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# Hepatic Insulin Signaling Is Required for Obesity-Dependent Expression of SREBP-1c mRNA but Not for Feeding-Dependent Expression

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## SUMMARY

**Dissecting the role of insulin in the complex regulation of triglyceride metabolism is necessary for understanding dyslipidemia and steatosis. *L*iver *i*nsulin *r*eceptor *k*nockout (LIRKO) mice show that in the physiological context of feeding, hepatic insulin signaling is not required for the induction of mTORC1, an upstream activator of the lipogenic regulator, SREBP-1c. Feeding induces SREBP-1c mRNA in LIRKO livers, though not to the extent observed in controls. A high fructose diet also partially induces SREBP-1c and lipogenic gene expression in LIRKO livers. Insulin signaling becomes more important in the pathological context of obesity, as knockdown of the insulin receptor in *ob/ob* mice, a model of Type 2 diabetes, using antisense oligonucleotides, abolishes the induction of SREBP-1c and its targets by obesity and ameliorates steatosis. Thus, insulin-independent signaling pathways can partially compensate for insulin in the induction of SREBP-1c by feeding but the further induction by obesity/Type 2 diabetes is entirely dependent upon insulin.**

## INTRODUCTION

In normal physiology, feeding triggers the release of insulin, which suppresses glucose production and activates lipogenesis in the liver. In obesity and Type 2 diabetes, insulin fails to suppress glucose production, and hence, these conditions are considered states of insulin resistance. However, lipogenesis is paradoxically increased. Excessive hepatic lipogenesis promotes the development of dyslipidemia and atherosclerosis, the leading cause of death in diabetic patients, and the development of nonalcoholic fatty liver disease (NAFLD). NAFLD affects more than 40% of diabetic patients (Williamson et al., 2011), but effective therapies for it have yet to be found (Cusi, 2009). It has even been argued that excessive hepatic lipogenesis, by promoting ectopic lipid deposition in the muscle, could compromise glucose uptake and produce hyperglycemia itself (McGarry, 1992). Despite the prevalence of obesity and Type 2 diabetes, and the significant morbidity and mortality associated with these disorders, we have yet to define the factors that drive hepatic lipogenesis in the insulin resistant state.

At the molecular level, the increase in lipogenesis observed in insulin-resistant states is due at least in part to dysregulation of the master transcriptional regulator of lipogenesis, sterol regulatory element binding protein (SREBP)-1c. SREBP-1c is capable of inducing the entire complement of genes necessary for the synthesis of monounsaturated fatty acids (Horton et al., 1998). Insulin activates SREBP-1c by at least two mechanisms: it increases SREBP-1c transcription (Foretz et al., 1999), and it increases the processing of SREBP-1c from an inactive membrane-bound precursor to a soluble fragment capable of translocating to the nucleus to activate transcription (Yabe et al., 2003). Thus, SREBP-1c is decreased in insulin-deficient states, such as fasting (Horton et al., 1998) and streptozotocin-diabetes (Shimomura et al., 1999), but increased in mouse models of insulin resistance, such as mice fed a high fat diet (Biddinger et al., 2005), *ob/ob* mice (Shimomura et al., 2000), and lipodystrophic mice (Shimomura et al., 2000).

There are two possible explanations for the increase in lipogenesis observed in the insulin-resistant state. The first possibility is that insulin, despite its inability to control glucose homeostasis, retains its ability to stimulate lipogenesis (Reaven, 1988). This is conceivable because of the complex nature of insulin signaling (Biddinger and Kahn, 2006). Upon binding to its receptor, insulin triggers a branching cascade of signaling events to exert its myriad effects upon the cell. As suggested by Brown and Goldstein, the signaling pathways utilized by

insulin to stimulate SREBP-1c could remain intact in obesity and Type 2 diabetes, even as the pathways that regulate glucose metabolism become resistant (Brown and Goldstein, 2008). Thus, insulin would fail to suppress glucose production, leading to hyperglycemia and a compensatory hyperinsulinemia. The hyperinsulinemia, acting through signaling pathways that remain sensitive to insulin, would drive SREBP-1c and lipogenesis to excess.

Alternatively, it is possible that in insulin resistant states, lipogenesis is driven by insulin-independent pathways. Particular attention has recently focused on dietary carbohydrates. The excessive consumption of carbohydrates, particularly in the form of sweetened beverages, has risen in parallel with the prevalence of obesity, diabetes, and fatty liver disease in our society. Dietary carbohydrates, particularly fructose, drive lipogenesis (Mayes, 1993) and excessive fructose intake is correlated with elevations in serum (Mayes, 1993) and hepatic (Ouyang et al., 2008) triglyceride levels, as well as obesity and other features of the metabolic syndrome (Johnson et al., 2007). Whether carbohydrates increase lipogenesis directly, or indirectly, by stimulating insulin secretion, has been difficult to dissect. However, the fact that carbohydrate loads in humans can induce hypertriglyceridemia even in the presence of somatostatin, which inhibits insulin secretion, indicates that at least some of the carbohydrate effect may be independent of insulin signaling (Ginsberg et al., 1982). Consistent with this, SREBP-1c can be induced by carbohydrate feeding even in streptozotocin-treated mice, which are insulin deficient (Matsuzaka et al., 2004). Moreover, SREBP-1c is not the only transcriptional regulator of lipogenesis. For example, *c*arbo*h*ydrate *r*esponse *e*lement *b*inding *p*rotein (ChREBP) induces lipogenic gene expression primarily in response to glucose and could potentially contribute to the insulin-independent regulation of the lipogenic genes (Denechaud et al., 2008).

Using mice with knockdown or knockout of the insulin receptor, we have dissected the roles of insulin-dependent versus insulin-independent pathways in the control of SREBP-1c and hepatic triglycerides under different physiological and pathological conditions. The livers of *l*iver *i*nsulin *r*eceptor *k*nock*o*ut (LIRKO) mice are entirely unable to respond to insulin, due to a lack of the insulin receptor, but are nonetheless capable of responding to nutrients or other stimuli. We have previously shown that LIRKO mice have decreased levels of SREBP-1c and its targets, decreased plasma triglycerides, and decreased VLDL-triglyceride content (Biddinger et al., 2008). The fact that humans with insulin receptor mutations show a similar phenotype, with decreased lipogenesis, decreased serum triglycerides and decreased VLDL-triglyceride content, demonstrates the utility of the LIRKO model for studying the effects of insulin in vivo (Semple et al., 2009). In the present studies, we show that, surprisingly, insulin signaling is not required for the control of lipogenic gene expression and hepatic triglycerides under the normal physiological stimuli of fasting and feeding. Moreover, lipogenic gene expression is induced by a fructose diet even in the absence of hepatic insulin signaling. However, under the pathological conditions of Type 2 diabetes, modeled by *ob/ob* mice, insulin signaling is required for the induction of SREBP-1c and the lipogenic genes and contributes to steatosis.

## RESULTS

### Regulation of the Lipogenic Program Is Largely Preserved in LIRKO Mice under Fasted and Ad Libitum Fed Conditions

The lipogenic program is under complex control by multiple factors, including insulin, other hormones, and nutrients themselves. In normal physiology, these factors suppress lipogenesis in the fasted state and activate it in the fed state. To dissect the specific role of insulin in the control of the lipogenic program, we used LIRKO mice. LIRKO mice and their littermate controls were maintained on a standard chow diet and studied after a 24 hr fast or in the ad libitum fed state.

Relative to their controls, LIRKO mice consumed similar amounts of food and were of normal weight (Table S1). However, consistent with the lack of hepatic insulin signaling, LIRKO mice were hyperglycemic, despite marked hyperinsulinemia, and were unable to suppress the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (*Pck1*) with feeding (Table S1). On the other hand, the ability of feeding to increase plasma glucose and thyroid hormone levels, and suppress plasma glucagon levels, was normal in LIRKO mice, though the suppression of corticosterone was blunted (Table S1).

In control livers, feeding increased *SREBP-1c* mRNA levels by 10-fold (Figure 1A). In LIRKO livers, *SREBP-1c* mRNA levels were increased 3-fold with feeding, despite their complete absence of hepatic insulin signaling. The lipogenic genes, acetyl CoA carboxylase (*Acc*), fatty acid synthase (*Fasn*), and stearoyl CoA desaturase 1 (*Scd1*), showed a similar expression pattern with *Acc*, *Fasn*, and *Scd1* increased 2- to 10-fold in both control and LIRKO mice (Figures 1B–1D). These data indicate that though insulin signaling is necessary for maximal induction, nutrients—either directly, or indirectly, via hormones other than insulin—are sufficient for the induction of the *SREBP-1c* and lipogenic gene transcripts.

ChREBP is another lipogenic transcription factor; it is activated by glucose (Postic et al., 2004). *ChREBP* mRNA, in parallel with blood glucose levels (Table S1), was induced to a similar level in control and LIRKO mice by feeding (Figure 1E), showing that the transcriptional regulation of *ChREBP* is independent of insulin. However, the activation of ChREBP requires its translocation to the nucleus, which in turn requires the metabolism of glucose to glucose-6-phosphate by glucokinase, an enzyme induced by insulin (Dentin et al., 2004). Thus, in control livers, feeding increased glucokinase expression by 50-fold, and this was associated with an increase in ChREBP protein in the nucleus (Figures 1F and 1G). In contrast, feeding induced glucokinase only 3-fold in LIRKO mice. Thus, glucokinase mRNA levels in the fed state were 50-fold lower than controls, and LIRKO livers showed no increase in nuclear ChREBP. Consequently, pyruvate kinase (*Pklr*), a relatively specific target of ChREBP, was not induced by feeding in LIRKO livers, though it was induced almost 10-fold in control livers (Figure 1H).

In the fasted state, nonesterified fatty acids are released from the adipocyte, taken up by the liver, and oxidized to generate ketones and fuel gluconeogenesis. Plasma nonesterified fatty acids were similar in control and LIRKO mice in the fasted state (Figure 1I), and the uptake of free fatty acids was uncompromised in LIRKO hepatocytes (Figure S1A). Direct measurements

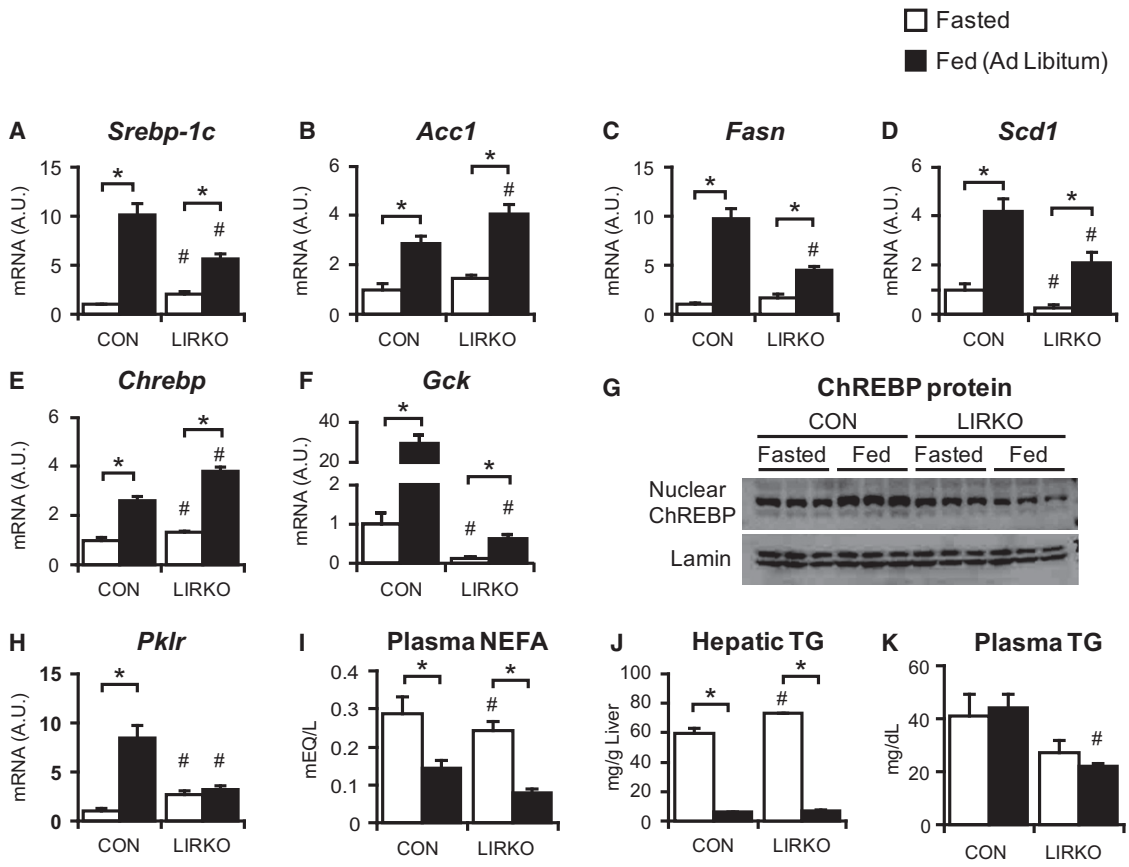


**Figure 1. Regulation of Lipogenic Gene Expression and Hepatic Triglycerides by Ad Libitum Feeding Is Largely Preserved in LIRKO Livers**

(A–K) LIRKO mice and their littermate controls (CON) were maintained on a chow diet and sacrificed after a 24 hr fast or in the ad libitum fed state. In (A–F) and (H), hepatic gene expression was measured using real-time PCR. ChREBP and lamin (G) were measured by immunoblotting nuclear extracts (each lane represents a sample taken from an individual mouse liver). Plasma nonesterified fatty acids (I), hepatic triglycerides (J), and plasma triglycerides (K) were measured at the time of sacrifice. Error bars represent SEM; n = 4–8; *p < 0.05 versus nonfasted mice of the same genotype; #p < 0.05 versus control mice in the same feeding state. See also Figure S1 and Table S1.

of fatty acid oxidation in LIRKO hepatocytes in vitro were normal (Figures S1B and S1C), and the respiratory exchange ratio, which reflects whole body fatty acid oxidation, was normal (data not shown). Consistent with this, hepatic triglycerides were normal in fasted LIRKO livers (Figure 1J).

In the fed state, insulin and other hormones act at the level of the adipocyte to suppress lipolysis and thereby decrease the flux of nonesterified fatty acids to the liver. Because insulin receptor expression is normal in the extrahepatic tissues of the LIRKO mouse (Figure S2C), the regulation of adipocyte lipolysis would be expected to be normal. Indeed, in response to feeding, LIRKO mice, like control mice, showed a 3-fold reduction in plasma nonesterified fatty acids and a 10-fold reduction in hepatic triglycerides. Plasma triglycerides were lower in LIRKO mice than controls, consistent with the decreased triglyceride secretion reported previously (Biddinger et al., 2008), but did not change with fasting (Figure 1K).

## Insulin-Independent Activation of mTORC1 Targets

The mTORC1 complex is an important regulator of SREBP-1c (Li et al., 2010; Porstmann et al., 2005; Jeon and Osborne, 2012). Insulin stimulates mTORC1 via a signaling cascade involving Akt. Upon activation, Akt becomes phosphorylated on residues Thr308 and Ser473 and, in turn, activates mTORC1(Biddinger and Kahn, 2006).

In fasted control livers, 5 min of insulin stimulation induced tyrosine phosphorylation of a 100 kDa protein, assumed to be the insulin receptor, and phosphorylation of Akt on Thr308 and Ser473 (Figure 2A). In contrast, in LIRKO hepatocytes, insulin was unable to activate Akt either in vivo or in vitro (Figures 2A and S2A). However, insulin signaling in LIRKO muscle and fat was not impaired (Figures S2B and S2C).

Feeding activates mTORC1 in vivo (Sengupta et al., 2010). Phosphorylation of the ribosomal S6 protein, a well-established marker of mTORC1 activation, was very low in the livers of fasted mice of either genotype (Figure 2B). Consistent with prior reports, 1 hr of feeding robustly induced phospho-S6 levels in control livers (Sengupta et al., 2010). Surprisingly, feeding was also able to induce phospho-S6 in LIRKO livers, despite their inability to respond to insulin. This induction was abolished by the mTORC1 inhibitor, rapamycin (data not shown).

In addition to stimulating lipogenesis, mTORC1 is required for the inhibition of ketogenesis upon feeding (Sengupta et al.,

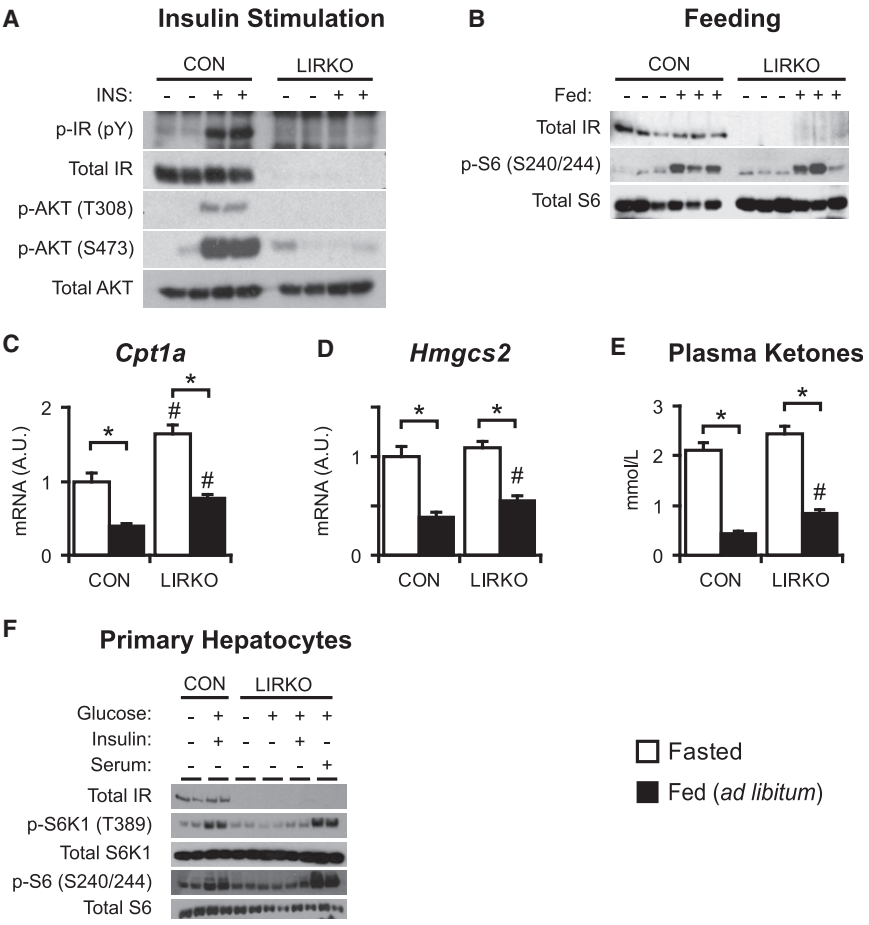


**Figure 2. Insulin-Independent Activation of mTORC1**

(A) Control and LIRKO mice were fasted overnight, injected with either vehicle or 3 units of insulin via the inferior vena cava, and sacrificed 5 min later. Livers were subjected to immunoblotting.

(B) Mice were fasted for 24 hr, or fasted for 24 hr and refed a high carbohydrate diet for 1 hr. Livers were subjected to immunoblotting.

(C–E) Mice were sacrificed in the ad libitum fed state or after a 24 hr fast. In (C and D), hepatic gene expression was measured by real-time PCR. In (E), ketone levels were measured in the blood at the time of sacrifice.

(F) Hepatocytes were isolated from 2-month-old female control and LIRKO mice. After plating, hepatocytes were incubated overnight in serum-free medium containing low glucose (5 mM) and then stimulated for 10 min with high glucose (25 mM), insulin (10 nM), and/or serum (10%). Error bars represent SEM; n = 4–8; *p < 0.05 versus nonfasted mice of the same genotype; #p < 0.05 versus control mice in the same feeding state. See also Figure S2.

2010). Upon feeding, mTORC1 is activated; *Cpt1a* and *Hmgcs2*, two genes required for ketone synthesis, are suppressed, and blood ketone levels fall. Thus, in the livers of ad libitum fed LIRKO mice, *Cpt1a* and *Hmgcs2* mRNA levels and blood ketone levels are decreased 2- to 3-fold relative to the livers of fasted mice (Figures 2C–2E). Taken together, these data suggest that mTORC1 activation in response to feeding is intact in LIRKO livers.

Previous studies have shown that nutrients alone are not sufficient for the activation of mTORC1 and that growth factors must also be present (Sancak et al., 2008). The insulin and IGF-1 receptors activate an overlapping, if not identical, set of downstream targets. Thus, it was conceivable that IGF-1 receptor could compensate for the lack of insulin receptor in LIRKO hepatocytes. To test this, we measured IGF-1 receptor expression in LIRKO livers. IGF-1 receptor was undetectable in control livers, as previously reported (Czech, 1982), as well as LIRKO livers (Figure S2D). Moreover, hepatocytes cultured from LIRKO livers failed to respond to IGF-1 (Figure S2A).

Nonetheless, it remained possible that growth factors other than insulin/IGF-1 could play a permissive role in the activation of mTORC1 by nutrients in LIRKO hepatocytes. To test this, primary hepatocytes were isolated from control and LIRKO livers, and cultured overnight in media containing low glucose and no serum. Subsequent stimulation of control hepatocytes with high glucose and insulin produced phosphorylation of the mTORC1 target S6 kinase (S6K1) and its downstream target ribosomal S6 (Figure 2F). LIRKO hepatocytes failed to respond to high glucose and insulin. However, stimulation of LIRKO hepatocytes with high glucose and serum, which contains numerous growth factors in addition to insulin, induced S6 kinase to the same level as control hepatocytes treated with high glucose and insulin. Thus, factors in the serum other than insulin can promote the activation of mTORC1.

## Acute Refeeding Fails to Induce Nuclear SREBP-1c Protein in LIRKO Livers

Refeeding a high carbohydrate diet after fasting is a strong lipogenic challenge that is thought to be primarily driven by insulin and SREBP-1c (Horton et al., 1998). Thus, in contrast to the ad libitum fed state, the refed state is characterized by an exaggerated insulin response, which induces SREBP-1c and lipogenesis to levels 5-fold greater than those observed in the ad libitum fed state (Horton et al., 1998). To examine the effects of insulin and nutrients on SREBP-1c under the stress of refeeding, we measured SREBP-1c expression in the livers of control and LIRKO mice fasted for 24 hr, and refed a high carbohydrate diet for 6–48 hr. In control mice, *SREBP-1c* mRNA levels increased within 6 hr of refeeding and reached a plateau by 12 hr (Figure 3A). In LIRKO livers, the same temporal pattern was observed, but *SREBP-1c* mRNA levels were induced to levels only 20% of controls.

SREBP-1c precursor and nuclear protein levels rose in parallel with mRNA levels in control mice, increasing after 6 hr of refeeding and reaching a plateau by 12 hr (Figure 3B). Precursor levels of SREBP-1 also increased in LIRKO livers in parallel with mRNA levels. Despite the increase in precursor SREBP-1, however, nuclear protein levels remained undetectable, even after 48 hr.

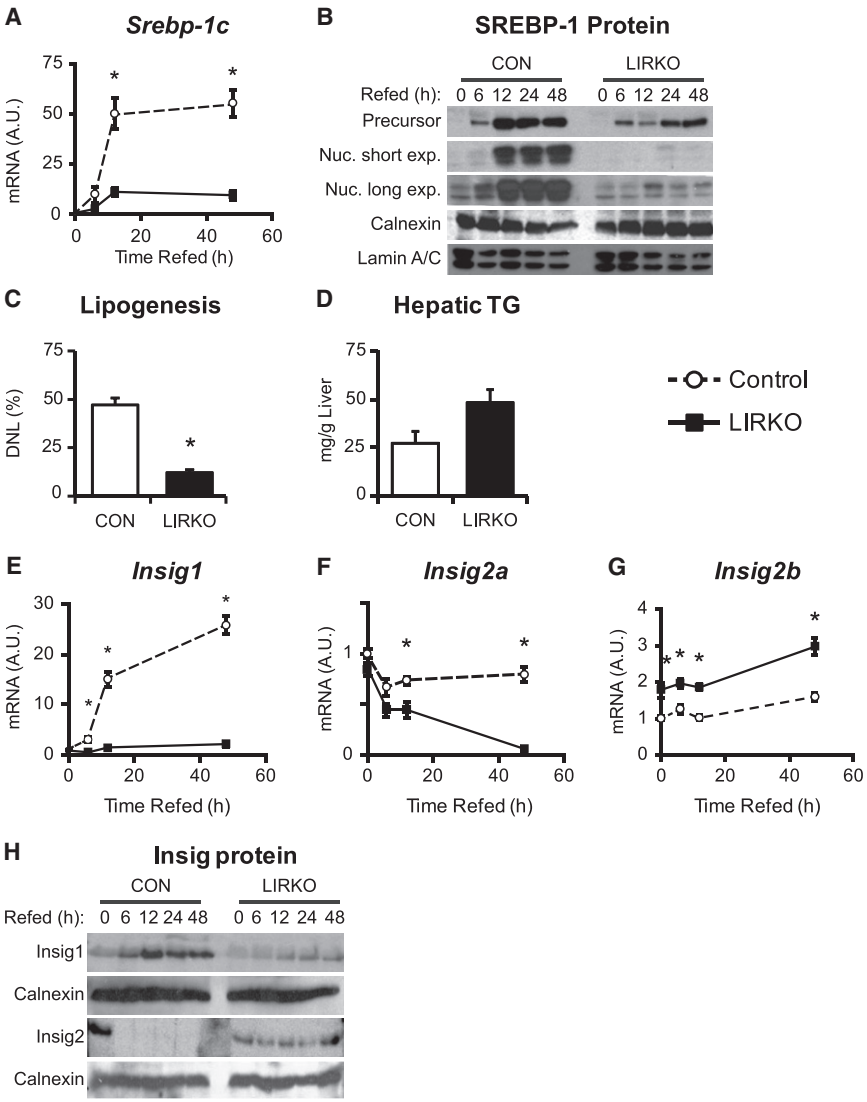


**Figure 3. LIRKO Livers Fail to Accumulate Nuclear SREBP-1c upon Acute Refeeding of a High Carbohydrate Diet**

(A–H) LIRKO mice and their littermate controls (CON) were fasted for 24 hr and then refed a high carbohydrate diet for 6–48 hr. Livers were used to prepare cDNA for real-time PCR analysis as well as microsomal and nuclear (Nuc.) extracts. SREBP-1c mRNA (A) was measured using real-time PCR. SREBP-1 precursor and nuclear protein levels (B) were measured by immunoblotting microsomal and nuclear extracts, respectively. Calnexin and lamin are shown as loading controls. Each lane represents extracts prepared from equal aliquots of liver from three mice. De novo lipogenesis (C) was measured as the fraction of newly synthesized palmitate present 24 hr after refeeding. hepatic triglycerides (D) were measured after 6 hr of refeeding. *Insig* mRNA (E–G) was measured using real-time PCR. Insig1 and Insig2 proteins (H) were measured by immunoblotting microsomal extracts. Each lane represents extracts prepared from equal aliquots of liver from three mice. Error bars represent SEM; n = 4–8; *p < 0.05 versus control mice. See also Figure S3.

Consistent with this, lipogenesis was markedly lower in refed LIRKO versus control mice (Figure 3C). Interestingly, however, hepatic triglyceride levels after 6 hr of refeeding tended to be increased (Figure 3D), suggesting a lag in the removal of triglycerides from the LIRKO liver, perhaps due to a decrease in triglyceride secretion (Biddinger et al., 2008).

The failure of LIRKO livers to accumulate the nuclear, i.e., active, form of SREBP1 upon refeeding suggests that insulin is necessary for the processing of SREBP-1c. SREBP proteins are synthesized as inactive, membrane-bound precursor proteins that become associated with SCAP, the SREBP cleavage-activating protein (reviewed in Jeon and Osborne [2012]). Insig proteins reside in the endoplasmic reticulum and bind to SCAP; in so doing, they retain the SCAP/SREBP complex in the endoplasmic reticulum and prevent SREBP processing. When SCAP dissociates from the Insig proteins, the SCAP/SREBP complex can translocate to the Golgi. Two proteins which reside in the Golgi, Site 1 and Site 2 protease, can then cleave the SREBP precursor, releasing a soluble fragment that can translocate to the nucleus to activate transcription. There are two Insig proteins. Insig1 is thought to mediate feedback inhibition of the SREBPs, as *Insig1* transcription is driven by the SREBP proteins (Engelking et al., 2004). Insig2 is encoded by two transcripts. *Insig2a* is the major *Insig* transcript in the fasted liver. It is induced by fasting and streptozotocin treatment and suppressed by feeding and insulin treatment (Yabe et al., 2003). *Insig2b* is a ubiquitous transcript (Yabe et al., 2003).

LIRKO livers showed normal mRNA levels of SCAP, Site 1 and Site 2 protease (Figure S3A), but marked derangements in Insig expression. In control livers, the increase in nuclear SREBP-1 observed with refeeding was associated with an increase in *Insig1* mRNA and protein (Figures 3E and 3H). In LIRKO livers, the failure to induce nuclear SREBP-1c was associated with a failure to induce *Insig1* mRNA and protein.

*Insig2a* mRNA decreased with refeeding in control mice, as expected (Figure 3F). Despite the lack of insulin signaling, *Insig2a* mRNA fell to an even greater extent with refeeding in LIRKO livers. *Insig2b* mRNA levels were relatively stable with refeeding in both control and LIRKO livers, though the expression in LIRKO livers was increased (Figure 3G). Insig2 protein was also higher in LIRKO versus control livers (Figure 3H).

Using an adenovirus encoding shRNA against *Insig2*, we knocked down Insig2 in the livers of nonfasted LIRKO mice (Figure S3B). Compared with LIRKO mice injected with an adenovirus encoding a scrambled shRNA sequence, LIRKO mice injected with shInsig2 adenovirus showed very low levels of Insig2, indicating the virus was effective. Though there was slight trend for the shInsig2 adenovirus to increase nuclear levels of SREBP-1 in LIRKO livers, it was not to the level observed in

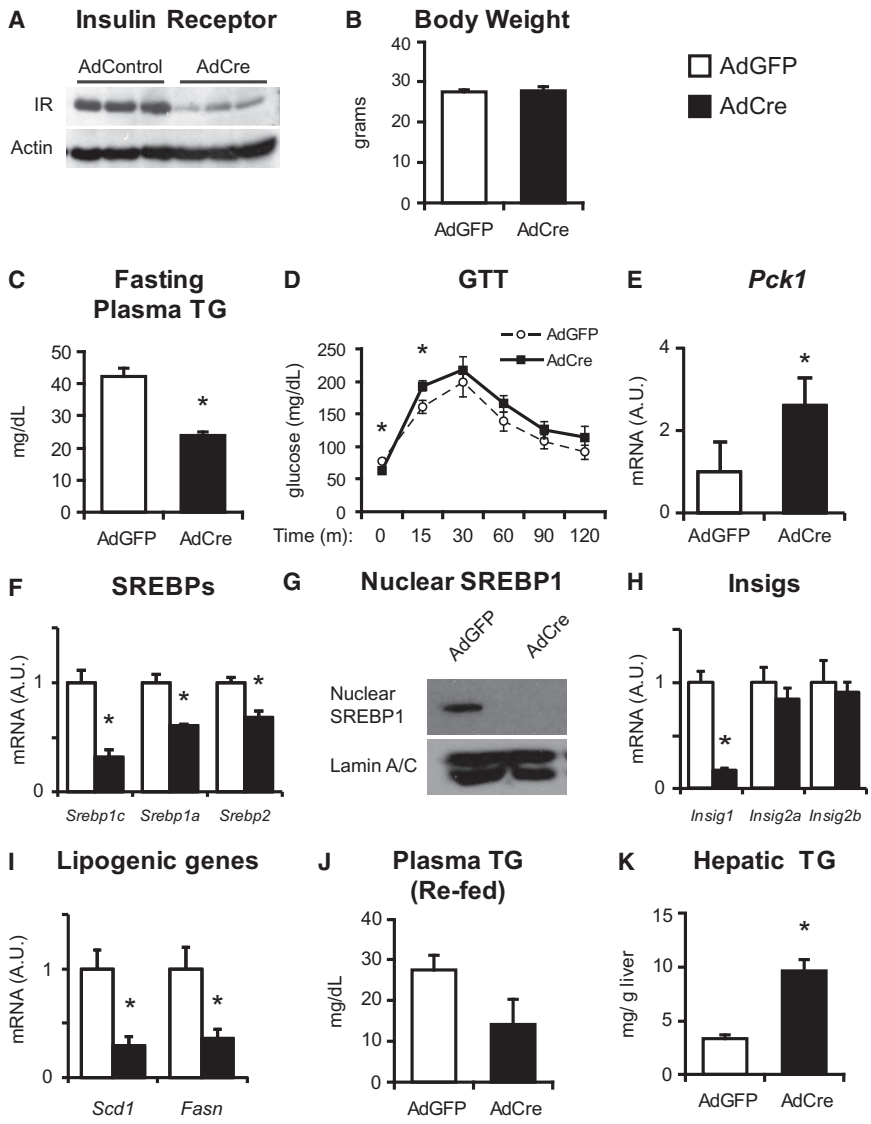


**Figure 4. Mice with Knockdown of the Insulin Receptor Fail to Accumulate Nuclear SREBP-1c upon Acute Refeeding of a High Carbohydrate Diet**

(A–K) Two-month-old mice homozygous for the floxed allele of the insulin receptor were injected with adenovirus encoding GFP or Cre recombinase and studied 2 to 3 weeks later. Mice were fasted for 24 hr and refed a high carbohydrate diet for 6 hr prior to sacrifice. Liver lysates (A) were subjected to immunoblotting with antibodies against the insulin receptor. Body weight (B) was measured at the time of sacrifice. Triglycerides (C) were measured in plasma taken after a 4 hr fast. Glucose tolerance testing (D) was performed by fasting mice overnight and then injecting them with 1 g/kg glucose, intraperitoneally. In (E, F, H, and I), RT PCR was used to measure hepatic gene expression. SREBP-1 protein (G) was measured in nuclear extracts (prepared from equal amounts of liver taken from at least three mice per group). Plasma (J) and hepatic triglycerides (K) measured in the refed state, at the time of sacrifice, are shown. Error bars represent SEM; n = 3–5, *p < 0.05 versus mice injected with adenovirus encoding GFP. See also Figure S4.

the livers of their littermate controls. Thus, the failure of LIRKO livers to accumulate nuclear SREBP-1c is due to increased Insig2 protein as well as other factors.

## Acute Refeeding Fails to Induce Nuclear SREBP-1c Protein in Mice with Knockdown of the Insulin Receptor

Because LIRKO mice lack hepatic insulin signaling from birth, it is conceivable that some of their phenotype is due to developmental defects or other chronic compensatory changes. Therefore, we knocked down the insulin receptor in 2- to 3-month-old control mice, homozygous for the floxed allele of the insulin receptor, using adenoviral mediated expression of Cre recombinase (AdCre). AdCre mice showed a very similar phenotype to LIRKO mice. Compared to mice infected with adenovirus encoding green fluorescent protein (AdGFP), AdCre treated mice showed a 90% decrease in hepatic insulin receptor protein, normal body weights, and a 50% decrease in fasting plasma triglycerides (Figures 4A–4C). AdCre-treated mice also showed fasting hypoglycemia, presumably due to limited glycogen reserves, and hyperglycemia after the injection of glucose (Figure 4D).

The response of AdCre treated mice to refeeding was almost identical to that of LIRKO mice. Thus, AdCre and AdGFP treated mice were fasted for 24 hr and refed for 6 hr with a high carbohydrate diet. Relative to AdGFP treated mice, AdCre-treated mice showed increased *Pck1* mRNA (Figure 4E) and a 75% decrease in SREBP-1c mRNA. The other SREBP isoforms, SREBP-1a and SREBP-2, were decreased 30%–40% in AdCre-treated mice (Figure 4F). Like LIRKO livers, the livers of AdCre treated mice showed markedly reduced nuclear SREBP-1, despite decreased *Insig1* mRNA, and a 60%–80% reduction in *Fasn* and *Scd1*(Figures 4G–4I). Neither AdCre (Figure 4H) nor LIRKO (Figure 3F) mice at the 6 hr refeeding time point showed significant changes in *Insig2a* mRNA. Finally, AdCre-treated mice in the refed state showed a trend toward reduced plasma triglycerides and increased hepatic triglycerides (Figures 4J and 4K).

## High Fructose Feeding Increases Lipogenic Gene Expression in LIRKO Mice

Dietary fructose is a strong stimulus for lipogenesis. Catalytic amounts of fructose activate glucokinase posttranscriptionally by promoting its dissociation from the glucokinase regulatory protein (Doiron et al., 1994; Mayes, 1993; Petersen et al., 2001). Fructose could therefore be particularly important in the insulin-independent induction of lipogenesis. Thus, LIRKO mice and their controls were subjected to a high fructose (60% fructose) versus standard chow diet for one week. In control

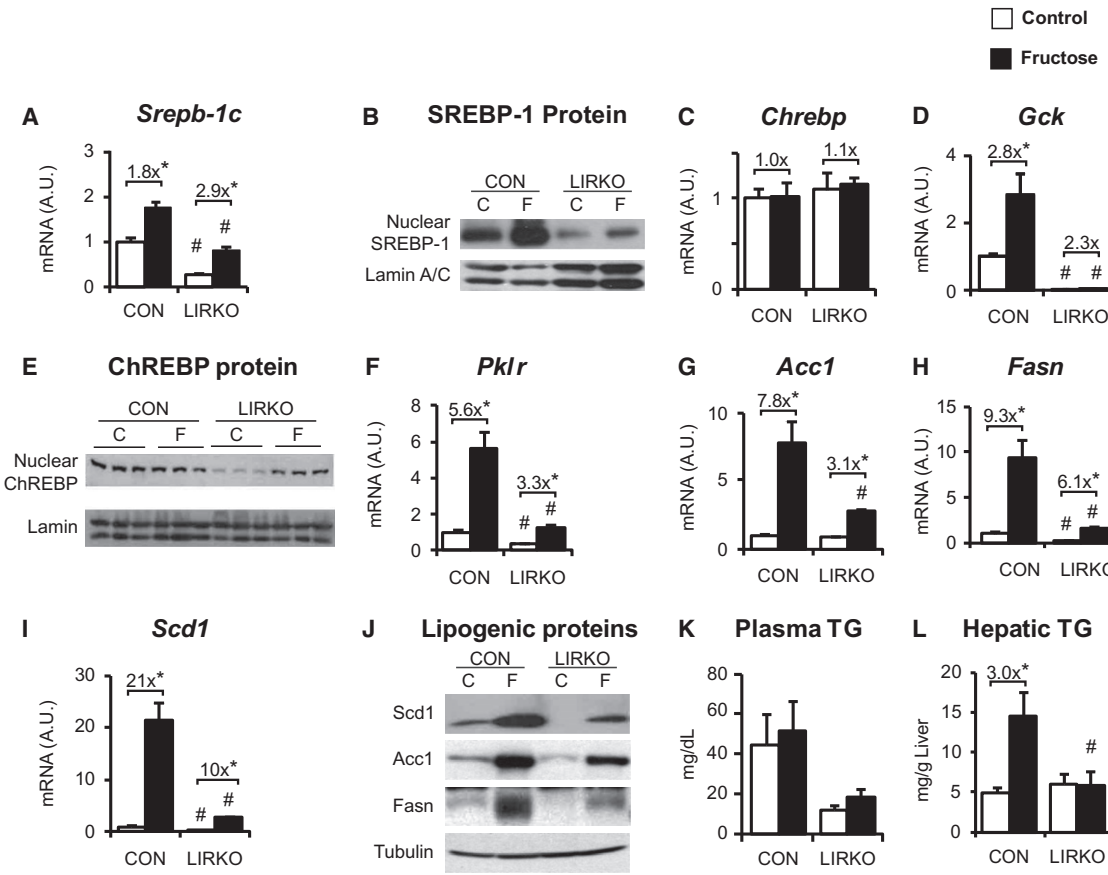


**Figure 5. Effects of Fructose Feeding on LIRKO Livers**

(A–L) LIRKO mice and their littermate controls (CON) were placed on either a chow (C, Chow) or high fructose diet (F, 60% fructose by weight) for 1 week and were sacrificed in the nonfasted state. Livers were analyzed for gene expression and triglyceride content. In (A, C, and D) and (F–I), gene expression was measured by real-time PCR. SREBP-1 (B) and ChREBP (E) proteins were measured by immunoblotting nuclear extracts. Acc, Fasn, and Scd1 (J) were measured by immunoblotting liver lysates. Plasma (K) and hepatic triglycerides (L) were measured at the time of sacrifice. Error bars represent SEM; n = 4–8, *$p < 0.05$ versus chow-fed mice of the same genotype; #$p < 0.05$ versus control mice on the same diet; equal amounts of liver from three mice were pooled to prepare samples for immunoblotting SREBP-1, Acc, Fasn, and Scd1; for ChREBP immunoblot, each lane represents an individual mouse liver. See also Figure S5.

mice, the high fructose diet produced a 75% increase in *Srebp-1c* mRNA and an increase in nuclear SREBP-1c (Figures 5A and 5B). In LIRKO mice, fructose feeding produced a 3-fold increase in *Srebp-1c* mRNA and a modest increase in nuclear SREBP-1c. Importantly, the levels of nuclear SREBP-1 protein in fructose-fed LIRKO livers were markedly lower than those found in even chow-fed controls, indicating the dominant role played by insulin in the accumulation of nuclear SREBP-1.

In control livers, *ChREBP* mRNA was not induced by fructose feeding, and glucokinase was increased more than 2-fold; no consistent change in ChREBP nuclear protein was observed (Figures 5C–5E). In LIRKO livers, neither *ChREBP* nor glucokinase mRNA was induced by fructose feeding. Nonetheless, fructose markedly increased the amount of nuclear ChREBP in LIRKO livers. In parallel, pyruvate kinase was increased by 3-fold in LIRKO livers upon fructose feeding (Figure 5F).

The induction of nuclear SREBP-1 in control livers and ChREBP in LIRKO livers was associated with an increase in lipogenic gene expression (Figures 5G–5I). In control mice, *Scd1* was increased 20-fold, and *Acc*, *Fasn*, and pyruvate kinase were increased 5- to 10-fold with fructose feeding. In LIRKO livers, *Scd1*, *Acc*, *Fasn*, and pyruvate kinase increased 3- to 10-fold with fructose feeding.

In parallel, fructose feeding increased the protein levels of Scd1, Acc and Fasn in both control and LIRKO mice (Figure 5J). Though lipogenic expression at the mRNA and protein levels were consistently lower in the LIRKO livers on either diet, fructose feeding induced lipogenic gene expression in fructose fed-LIRKO mice to levels similar to or higher than those found in control mice on the chow diet. This indicates the ability of a high fructose diet to drive lipogenic gene expression even in the complete absence of hepatic insulin signaling.

Plasma triglyceride levels were not significantly changed by the high fructose diet in either control or LIRKO mice (Figure 5K). Hepatic triglycerides were increased 3-fold in control mice subjected to the fructose diet (Figure 5L). In contrast, LIRKO mice showed no change in hepatic triglycerides despite the increase in lipogenic gene expression. These data indicate that insulin is also necessary for some other aspect of triglyceride metabolism required for steatosis.

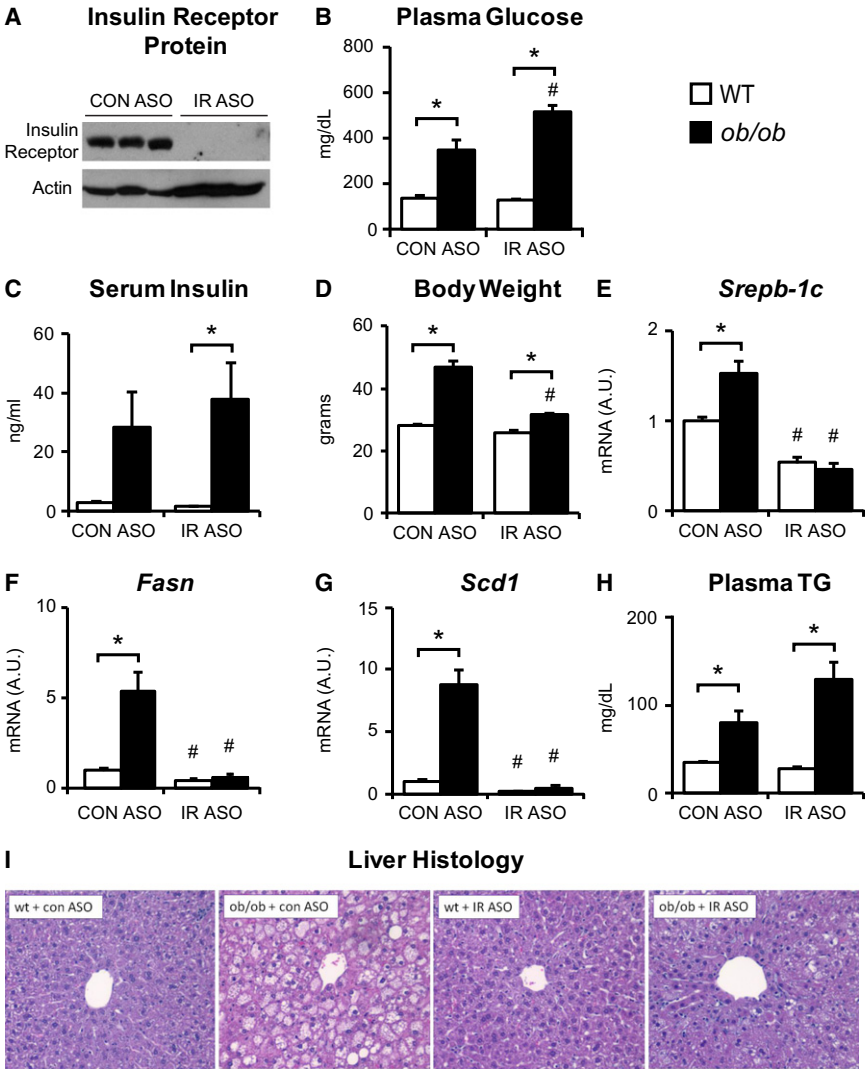


**Figure 6. The Induction of Lipogenic Gene Expression in *ob/ob* Mice Requires Insulin Signaling**

(A–I) Five- to six-week-old male *ob/ob* mice and their lean controls (WT) were treated with chemically modified antisense oligonucleotides (ASO) for 4 weeks and sacrificed in the nonfasted state. Insulin receptor expression (A) was measured by immunoblotting liver lysates. Blood glucose levels (B) were measured after a 4 hr fast. Plasma insulin (C) and body weights (D) were measured at the time of sacrifice. Livers (E–G) were analyzed for gene expression using real-time PCR. Plasma triglycerides (H) were measured after a 4 hr fast. Livers (I) were subjected to hematoxylin and eosin staining. Error bars represent SEM; n = 3–6; *p < 0.05 versus WT mice treated with the same ASO; #p < 0.05 versus mice of the same genotype treated with control ASO. See also Figures S6 and S7.

receptor (IR ASO) or a control antisense oligonucleotide (CON ASO). Treatment with the IR ASO reduced liver insulin receptor protein to near undetectable levels (Figure 6A). There was also a decrease in insulin receptor content in the adipose tissue, but this did not significantly alter phosphorylation or expression of hormone sensitive lipase, expression of adipocyte triglyceride lipase, plasma nonesterified free fatty acids or plasma adiponectin (Figures S6A–S6D).

In mice treated with the CON ASO, leptin deficiency produced hyperglycemia, raising fasting blood glucose levels from 136 mg/dl to 347 mg/dl (Figure 6B). In mice treated with the IR ASO, the effect was exacerbated, as fasting blood glucose levels increased from 127 to 519 mg/dl, though *Pck1* gene expression was not significantly changed (Figure S6E). Leptin deficiency produced hyperinsulinemia during treatment with either the CON or IR ASO, even though the livers of mice treated with the IR ASO were unable to respond to the hyperinsulinemia (Figure 6C). Consistent with the worsening of diabetes, *ob/ob* mice treated with the IR ASO accumulated less weight than *ob/ob* mice treated with the control ASO (Figure 6D).

In the presence of the control ASO, leptin deficiency produced a 40% increase in *SREBP-1c* (Figure 6E), a 5-fold induction in *Fasn* (Figure 6F), and an 8-fold induction in *Scd1* (Figure 6G). However, in the presence of the IR ASO, these effects were entirely abolished, and the expression of *SREBP-1c*, *Fasn*, and *Scd1* was similar in wild-type and *ob/ob* mice. Even the ability of leptin deficiency to induce glucokinase, *ChREBP*, and pyruvate kinase was abolished by treatment with the IR ASO (Figures S6G–S6I). Consequently, in the presence of the CON ASO, leptin deficiency produced severe hepatic steatosis; in the presence of the IR ASO, the steatosis was markedly attenuated (Figure 6I).

The acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes, which are encoded by two genes, catalyze the committed step in triglyceride biosynthesis and are important for the development of steatosis (Yen et al., 2008). Interestingly, LIRKO mice showed decreased levels of DGAT2 mRNA, which could contribute to the failure of LIRKO livers to accumulate hepatic triglycerides on the fructose diet (Figure S5B). However, DGAT1 mRNA (Figure S5A) and DGAT activity (Figure S5C) were normal in LIRKO livers.

## IR ASO Therapy in *ob/ob* Mice

In Type 2 diabetes, plasma insulin levels rise as the β-cells of the pancreas attempt to maintain glucose homeostasis. This increase in insulin levels could potentially drive *SREBP-1c* and the lipogenic genes. To test this directly, we used *ob/ob* mice. In these mice, leptin deficiency leads to hyperphagia, obesity and severe hyperinsulinemia. To block the effects of hyperinsulinemia on the liver, we knocked out the insulin receptor using chemically modified antisense oligonucleotides.

Wild-type and *ob/ob* mice on the C57BL/6J background were treated with an antisense oligonucleotide against the insulin

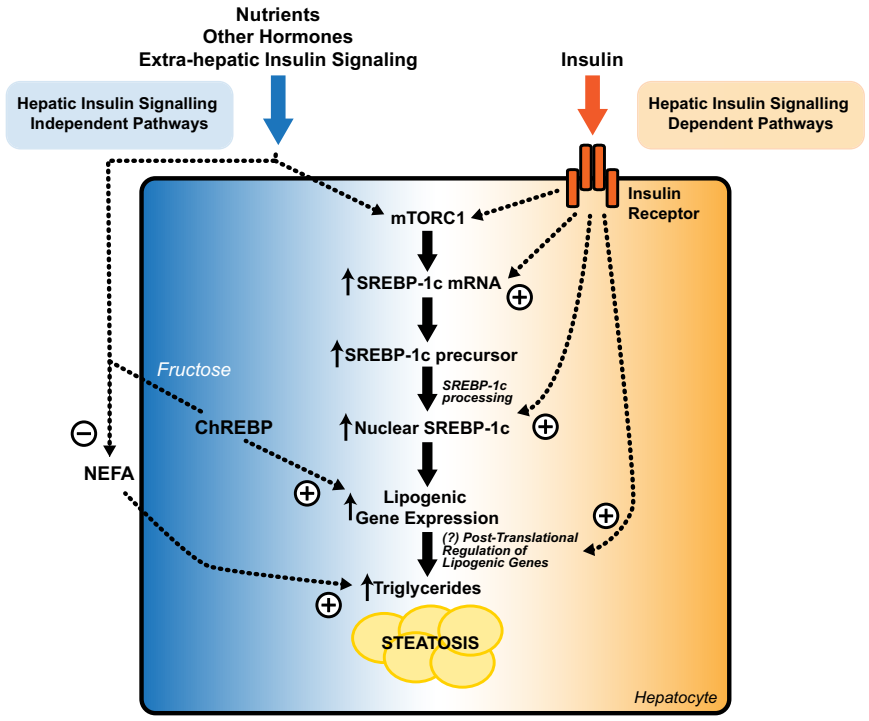


**Figure 7. Interaction between Insulin-Dependent and Insulin-Independent Signaling Pathways in the Control of SREBP-1c and Hepatic Triglycerides**

There are multiple nodes at which insulin-dependent and -independent signaling pathways can crosstalk in the regulation of SREBP-1c and hepatic triglycerides. Hepatic insulin signaling independent pathways involve the direct effects of nutrients, other hormones, and other tissues. These pathways are able to activate mTORC1 and increase SREBP-1c mRNA. In the case of fructose, they are also able to activate ChREBP. Moreover, insulin and other hormones, acting on the adipocyte, can regulate the flux of nonesterified fatty acids to the liver. Insulin-dependent signaling pathways are required for the processing of SREBP-1c, maximal lipogenic gene expression, and the induction of steatosis. The relative roles played by the insulin-dependent and insulin-independent signaling pathways vary in different physiological and pathophysiological states.

The ability of leptin deficiency to increase plasma triglycerides, however, remained intact (Figure 6H).

Another commonly used mouse model of Type 2 diabetes is diet-induced obesity (DIO). To determine whether the ablation of hepatic insulin signaling had similar effects in DIO mice, control and LIRKO mice were fed a high fat diet for 18 weeks. On the high fat diet, body weight and fat mass were similar in control and LIRKO mice, but LIRKO mice were more glucose intolerant (Figures S7A–S7C). LIRKO mice also showed reduced levels of nuclear SREBP-1, lipogenic gene expression, and hepatic triglycerides (Figures S7D–S7G). Similarly, the injection of AdCre into DIO mice homozygous for the floxed allele of the insulin receptor also worsened glucose tolerance, but lowered nuclear SREBP-1c, lipogenic gene expression, and plasma triglycerides and tended to lower hepatic triglycerides (Figure S4). Thus, the interruption of hepatic insulin signaling is sufficient to lower SREBP-1c and its targets in both the *ob/ob* and DIO models of Type 2 diabetes.

## DISCUSSION

Insulin is one of several factors regulating SREBP-1c and hepatic triglyceride metabolism (Figure 7). Our data, together with data in mice with liver-specific knockout of both IRS-1 and IRS-2 (Guo et al., 2009), or Akt2 (Leavens et al., 2009), show that feeding is able to activate mTORC1 and induce SREBP-1c mRNA via insulin-independent signaling pathways. The activation of ChREBP and lipogenic gene expression by fructose is also independent of hepatic insulin signaling, as is the regulation of nonesterified fatty acid flux from the adipocyte, an important driver of hepatic triglycerides. Hepatic insulin signaling is, however, required for the accumulation of nuclear SREBP-1c protein. Since SREBP-1c activates its own transcription, completing a feed-forward loop (Chen et al., 2004; Dif et al., 2006), insulin is also necessary for the full induction of SREBP-1c mRNA. These insulin-dependent signaling pathways are critical for the pathological induction of SREBP-1c and lipogenic gene expression observed in obesity.

One important regulator of SREBP-1c is Insig2a. In vitro, insulin increases degradation of the *Insig2a* transcript (Yellaturu et al., 2009), but it may also increase *Insig2a* transcription, since activation of Liver X Receptor, a nuclear receptor that is induced by insulin, increases *Insig2a* mRNA (Hegarty et al., 2005). Our data add further complexity. First, *Insig2a* expression in vivo can be suppressed independently of hepatic insulin signaling, as *Insig2a* mRNA levels are decreased in the livers of refed LIRKO mice (Figure 3F), AdCre-treated mice on the high fat diet (Figure S4F), and mice treated with IR ASO (Figure S6K). Such in vivo regulation by feeding could be mediated by a factor other than insulin. One intriguing possibility is that in vivo *Insig2a* is suppressed by leptin, as *Insig2a* mRNA is increased by leptin deficiency (Kammoun et al., 2009), even after knockdown of the insulin receptor by ASO treatment (Figure S6K). Second, insulin may regulate Insig2 posttranscriptionally, as Insig2 protein is increased in LIRKO livers despite lower levels of *Insig2a* mRNA. Moreover, insulin is required for other aspects of SREBP-1c processing, nuclear import or stability, as knockdown of Insig2 does not fully restore nuclear SREBP-1c protein in LIRKO livers (Figure S3B).

Insulin signaling plays a key role in the induction of SREBP-1c and the lipogenic genes by obesity/Type 2 diabetes. That is, though feeding induces mRNA levels of SREBP-1c and its targets via insulin-independent signaling pathways, the further induction which occurs in obesity is entirely insulin dependent. Neither leptin deficiency, nor any of the other metabolic or hormonal changes associated with the Type 2 diabetic state is capable of inducing lipogenic gene expression in the absence of hepatic insulin signaling. This is consistent with concept of

"selective insulin resistance." Although the specific signaling pathways that remain sensitive to insulin in obesity remain unclear, continued insulin signaling through phosphatidylinositol 3-kinase (Anai et al., 1999) and its downstream targets mTORC1 (Um et al., 2004; Khamzina et al., 2005), as well as PKC-λ (Standaert et al., 2004), have been reported.

Both mTORC1-dependent and -independent signals are necessary for the full activation of SREBP-1c (Yecies et al., 2011; Wan et al., 2011). We therefore propose a model of obesity in which over-nutrition, independently of insulin, drives mTORC1, while insulin signaling through a distinct pathway, such as PKC-λ, permits the accumulation of nuclear SREBP-1c and activation of its feed-forward transcriptional loop. However, our data do not rule out the possibility that insulin, in the setting of hyperinsulinemia and obesity, becomes an important driver of mTORC1, even though it is not required for the induction of mTORC1 by feeding.

Fructose can bypass the requirement for insulin in the regulation of the lipogenic genes, as the fructose diet increases *Acc*, *Fasn*, and *Scd1* mRNA and protein in LIRKO livers to levels equal to or higher than those found in chow-fed controls. This is due, in part, to activation of ChREBP, as nuclear ChREBP levels and expression of the ChREBP target, pyruvate kinase, are increased in the livers of fructose-fed LIRKO mice. The activation of ChREBP requires glucokinase, which in turn requires insulin for its transcription (Dentin et al., 2004). However, fructose, which activates glucokinase posttranscriptionally by promoting its translocation from the nucleus to the cytosol, could drive ChREBP independently of insulin (Doiron et al., 1994; Mayes, 1993; Petersen et al., 2001).

In human Type 2 diabetes, hyperinsulinemia, dietary fructose, and other lipogenic stimuli are present. Our data suggest that identifying and targeting the specific signaling pathways by which insulin stimulates lipogenesis, as well as decreasing dietary fructose, could be extremely effective in reducing SREBP-1c and ameliorating hepatic steatosis in Type 2 diabetes.

## EXPERIMENTAL PROCEDURES

A detailed description of the Experimental Procedures can be found in the Supplemental Information.

### Animals, Diets, and Treatments

Generation and genotyping of LIRKO ($Cre^{+/-}$, IR $^{lox/lox}$) mice and their littermate controls ($Cre^{-/-}$, IR $^{lox/lox}$) has been described previously (Michael et al., 2000). LIRKO mice were maintained on a mixed genetic background. Unless otherwise indicated, the mice used in these experiments were male, eight to ten weeks of age, and sacrificed at 2 p.m. For fasting and refeeding studies, mice were sacrificed under the following conditions: ad libitum fed; after a 24 hr fast; or after a 24 hr fast followed by refeeding a high carbohydrate diet (TD. 88122, Harlan Teklad Diets). For adenoviral-mediated knockdown of the insulin receptor, 2-month-old mice homozygous for the floxed allele of the insulin receptor were injected via tail vein with $5 \times 10^9$ pfu of adenovirus encoding Cre or GFP (Viraquest), and sacrificed 21 days later. For the fructose feeding experiments, mice were fed ad libitum with a 60% fructose diet for 1 week (TD. 00202, Harlan Teklad Diets). For the antisense oligonucleotide-mediated knockdown of the insulin receptor, C57BL/6J mice and *ob/ob* mice (Jackson Labs) were given 50 mg/kg IP of the chemically modified ASO each week for 4 weeks and sacrificed 1 day after the final dose. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Children's Hospital Boston.

### Gene Expression Analysis

Gene expression was measured using real-time PCR. Results were normalized to the housekeeping gene, *Tbp*, and the value of the control group was set to 1.

### Western Blotting

Microsomal and nuclear protein extracts for measurements of SREBP-1 (Horton et al., 1998), microsomal extracts for the measurements of the Insig proteins (Engelking et al., 2004), and nuclear extracts for the measurements of ChREBP (Miao et al., 2009) were prepared as previously described, and subjected to western blotting.

### Phenotypic and Histological Characterization

Blood glucose and ketone levels were measured using a glucometer and ketone meter. Plasma insulin (ALPCO) was measured using a commercial kit. Plasma measurements of nonesterified fatty acids (Wako Chemicals) and total triglycerides (ThermoScientific) were made using colorimetric assays. Hepatic triglycerides were measured as previously described (Biddinger et al., 2008). Hematoxylin and eosin staining of the liver was performed by the Dana-Farber/Harvard Cancer Center Rodent Histopathology Core.

### De Novo Lipogenesis

Mice were fasted for 24 hr, refed a high carbohydrate diet for 24 hr, and then sacrificed. One hour prior to refeeding, mice were injected IP with 24 μl/g body weight of deuterated normal saline, and their drinking water was replaced with 4% D2O. The fraction of newly synthesized palmitate was measured using gas chromatography-electron impact ionization mass spectrometry as previously described (Leavens et al., 2009).

### Primary Hepatocytes

Primary hepatocytes were isolated from 8-to 10-week-old female control and LIRKO mice as previously described (Biddinger et al., 2008). After plating the hepatocytes, they were cultured overnight in DMEM (5 mM glucose), without serum or insulin. They were then stimulated for 10 min with DMEM (5 or 25 mM glucose), with or without 10 nM insulin, or 10% fetal bovine serum.

### Statistics

Differences between groups were assessed by Student's t test using the Bonferroni correction for multiple testing. Bars and error bars correspond to the mean and SEM, respectively.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2012.05.002.

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