by oil-red staining is partitioned into the steroid-producing Leydig cells in testes. In ovaries, the lipid accumulation was much more generalized, sparing follicles and accumulating in corpora luteum and in vacuolated theca and stromal cells. These mice showed reduced fecundity when knockout females and males were mated; detailed breeding studies should be performed to localize the fertility defect to males, females, or both. Further studies are required to study the potential role of Insig-1 in regulating lipid homeostasis in gonadal steroid-producing cells and whether the florid lipid accumulation is due to alterations in lipid synthesis rates resultant from a loss of Insig-mediated regulation of the SCAP/SREBP complex and HMG-CoA reductase or by other, yet undefined, mechanisms.

These gonadal changes represent another example of nonredundant Insig function, since they are not noted in Insig-2 knockout animals. This functional specificity may be due to the sole expression of Insig-1 in the affected subset of gonadal cells, which can be tested

by *in situ* analysis of Insig-1 and Insig-2 mRNA. If no isoform-specific expression pattern is noted, intrinsic functional differences between Insig-1 and Insig-2 may account for absence of the phenotype in Insig-2 knockout animals although the nature of such differences in the context of steroidogenic cells is totally undefined.

Insigs during Craniofacial Development

As shown in Table 3-1, mice homozygous for germline deletions in Insig-1 and Insig-2 are nonviable. These mice die early in the neonatal period and present with a highly penetrant cleft palate or cleft face / cleft palate malformation (Appendix H). Gene knockouts in other elements of the SREBP pathway do not show this malformation. Unexpectedly, tissues of Insig deficient embryos accumulate not only high levels of cholesterol but also high levels of sterol intermediates of the cholesterol biosynthetic pathway such as 7-dehydrocholesterol, desmosterol, lathosterol, zymosterol, and lanosterol (data not shown). Curiously, the lipid-laden ovaries of *Insig-1*$^{-/-}$ mice also accumulate high levels of these intermediates. The accumulation of such intermediates is usually attributed to a downstream block in the biosynthetic pathway; i.e. mice with deficiencies in 7-dehydrocholesterol reductase (*DHCR7*) accumulate 7-dehydrocholesterol. Although the nature of such a downstream block in *Insig-1*$^{-/-}$*2*$^{-/-}$ tissues is unclear; it is unlikely to be transcriptional since the genes responsible for metabolizing these sterol intermediates are SREBP targets (Horton et al., 2003) and nSREBPs are predicted to be elevated in these Insig-null embryos. Alternatively, the accumulation of cholesterol precursors may be secondary to an increase in the abundance of HMG-CoA reductase in Insig-null embryos; *Saccharomyces cerevisiae* strains which

overexpress the catalytic domain of yeast HMG-CoA reductase, Hmg1p, do not accumulate ergosterol but rather accumulate squalene and other sterol intermediates (Donald et al., 1997; Veen et al., 2003).

Smith-Lemli-Opitz Syndrome (SLOS), a human autosomal recessive disorder associated with multiple malformations including cleft palate, is caused by mutations in *DHCR7*. Gene knockouts in mice have been generated for *DHCR7*; homozygous null pups die in the early neonatal period and approximately 10% present with cleft palate (Wassif et al., 2001; Fitzky et al., 2001). Lathosterol-5-desaturase (*Sc5D*) null pups accumulate lathosterol and display a strongly penetrant cleft palate (Krakowiak et al., 2003). Human X-linked dominant chondrodysplasia punctata (CDPX2), another multiple malformation syndrome, is caused by mutations in mutations in emopamil binding protein (*EBP*, Δ^8 Δ^7-sterol isomerase). *Tattered* mice, deficient in *EBP,* accumulate zymosterol; affected hemizygous males die late in gestation and display cleft palate (Derry et al., 1999). Whether cholesterol deficiency or accumulation of potentially toxic precursor sterols mediate the developmental abnormalities in these disorders is a controversial subject. The observation that mouse embryos deficient in Insig-1 and Insig-2, DHCR7, Sc5D, and EBP have cleft palates and high levels of sterol intermediates may favor the latter interpretation.

Alternatively, the disruption of palate development in Insig double knockout embryos may reflect the loss of Insig-mediated regulation of other SSD-containing proteins, such as Patched, the receptor for the morphogen Sonic Hedgehog (Shh), or DHCR7 (Kuwabara and Labouesse., 2002; Bae et al., 1999). Deficiencies in Shh signaling cause holoprosencephaly, which is frequently associated with palate defects in addition to other abnormalities in

forebrain development (Wallis and Muenke, 2000). Recent studies by Beachy have suggested that deficiencies in Shh signaling may account for the malformations seen in mouse models of SLOS and lathosterolosis (Cooper et al., 2003). Uncovering the nature of the disruption of palate development in Insig null embryos will require many studies, but may shed light on the curious interactions between cholesterol metabolism and craniofacial development. .

APPENDIX A

Effects of feeding various amounts of cholesterol (0.02 to 0.05%) to wild type and TgInsig-1 mice

Parameter	Wild type				TgInsig-1			
	0.02%	0.05%	0.15%	0.5%	0.02%	0.05%	0.15%	0.5%
Body weight (g)	20.5 ± 0.6	20.4 ± 0.8	20.2 ± 0.4	20.4 ± 0.9	19.5 ± 0.2	19.7 ± 0.3	19.4 ± 0.4	19.4 ± 0.3
Liver weight (g)	1.2 ± 0.04	1.2 ± 0.03	1.3 ± 0.02	1.2 ± 0.04	1.1 ±- 0.05	1.1 ± 0.03	1.2 ± 0.05	1.2 ± 0.02
Liver cholesterol content (mg/g)	2.3 ± 0.05	2.7 ± 0.15	2.8 ± 0.1	4.5 ± 0.2	1.9 ± 0.05**	1.9 ± 0.05**	2.3 ± 0.1*	3.8 ± 0.3
Liver triglyceride content (mg/g)	7.2 ± 1.6	6.9 ± 1.5	8.6 ± 0.6	10.1 ± 1.4	5.0 ± 0.2	4.9 ± 1.1	3.4 ± 0.1**	7.8 ± 0.8
Total plasma cholesterol (mg/dl)	70 ± 5	72 ± 2	81 ± 2	94 ± 6	49 ± 4*	46 ± 3**	42 ± 4**	67 ± 8*
Total plasma triglycerides (mg/dl)	131 ± 23	104 ± 9	85 ± 6	67 ± 8	107 ± 18	71 ± 8	92 ± 16	66 ± 2
Plasma insulin (ng/ml)	0.53 ± 0.02	0.50 ± 0.04	0.50 ± 0.19	0.55 ± 0.09	0.43 ± 0.11	0.46 ± 0.02	0.62 ± 0.08	0.51 ± 0.05
Plasma glucose (mg/dl)	205 ± 9	193 ± 10	190 ± 8	190 ± 1	184 ± 8	182 ± 16	213 ± 7	200 ± 4
Plasma free fatty acids (mM)	0.61 ± 0.08	0.62 ± 0.1	0.66 ± 0.09	0.47 ± 0.07	0.52 ± 0.04	0.44 ± 0.05	0.49 ± 0.04	0.54 ± 0.02

8-week old female mice (4 per group) were fed an ad libitum chow diet supplemented with the indicated amount of cholesterol for 2 days prior to study. The wild type mice were littermates of the transgenic mice. Each value represents the mean ± SEM of 4 mice. Asterisks denote the level of statistical significance (Student's *t* test) between the wild type and TgInsig-1 groups. *, $p < 0.05$; **, $p < 0.01$.

APPENDIX B

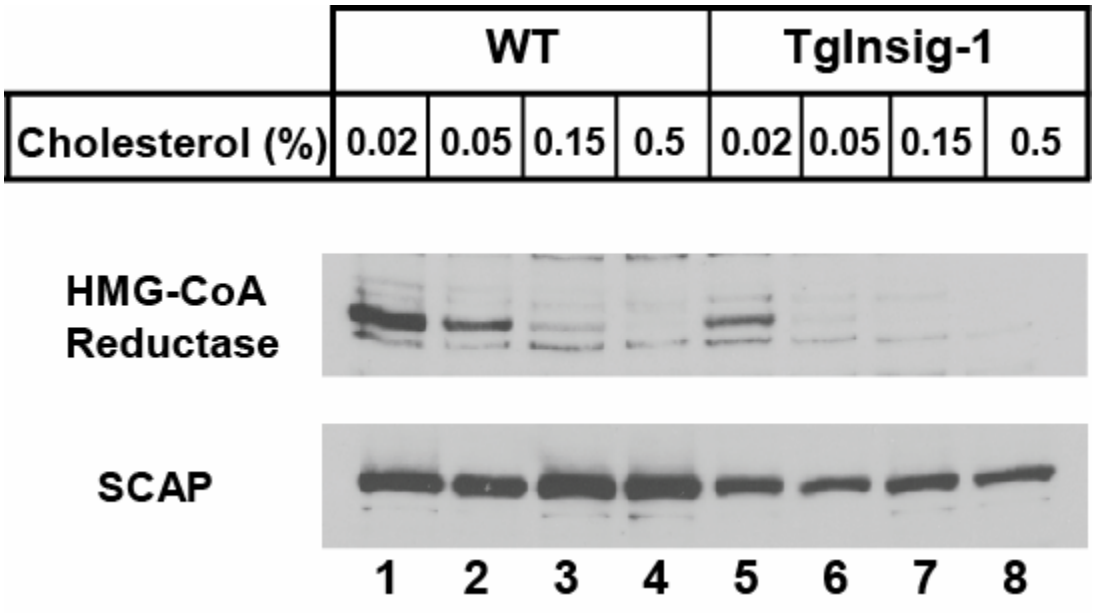
Effects of fasting and refeeding in wild type and TgInsig-1 mice

Parameter	Wild type			TgInsig-1		
	Nonfasted	Fasted	Refed	Nonfasted	Fasted	Refed
Body Weight (g)	28.8 ± 0.2	26.7 ± 0.8	28.1 ± 0.9	27.0 ± 1.1	23.9 ± 0.5*	27.4 ± 1.2
Liver Weight (g)	1.5 ± 0.1	1.2 ± 0.1	2.2 ± 0.0	1.5 ± 0.1	1.0 ± 0.0*	2.0 ± 0.1
Liver cholesterol content (mg/g)	2.4 ± 0.1	3.4 ± 0.3	1.6 ± 0.1	2.0 ± 0.1**	2.6 ± 0.1*	1.4 ± 0.1*
Liver triglyceride content (mg/g)	5.4 ± 0.6	41 ± 7	4.5 ± 0.3	2.6 ± 0.1**	20 ± 13	2.0 ± 0.3**
Total plasma cholesterol (mg/dl)	100 ± 7	119 ± 6	90 ± 8	69 ± 4**	70 ± 5**	47 ± 3**
Total plasma triglycerides (mg/dl)	70 ± 7	57 ± 9	293 ± 25	64 ± 5	38 ± 11	60 ± 14**
Plasma insulin (ng/ml)	0.87 ± 0.1	0.13 ± 0.02	3.5 ± 0.78	0.57 ± 0.05	0.22 ± 0.04	1.9 ± 0.36
Plasma glucose (mg/dl)	222 ± 16	133 ± 14	213 ± 20	195 ± 13	128 ± 20	187 ± 16
Plasma free fatty acids (mM)	0.35 ± 0.02	0.78 ± 0.13	0.24 ± 0.02	0.34 ± 0.02	0.73 ± 0.02	0.12 ± 0.02 **

16-week old male mice (5 per group) were subjected to fasting and refeeding as described in Methods of Chapter 2. The wild type mice were littermates of the transgenic mice. Each value represents the mean ± SEM of 5 mice. Asterisks denote the level of statistical significance (Student's *t* test) between the wild type and TgInsig-1 groups. *, $p < 0.05$; **, $p < 0.01$.

APPENDIX C

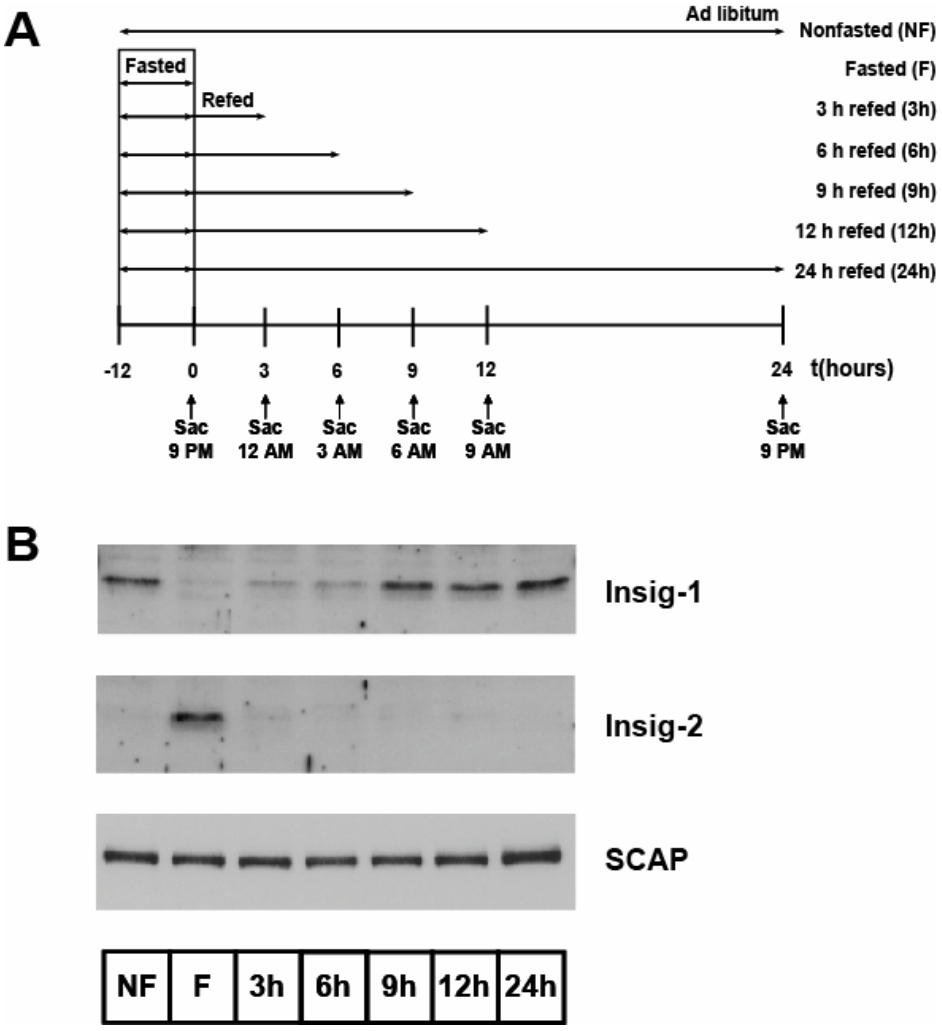
Effects of feeding cholesterol feeding on HMG-CoA reductase and SCAP proteins in the livers of WT and TgInsig-1 mice



8-week old female mice (4 per group) were fed an ad libitum chow diet supplemented with the indicated amount of cholesterol for 2 days prior to study. The wild type mice were littermates of the transgenic mice. A detailed description of these mice is provided in Appendix B and are the same as those shown in Figure 2-2. (A) Immunoblot analysis. Livers from each group (4 mice per group) were pooled, and 30-μg aliquots of the membrane fractions were subjected to SDS-PAGE and immunoblotted with antibodies against HMG-CoA reductase and SCAP

APPENDIX D

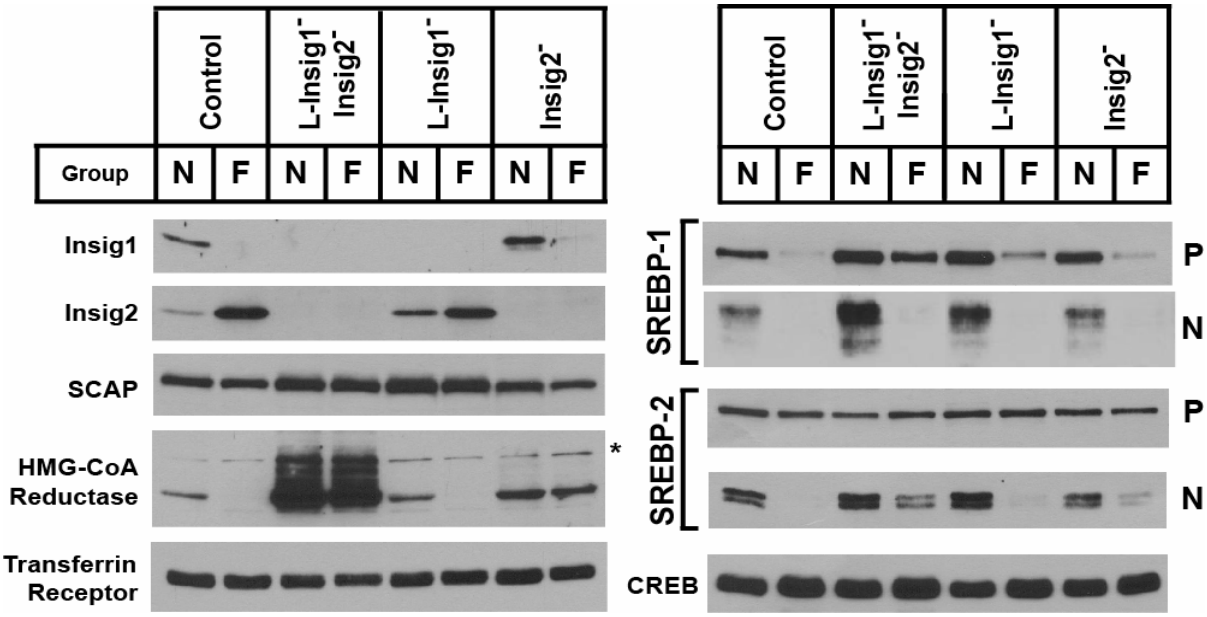
Immunoblot analysis of Insig-1 and Insig-2 protein in the livers of WT mice subjected to fasting and various time intervals of refeeding



13-week old male mice (4 per group) were fed an ad libitum chow diet until the day of study. (A) Schematic of the fasting and refeeding timecourse. NF: nonfasted mice were continued on the ad libitum chow diet. F: fasted mice were fasted for 12 hours prior to sacrifice. 3h, 6h, 9h, 12h, 24h: mice were fasted for 12 hours and then refed a high-carbohydrate diet for the indicated number of hours. All times of sacrifice are indicated and all mice were maintained in a single facility with a 12 hour light/dark cycle (9AM to 9PM lights on). (B) Immunoblot analysis. Livers from each group (4 mice per group) were pooled, and 45-μg aliquots of the membrane fraction was subjected to SDS-PAGE and immunoblotted with polyclonal antibodies against Insig-1 and Insig-2.

APPENDIX E

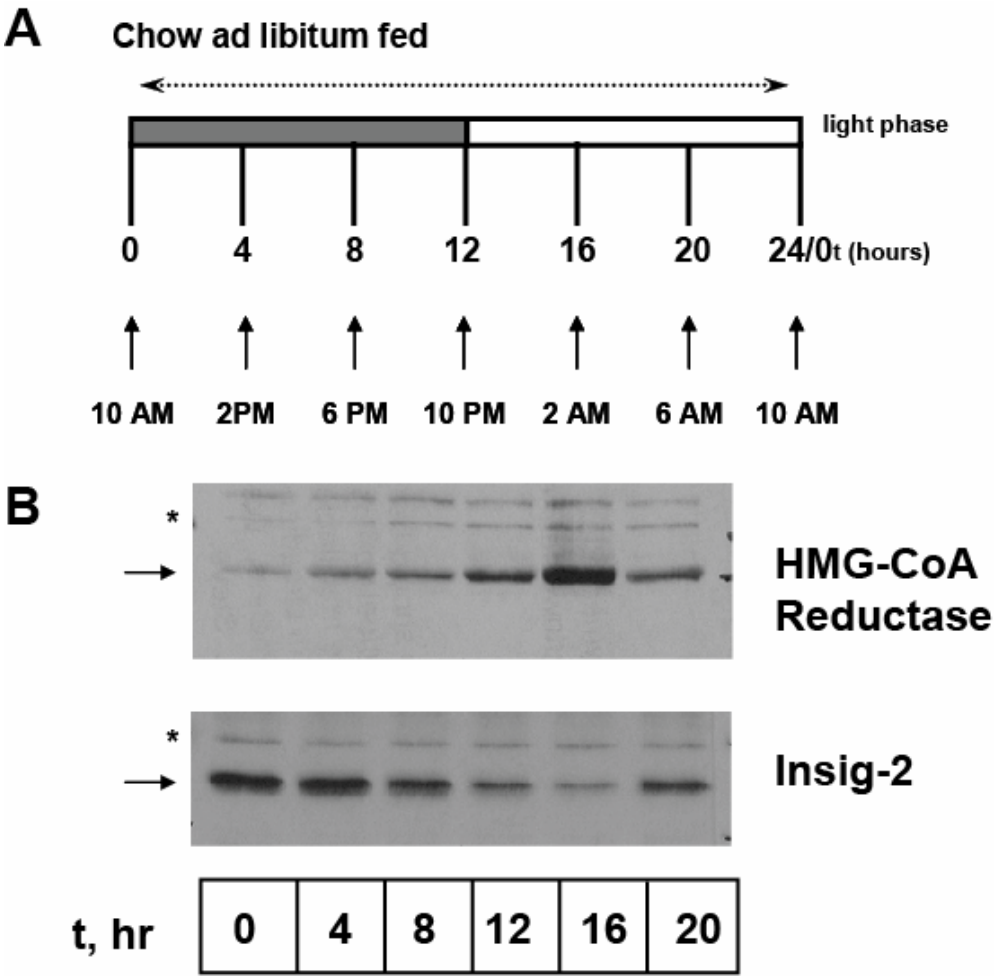
Effects of fasting on SREBP, HMG-CoA reductase, and Insig proteins in the livers of control and Insig-deficient mice



Immunoblot analysis of nuclear extract and membrane fractions obtained from 10 to 12-week old male mice with the following four genotypes subjected to fasting: 1) *Insig-1*$^{f/f}$ (designated Control); 2) *Insig-1*$^{f/f}$; *Insig-2*$^{-/-}$;*MX1-Cre* (designated *L-Insig-1*$^{-/-}$*;Insig-2*$^{-/-}$); 3) *Insig-1*$^{f/f}$;*MX1-Cre* (designated *L-Insig-1*$^{-/-}$); and 4) *Insig-1*$^{f/f}$;*Insig-2*$^{-/-}$ (designated *Insig-2*$^{-/-}$). Each mouse was treated with 4 intraperitoneal injections of pIpC (300 μg/injection), and tissues were obtained 14 days after the final injection. N denotes nonfasted mice fed a chow diet ad libitum prior to sacrifice. F denotes mice which were fasted for 12 hours prior to sacrifice. Livers (5 mice per group) were separately pooled, and 45 μg aliquots of the pooled membrane and nuclear extract fractions were subjected to SDS-PAGE and immunoblot analysis. The precursor and nuclear forms of SREBPs are denoted as P and N, respectively. CREB protein (cAMP-responsive element binding protein) and the transferrin receptor were used in the immunoblots as loading controls for the nuclear extract and membrane fractions, respectively. Asterisks denote non-specific bands.

APPENDIX F

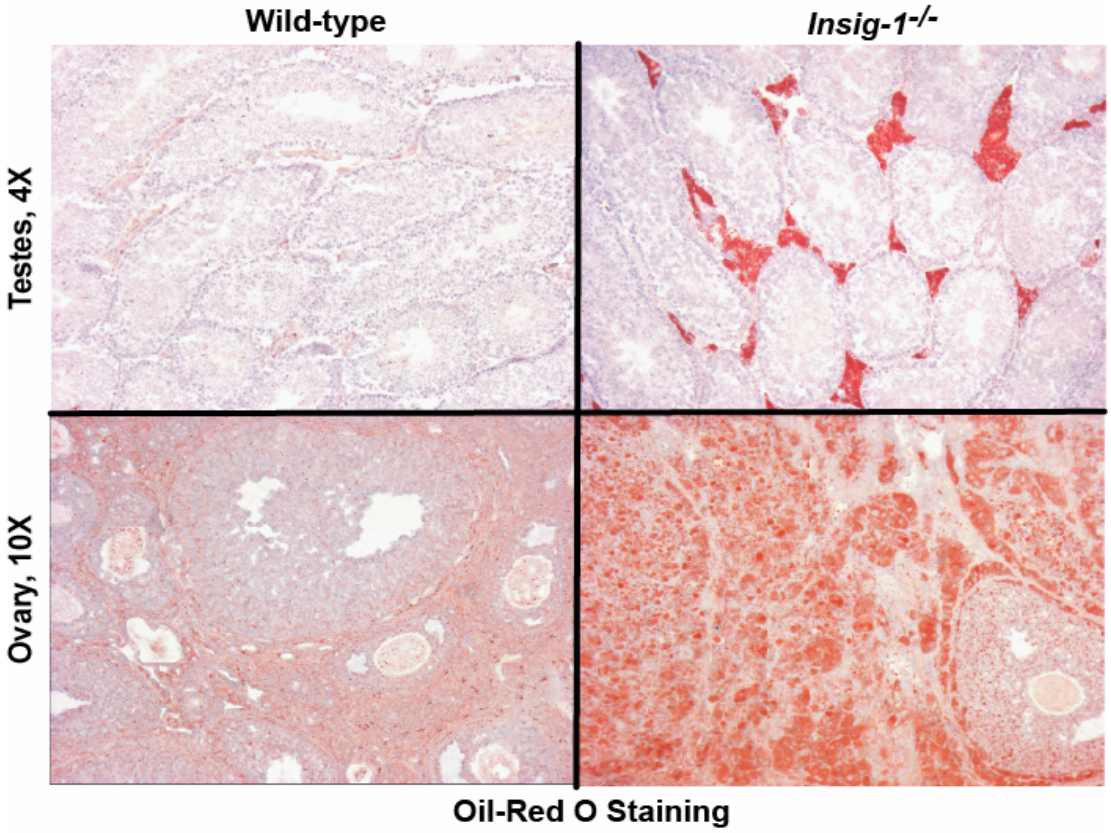
Immunoblot analysis of HMG-CoA reductase and Insig-2 proteins in the livers of WT mice killed at regular intervals across the diurnal cycle



4-month old male mice (4 per group) were fed an ad libitum chow diet throughout the study. (A) Schematic of the diurnal timecourse study. All times of sacrifice are indicated and all mice were maintained in a single facility with a 12 hour light/dark cycle (9AM to 9PM lights on). (B) Immunoblot analysis. Livers from each group (4 mice per group) were pooled, and 45-μg aliquots of the membrane fraction was subjected to SDS-PAGE and immunoblotted with polyclonal antibodies against HMG-CoA reductase and Insig-2. Asterisks denote non-specific bands. Study performed with B. Evers.

APPENDIX G

Oil-Red O staining of histologic sections from gonads of WT and Insig-1$^{-/-}$ mice

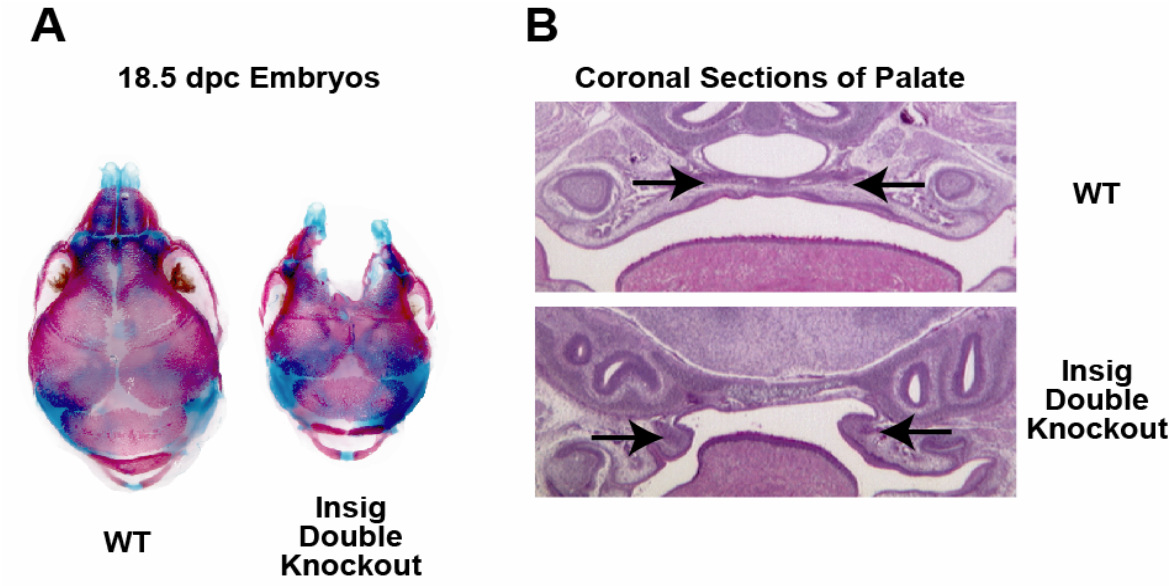


Lipid accumulation in the gonads of WT and Insig-1$^{-/-}$ mice. Testes and ovaries from adult male and female WT and Insig-1$^{-/-}$ mice, respectively, were harvested, fixed in 10% formalin, and cryosections were stained with Oil-Red O. Magnification is indicated.

(sectioning and Oil-Red O staining by the UTSW Pathology Core - Jim Richardson)

APPENDIX H

Craniofacial structure of 18.5 dpc WT and Insig-1$^{-/-}$2$^{-/-}$ embryos



Clefting malformations in Insig-1$^{-/-}$2$^{-/-}$ embryos. Male and female Insig-1$^{+/-}$2$^{-/-}$ mice were interbred and mated females were identified by the detection of a vaginal plug. 18.5 dpc embryos were harvested by caesarian section and (A) soft tissues were dissolved and skeletons were stained with alizarin red and alcian blue or (B) fixed in 10% formalin and histologic sections were stained with H&E. Arrows indicate locations of the palatal shelf.

(sectioning and H&E staining by the UTSW Pathology Core - Jim Richardson)

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VITAE

Luke James Engelking was born in Houston, Texas, on April 20, 1978, the son of Cynthia Wilderman Engelking and James Culberson Engelking. After completing his work at Cypress Creek Senior High School, Houston, Texas in 1996, he entered Texas A&M University at College Station, Texas. There he worked in the laboratory Max D. Summers from 1998 to 2000. He completed summer research fellowships at University of Texas Southwestern Medical Center and Baylor College of Medicine in the summers of 1998 and 1999, respectively. He received the degree of Bachelor of Science with a double major in biochemistry and genetics from Texas A&M University in May 2000. Later that summer, he entered the Medical Scientist Training Program at the University of Texas Southwestern Medical Center at Dallas and joined the laboratory of Michael S. Brown and Joseph L. Goldstein. On July 14, 2001, he married Stephanie Ann Vierus of Houston, Texas.

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