SREBP-regulated adipocyte lipogenesis is dependent on substrate availability and redox modulation of mTORC1

Clair Crewe, …, Jay D. Horton, Philipp E. Scherer

*JCI Insight.* 2019. [https://doi.org/10.1172/jci.insight.129397](https://doi.org/10.1172/jci.insight.129397).

**Graphical abstract**

Find the latest version:

[http://jci.me/129397/pdf](http://jci.me/129397/pdf)
SREBP-regulated Adipocyte Lipogenesis is Dependent on Substrate Availability and Redox Modulation of mTORC1

Clair Crewe¹, Yi Zhu¹, Vivian A. Paschoal¹, Nolwenn Joffin¹, Alexandra L. Ghaben¹, Ruth Gordillo¹, Da Young Oh¹, Guosheng Liang², Jay D. Horton²,³ and Philipp E. Scherer¹,*

¹Touchstone Diabetes Center, ²Department of Molecular Genetics, ³Center for Human Nutrition, University of Texas Southwestern Medical Center, Dallas, Texas, USA

*Corresponding author:
5323 Harry Hines Blvd. L5206; MC:8549 Dallas, TX 75390
philipp.scherer@utsouthwestern.edu, Tel.: (214) 648-8715; Fax: (214) 648-8720

Declaration of Interests
The authors have declared that no conflict of interest exists.

Running title: SREBP1-mediated Regulation of Adipocyte Lipogenesis
ABSTRACT

The synthesis of lipid and sterol species through *de novo* lipogenesis (DNL) is regulated by two functionally overlapping but distinct transcription factors: the sterol regulatory element-binding proteins (SREBPs) and carbohydrate response element binding protein (ChREBP). ChREBP is considered to be the dominant regulator of DNL in adipose tissue (AT); however, the SREBPs are highly expressed and robustly regulated in adipocytes, suggesting that the model of AT DNL may be incomplete. Here we describe a new mouse model of inducible, adipocyte-specific overexpression of the insulin-induced gene 1 (Insig1), a negative regulator of SREBP transcriptional activity. Contrary to convention, Insig1 overexpression did block AT lipogenic gene expression. However, this was immediately met with a compensatory mechanism triggered by redox activation of mTORC1 to restore SREBP1 DNL gene expression. Thus, we demonstrate that SREBP1 activity sustains adipocyte lipogenesis, a conclusion that has been elusive due to the constitutive nature of current mouse models.
INTRODUCTION

Cellular lipid synthesis is essential for organismal metabolic homeostasis, yet can also be destructive if left unrestrained. For this reason, lipid production is tightly regulated. Lipid is generated through the process of de novo lipogenesis (DNL), whereby acetyl-coA derived from the mitochondrial oxidation of carbohydrates is converted into fatty acids or cholesterol species. Adipose tissue (AT) and liver are major lipogenic organs, coordinating the conversion of dietary sugars into fatty acids and subsequent storage in AT as triglycerides. DNL in AT is not considered a significant source of circulating lipids, compared to the liver, but is essential to whole body homeostasis due its pertinent role in storing potentially toxic excess sugars as chemically stable lipids (1). In obese mice and humans, AT lipogenic enzyme expression is reduced, leading to suppression of DNL flux (2-6). It is widely accepted that insulin resistance is positively correlated to DNL in the liver and negatively correlated to DNL in AT (3, 7, 8).

Two pathways coordinate the regulation of lipid biosynthesis genes: the sterol regulatory element-binding proteins (SREBPs) and carbohydrate response element binding protein (ChREBP) (9, 10). The SREBP family of basic helix-loop helix transcription factors consists of SREBP1a, SREBP1c and SREBP2, which promote fatty acid production (SREBP1a/c) and cholesterol synthesis (SREBP2). SREBP proteins are synthesized as a membrane-bound, inactive form sequestered in the ER by association with Scap. The Scap protein senses an increase in cellular sterol concentration and maintains retention of the SREBPs in the ER through interaction with the insulin-induced gene 1 or 2 (Insig1,2). When sterol levels decline, such as in the postprandial state, conformational changes in Scap result in its dissociation from Insig1, freeing Scap to escort the SREBPs to the Golgi. Here, the SREBPs are cleaved into an active form and transported to the nucleus to stimulate the transcription of lipogenesis genes. In addition to sterol regulation of protein processing, SREBP1c mRNA is strongly induced by insulin through LXR transcriptional activation (11, 12). Downstream of insulin-stimulated AKT activation, the mechanistic target of rapamycin complex 1 (mTORC1) is a key positive regulator of SREBP1 mRNA transcription and SREBP1 processing (13).

DNL capacity is acutely regulated by glucose and fructose availability, both serving as lipogenic substrates. This occurs through the activation of the ChREBP transcription factor by glycolytic intermediates, such as xylulose 5-phosphate and glucose-6-phosphate. ChREBP induces the transcription of genes in both the
DNL and glycolytic pathways (14, 15). Therefore, the presence of both insulin and lipogenic precursors generate a synergistic response through the actions of these two overlapping, yet distinct lipogenic transcriptional pathways (14).

Regulation of DNL in AT is considered to be the exclusive responsibility of the ChREBP pathway as, ChREBP knock-out mice exhibit complete repression of glucose- and fructose-stimulated lipogenesis in AT (15). In contrast, SREBP1 null mice do not display a reduction in AT lipogenic gene expression (8, 16). However, SREBP proteins are highly expressed in AT tissue and are greatly affected by re-feeding, similar to their regulation in the liver (17). This implies that the SREBPs may participate in AT DNL, yet current experimental models have not detected a significant role for SREBPs to support this notion. To date, mouse models used to genetically suppress lipogenesis have either been whole-body knock-outs of essential lipogenic regulators or adipocyte-specific knock-outs using a constitutively expressed Cre-recombinase. These models have been pivotal for our current understanding of lipogenesis in AT; however, by nature, constitutive models are prone to developmental compensation. Here we use a mouse model of inducible, adipocyte-specific Insig1 overexpression as a tool to study the effect of acutely obstructing SREBP-mediated lipogenesis in adult AT. Others have demonstrated that overexpression of Insig1 at high levels in cultured epithelial-like cells or in vivo hepatocytes can trap the SCAP/SREBP complex in the ER, independent of sterol concentrations, thereby precluding lipogenic gene expression (18, 19). Therefore, Insig-1 overexpression can mimic the natural inhibition of DNL. We found that within 12 hr of Insig1 induction, lipogenic enzyme levels are significantly reduced, followed by a striking increase in the same proteins at 24 hr, and finally a return of these enzymes to control levels within 3 d. Initial inhibition of lipogenesis by Insig1 overexpression results in mitochondrial dysfunction, likely due to excess carbons entering oxidative phosphorylation, which would otherwise be shunted via the citrate transporter into the lipogenic pathway. The ensuing mitochondrially-generated reactive oxygen species (ROS) results in mTORC1 activation and thus, restoration of DNL enzyme expression. mTORC1 activation is transient, resolving within 3d of Insig1 overexpression, but chronic compensation was facilitated by upregulation of the fructose transporter GLUT5. We demonstrate that chronic compensation does not occur in the absence of DNL substrates. Thus, similarly to ChREBP, the SREBP proteins are also regulated by substrate availability in AT through this mitochondria-mediated mTORC1 activation. In fact, our data suggests that this mechanism ensures
there is never a brake in AT lipogenesis in the presence of substrate. Furthermore, an important lesson from the current work is that if essential pathways are genetically modified, rapid compensation may take effect through partially redundant pathways, necessitating a detailed analysis of the system during the immediate aftermath of the genetic intervention.
RESULTS

Inducible, tissue-specific over-expression of Insig1 is accomplished through the generation of a transgenic mouse carrying the Insig1 gene driven by the tetracycline response element (TRE). This mouse is bred to another mouse that carried the reverse tetracycline trans-activator (rtTA) under the control of the adiponectin promoter to restrict transgene expression to adipocytes (Figure 1A). Mice were provided with a diet containing doxycycline (dox) to induce Insig1 gene expression (Figure 1A). The resulting overexpression model is referred to as ad-Insig1. Insig1 is successfully overexpressed in adipose tissue (AT), but not in the liver, and expression is strictly dependent on dox (Figure 1B). Furthermore, Insig1 protein expression is successfully increased in subcutaneous adipose tissue (sWAT) 24 hr and 2 wk post dox administration (Figure 1C). For the initial experiments, control and ad-Insig1 mice were maintained on high fat diet containing dox for 16 wks. Body weight, oral glucose tolerance, and triglyceride (TG) clearance measurements are un-altered by adipocyte overexpression of Insig1 (Figure 1D-F). We found that, although Insig1 mRNA remains highly expressed in sWAT after 16 wk HFD, lipogenic genes are not suppressed as would be expected (Figure S1A). Scd1 mRNA is the exception, being reduced by 60% compared to control tissue (Figure S1A). We hypothesized that the perceived lack of Insig1 function is the result of a compensatory mechanism that restores lipogenic gene expression. Thus, we took advantage of our inducible expression system and harvested AT at 12 hr following dox injection or 3d to 3wk of dox chow feeding. We observed an overall reduction in lipogenic genes at the 12 hr time-point (Figure 1G), an effect that is reversed at 1 d when DNL genes were unchanged (Figure S1B) and at 3d when DNL genes trended towards an increase (Figure S1C). The suppression of DNL gene expression was completely lost at 3 wk (Figure 1H). Again, the exception was SCD1 mRNA, which remained suppressed (Figure 1H). Interestingly, this compensation observed at 3 d does not occur in brown AT (Figure S1D). At the protein level, two rate-limiting enzymes in DNL, ACC and FASN, are decreased in sWAT after 12 hr dox treatment, but significantly increased following 1d dox feeding and finally normalized to control levels at the 3d and 3 wk time points (Figure 1I). Similarly, fasted ad-Insig1 mice re-fed with dox-containing diet for 12 hr display less nuclear/processed SREBP1 in sWAT compared to control mice (Figure 1J). In contrast, if the re-feeding period is extended to 1 d, ad-insig1 mouse sWAT contained much higher levels of nuclear SREBP1 (Figure 1J). Furthermore, dox administration for 2 wks resulted in normalization of SREBP1 levels (Figure 1J). sWAT ChREBP protein content trended towards an increase at 1d dox, however, expression levels were highly variable
in ad-Insig1 mice (Figure S2). At 2 wk of dox feeding ChREBP levels were slightly decreased (Figure S2), suggesting ChREBP may not be a robust mediator of lipogenic gene restitution in ad-Insig1 mice. Taken together, this data validates that Insig1 overexpression indeed suppresses SREBP1-mediated lipogenesis in white AT; however, this block in lipogenesis is promptly reversed by some compensatory mechanism.

We went on to determine if the transient shifts in AT lipogenesis promoted by Insig1 overexpression are sufficient to alter whole body metabolism. 3 timepoints were chosen for basic phenotyping with the caveat that these are snapshots in time while the adipose tissue is undergoing dramatic changes in signaling. 12 hr post dox injection, a time point at which AT lipogenesis is reduced, ad-Insig1 mice display a slight impairment in an oral glucose tolerance test (OGTT) (Figure 2A), and a significant improvement in TG clearance (Figure 2B). The sWAT fat pad weight is increased and the visceral epididymal fat pad (eWAT) weight is not changed in ad-Insig1 mice at the 12 hr timepoint (Figure 2C). Although this result is unexpected for AT with reduced lipogenesis, it is likely that a longer period of DNL blockade is required to detect the expected decrease in fat pad weight. Additionally, no change in total body weight is observed (Figure 2D). Upon 3 d dox feeding, AT lipogenesis is in the process of compensating (Figure 1I and Figure S1C). At this time point, ad-Insig1 mice trended toward improved oral glucose tolerance (Figure 2E) and improvements observed in TG clearance at 12 hr were reversed at 3d (Figure 2F). Furthermore, ad-Insig1 sWAT and eWAT mass are reduced (Figure 2G) without a change in body weight (Figure 2H) at 3d. Reductions in fat pad mass can be attributed to both Insig1-mediated inhibition of lipogenesis as well as enhanced lipolysis, cellular processes that are known to be reciprocally regulated. At 3d dox, ad-Insig1 mice exhibit a lipolytic phenotype as evidenced by higher levels of un-stimulated hormone sensitive lipase (HSL) phosphorylation in sWAT (Figure S3A and B), increased glycerol release during an ex vivo lipolysis assay (Figure S3C), increased lipase and fatty acid transporter mRNA expression (Figure S3D), and slightly smaller adipocytes (Figure S3E). At 3 wk dox, when the AT has completely restored the ability to do DNL, glucose tolerance, TG clearance, fat pad weight and body weight are not different between control and ad-Insig1 mice (Figure 2I-L). Mouse models of liver DNL deficiencies have presented with compensatory enhancements in AT lipogenesis (20). Here, we find that such a level of inter-organ compensation does not hold true for impairments in AT DNL, i.e., the lack of a phenotype at 3 wk of dox feeding is not the result of enhanced liver lipogenesis (Figure 2M). These data suggest that changes in SREBP1-mediated DNL in AT would have a
functional consequence for whole body metabolic homeostasis if it was not for the self-correcting normalization of DNL at the level of AT itself.

Insulin is a major regulator of lipogenesis in AT and liver through the PI3-kinase/AKT-mediated mTORC1 pathway (21). mTORC1 is required for AKT-stimulated SREBP1 expression and processing (22). Blockade of mTORC1 activity results in loss of lipogenic gene expression (21-24). We determined whether mTORC1 participates in the observed compensatory mechanism. At 12 hr post dox injection, ad-Insig1 mice display a striking increase in phosphorylation of S6 ribosomal protein, which is a surrogate read-out for mTORC1 activity (Figure 3 A and B). Phospho-S6 remains significantly elevated in ad-Insig1 mice compared to controls for a full 24 hr of dox feeding, then it returns to control levels by 3 d (Figure 3B), a time-course similar to that observed for the re-establishment of lipogenic enzyme expression (Figure 1). mTORC1 activation did not correlate with AKT activity, as basal levels of phospho-AKT (Ser473 and Thr308) were not altered at any time-point on diet in ad-Insig1 mice (Figure 3C). To determine if mTORC1 played a causative role in lipogenic compensation, we treated control and ad-Insig1 mice with rapamycin, an inhibitor of mTORC1 activity. We validated the dosage of rapamycin by demonstrating that at the dose chosen, rapamycin is able to suppress mTORC1 activity in AT of high fat diet-induced obese mice, a condition where mTORC1 activity is typically enhanced (Figure S4A). We found that in the absence of mTORC1 signaling by rapamycin treatment, 2 wk of Insig1 over-expression maintained the suppression of SREBP1 and ChREBP lipogenic genes, whereas SREBP2 and downstream targets HMGCR and HMGCS were unaltered (Figure 3D). Furthermore, the 2 wk rapamycin regime resulted in decreased protein levels of FASN and ACC compared to vehicle treated mice regardless of genotype (Figure 3E). Insig1 overexpression was synergistic with rapamycin treatment, as ad-Insig1 mice injected with rapamycin displayed greater suppression of lipogenic protein levels compared to control mice treated with rapamycin. (Figure 3E). The combination of Insig1 overexpression and rapamycin treatment led to a significant reduction in white fat pad weight (Figure 3F) and marked improvements in both TG clearance (Figure 3G) and glucose tolerance (Figure 3H). The glucose and triglyceride clearance curves in the control mice are reminiscent of a diabetic mouse, likely because rapamycin has been shown to cause β-cell toxicity, resulting in peripheral insulin resistance (25). Even so, we can demonstrate that improvements in oral and triglyceride clearance in ad-Insig1 mice are not due to differential effects of rapamycin on control and ad-Insig1 mice. Glucose-stimulated insulin
secretion is not different between groups, if fact, insulin secretion in ad-Insig1 mice trends lower than control mice (Figure 3I). Furthermore, islet size was similar between genotypes (Figure 3J). We confirmed the rapamycin results with a genetic mouse model in which we simultaneously over-expressed Insig1 and knocked-out the regulatory-associated protein of mTOR (raptor), the regulatory protein required for mTORC1 activity. We did this by crossing ad-Insig1 mice with raptor floxed mice carrying the TRE-cre transgene (raptor aKO) to produce an adipocyte-specific, inducible model of insig1 overexpression in a raptor KO background (Figure S4B and C). Overexpression of Insig1 in a raptor aKO background for 2 wk resulted in suppression of most triglyceride synthesis genes, but not HMGCR and HMGCS, compared to raptor aKO mice alone (Figure S4D), a result consistent with the rapamycin treated mice (Figure 3D). Furthermore, raptor aKO mice overexpressing insig1 displayed a reduction in sWAT depot weight (Figure S4E) and mild improvements in both glucose tolerance (Figure S4F) and triglyceride clearance (Figure S4G). However, unlike the rapamycin experiment, Insig1 overexpression in raptor aKO mice resulted in significant induction of Chrebpα and β transcripts (Figure S4D). Activation of ChREBP in this context is likely partially masking the Insig1 effect, accounting for the difference in phenotype intensity between the rapamycin treatment and raptor KO experiments (Figure 3 and S4). Regardless, these data suggest mTORC1 activity is essential for the compensatory mechanism observed in ad-Insig1 mice.

Adipocytes have a high basal flux through lipogenesis, so it is plausible that a sudden blockade of this pathway may result in enhanced TCA cycle flux and mitochondrial ROS production, as carbons (citrate) that are normally siphoned off for lipogenesis are now forced into ATP production. As a result, this may initiate a mechanism to activate mTORC1, since the kinase activity of this complex can be regulated by glycolytic flux, glucose and fructose concentrations, oxidative stress, and cellular energy levels (13, 26, 27). In support of this hypothesis, the mRNA expression of the mitochondrial citrate transporter (CiC) is significantly reduced in ad-Insig1 AT 12 hr post Insig1 induction and increased in expression during the compensatory phase (3 d dox), then returned to control levels by 2 wk (Figure 4A). This is a similar expression pattern as seen for other lipogenic genes (Figure 1G and H, Figure S1C), which is a result consistent with previous literature that demonstrates CiC expression is under the regulation of SREBP1 (28). A predicted consequence of reduced CiC expression is a build-up of TCA cycle and glycolytic intermediates. Mice were treated with dox for 4 hr. During the last 30 min
mice were gavaged with a 1:1 glucose:fructose solution and sWAT was analyzed for glycolytic and TCA cycle intermediate concentrations. Under these conditions we detected a significant increase in fructose-1-phosphate and fructose-6-phosphate in ad-Insig1 sWAT compared to controls (Figure 4B). Furthermore, both isocitrate and pyruvate concentrations were increased in ad-insig1 sWAT compared to that of the control (Figure 4B). This metabolite profile is to be expected as citrate is converted to isocitrate in the next step of the TCA cycle. The reaction immediately downstream of isocitrate is rate-limiting (isocitrate dehydrogenase) explaining the steady-state increase in isocitrate. Furthermore, preventing citrate efflux from the mitochondria would also induce inhibition of upstream pyruvate dehydrogenase through acetyl-coA mediated product inhibition, allowing pyruvate and fructose -1, and -6 phosphate concentrations to rise. To establish a direct connection between high glycolytic activity and mTORC1 activation in AT, we treated wild-type mice with fructose in the drinking water for 12 hr and analyzed sWAT for pS6 levels. Fructose-treated mice displayed significantly higher levels of pS6 and pAKTThr308 (Figure 4C). To determine if the observed alterations in glycolytic and TCA cycle activity is associated with oxidative stress we treated control and ad-Insig1 mice with dox for 8 hr, a timepoint before the compensatory mechanism is activated. We found that protein carbonylation trended towards an increase in ad-insig1 mice, a secondary measure of oxidative stress (Figure 4D). Moreover, ad-Insig1 AT contained significantly higher antioxidant enzyme mRNA expression (Figure 4E), further indication of active oxidative stress. Previous studies have shown that mTORC1 can be positively or negatively regulated by ROS in a cell type- and ROS dose-dependent manner (29). To determine if mitochondrial ROS production during initial Insig1 overexpression is responsible for mTORC1 activation, we treated control and ad-Insig1 mice with mitoQ, a mitochondrially-targeted antioxidant compound. During the phase of over-compensation (1d dox exposure) sWAT from ad-Insig1 mice treated with mitoQ for 1d displayed a significant reduction in pS6 levels compared to that of control mice under the same regimen (Figure 4F). This is in contrast to the observed increase in pS6 level at 1 d dox without mitoQ treatment (Figure 4 F). MitoQ treatment resulted in significantly lower sWAT and eWAT mass (Figure 4G) and suppression of ACC protein expression in ad-Insig1 mice (Figure 4H). However, FASN levels were unaltered (Figure 4H). As further evidence for a substrate-driven mechanism of mTORC1 activation, 2-deoxy-glucose (2-DG) treatment, to reduce glycolytic flux, prevented the increase in pS6, ACC or FASN observed following 1 d dox exposure (Figure 4F and H). Thus, both MitoQ and 2-DG treatment are sufficient to prevent the compensatory activation of mTORC1 and the over-compensation of lipogenic enzyme expression characteristic
of the 1d time-point. However, these treatments did not fully suppress the compensation, suggesting the ROS-mTORC1 pathway is not the only route to compensation. ChREBP is a potential parallel pathway that is activated by high glycolytic intermediate concentrations and may participate in enhancing lipogenic enzyme expression at the 1d timepoint (Figure S2).

The above data suggests substrate flux through glycolysis and the TCA cycle is required for the redox signal responsible for mTORC1-mediated compensation of the adipocyte DNL suppression. However, mTORC1 activation is resolved within 3d of Insig1 overexpression suggesting another mechanism takes over for chronic compensation. We found that ad-Insig1 sWAT had higher fructose transporter (GLUT5) mRNA content compared to control at 3 d and 2 wk dox administration (Figure 5A), suggesting DNL substrate availability may also be important for chronic compensation. At the protein level, GLUT5 was not changed at 1 or 3d feeding but is significantly increased at 2 wk on dox diet (Figure 5B). Protein levels of GLUT4, the insulin sensitive glucose transporter, was unaltered in ad-Insig1 sWAT regardless of time on dox diet (Figure 5B). This data suggests that the up-regulation of GLUT5 does not participate in the initial compensatory mechanism, but may be important for chronic compensation in response to DNL suppression. In keeping with this hypothesis, GLUT5 upregulation is maintained in ad-Insig1 sWAT even during rapamycin treatment, suggesting GLUT5 expression is not regulated by mTORC1 activity (Figure 5C). Additionally, fructose treatment of WT mice moderately increases the expression of GLUT5, but did not affect the expression of GLUT4 (Figure 5D). We went on to determine if feeding ad-Insig1 mice a dox-containing ketogenic diet, which lacks DNL substrates, will prevent the compensation. Control mice fed a ketogenic diet for 3 wk inherently displayed lower ACC and FASN gene expression compared to those on a chow diet (Figure S5A). We observed that Insig1 overexpression during the ketogenic diet reduced sWAT fat pad mass (Figure 5E) and suppressed all triglyceride synthesis gene expression to a greater extent than the ketogenetic diet in control mice (Figure 5F and S5). Interestingly this dietary regime did not promote Insig1 actions on the cholesterol synthesis pathway (Figure 5F). To ensure that these results are due to the lack of lipogenic substrates, not ketosis per se, we treated mice with 2-DG in the drinking water, in addition to dox, for 2 wk. 2-DG did not alter lipogenic gene expression in control mice (Figure S5). However, similarly to the ketogenic diet, 2-DG feeding resulted in a reduction of fat pad mass in ad-Insig1 mice (Figure 5G). This was accompanied by a significant decrease in triglyceride synthesis genes, but not
cholesterol synthetic genes in ad-Insig1 sWAT (Figure 5H). This data provides further evidence that substrate availability is essential for chronic compensation of the adipocyte to a block in SREBP1-mediated lipogenesis (Figure 6).
DISCUSSION

ChREBP is considered to be a more robust regulator of lipogenic gene expression in adipocytes compared to SREBP1. This is primarily based on knock-out mouse models. Here, we reveal new insights into AT DNL by perturbing the system transiently in the adult mouse and analyzing the adaptation of the adipocyte. 12 hrs post Insig1 induction, we observe a robust suppression of DNL enzyme levels in AT (Figure 1G and I). Interestingly, this effect is immediately followed by reactive oxygen species-mediated activation of mTORC1. This mechanism results in enhanced processing of SREBP1 (Figure 1J) and restoration of lipogenic gene expression. This cannot, however, be the only compensatory mechanism, because AT from whole body SREBP1 null mice have normal levels of lipogenic gene mRNAs, suggesting the activation of a redundant pathway. Therefore, it is likely that ChREBP also participates in restoring lipogenesis in the context of SREBP1 pathway inhibition. Our data provides some evidence for this, as ChREBP levels trend towards an increase in ad-Insig1 mice treated with dox for 1 d (Figure S2). However, the data is highly variable, precluding any firm conclusions.

Based on mouse models with an adipocyte-specific ChREBP knock-out, we know that SREBP1 does not compensate for ChREBP activity, as suppression of lipogenic genes in AT is maintained (15). We suggest this observation cannot serve as evidence for a greater importance of ChREBP in AT DNL regulation. Rather, it reflects the dual role of ChREBP in regulating both DNL and glycolysis (14, 15). In this study, we have found that the redox-regulation of the mTORC1/SREBP1 pathway is dependent on DNL substrate availability, as fructose feeding enhances adipocyte pS6 levels, and 2DG treatment prevents the initial increase in pS6 observed after dox induction of Insig1. Therefore, we predict that the strong suppression of AT lipogenesis in ChREBP knock-out mice and the inability of SREBP1 to compensate is by virtue of the additional blockade of glycolysis. In fact, if we mimic inhibition of glycolysis by feeding ad-Insig1 mice with a ketogenic diet, devoid of glycolytic substrates, or 2-DG we could maintain suppression of lipogenic gene expression to a similar level as ChREBP null mice (15). Therefore, the relationship between ChREBP and SREBP1 is likely complementary in AT, as has been described in the liver.

We found that the initiating mechanism of compensation post Insig1 overexpression involves ROS-mediated activation of mTORC1. ROS can stimulate mTORC1 activity through oxidation of cysteine residues in the complex (29). This may explain why mTORC1 activation in our model is independent of AKT activity (Figure
Interestingly, this mechanism is likely specific to the adipocyte, as constitutive models of liver DNL blockade, such as Insig1 overexpression or whole body SCAP knock-out, do not show signs of a cell-autonomous mechanism for compensation (19, 30). Interestingly, mice fed fructose display a reduction in liver pS6 levels (27), the opposite of our finding reported here in adipocytes. It is likely that inherent differences between hepatocytes and adipocytes give way to divergent regulation of mTORC1 activity, such as differences in basal antioxidant capacity. Consequently, hepatocytes do not activate mTORC1 to compensate for obstructed lipogenesis. It is also likely that both cell types differentially regulate mTORC1 in a ROS concentration-dependent manner, as previous studies have shown that low concentrations of ROS stimulate mTORC1 activity, whereas high doses suppress activity in various cell lines (31). In fact, a mouse model of adipocyte-specific glutathione depletion, which results in oxidative stress, displays a reduction in SREBP1-regulated DNL mRNA expression (32). Although mTORC1 activity was not measured in this study, the changes in SREBP1 transcriptional activity could be explained by mTORC1 inhibition via high ROS doses, an effect opposite from what would be expected at doses within the lower physiological range. Thus, differences in the response of liver and AT to suppression of lipogenesis likely have a multifactorial explanation involving lipogenic substrate flux, ROS generation rates and basal antioxidant capacity. This may also be true for the response between different adipose depots, as BAT did not compensate for Insig1 overexpression at 3 days of dox treatment (Figure S1C).

The transient changes in AT lipogenic capacity produced in ad-Insig1 mice result in changes in whole-body glucose and TG metabolism (Figure 2). The prevailing claim is that whole-body insulin sensitivity positively correlates with AT DNL (8, 33-35). Functionally, this is true for ChREBP-mediated DNL, as mice overexpressing ChREBP in the adipocyte display lower body weight on a Western diet, improved insulin sensitivity, reduced hepatic TG content, and a beige adipocyte gene expression profile (36). Conversely, adipocyte-specific ChREBP knock-out mice are insulin resistant (15). Consistent with these results, we report here that Insig1-mediated suppression of AT DNL results in slight glucose intolerance before compensation is fully initiated (Figure 2A). However, when contemplating the question of whether we should be therapeutically activating or inhibiting AT DNL to improve metabolic homeostasis in disease states, we need to consider all effects of AT DNL on physiology. The most striking phenotypic change we observed is an improvement in triglyceride clearance with Insig-1 overexpression before compensation or under conditions that prevent compensation (Figure 2B, Figure
3G and Figure S4G). This data suggests AT SREBP1-regulated DNL may negatively regulate whole-body lipid clearance. In keeping with this, previous studies have shown that a knock-out of FASN in adipocytes displays a beneficial effect on whole body metabolism: improved glucose tolerance, increased energy expenditure, adipocyte beiging, and resistance to diet-induced obesity (37). In contrast, activating adipocyte DNL by over-expressing the nuclear form of SREBP1a using an aP2 promoter, increases the release of fatty acids, resulting in hepatic steatosis (38). A further consideration for the role of AT DNL in metabolic syndrome is its involvement in adipogenesis, as production of new adipocytes in obesity is one hallmark of healthy AT expansion (39). FASN is required for adipogenesis, and overexpression nuclear SREBP1c inhibits adipocyte differentiation causing lipodystrophic and diabetic phenotype (37, 40). Therefore, further work is required to determine if enhancing or suppressing AT lipogenesis is a viable approach to treat metabolic conditions, such as type 2 diabetes. The answer is likely dependent on the pathway of DNL that is to be manipulated. Although both ChREBP and SREBP1 pathways regulate DNL gene expression in AT, as explained above, they do diverge in their metabolic function at both the cellular and organismal levels. Particularly important are potential differences in the specific bio-active lipid species produced by each pathway and how each pathway regulates adipogenesis.

Our study makes evident the utility of inducible genetic systems to establish the importance of a pathway in cellular homeostasis in vivo. Identifying mechanisms of compensation following a perturbation in the adult animal can be a highly valuable endeavor to determine if a pharmacological intervention will have sustained efficacy in the targeted tissue. For example, treatment of mice with neuropeptide Y (NPY) has been shown reduce food intake and promote weight loss in rodents; however whole-body knockout of this peptide in mice does not affect body weight regulation. By using a dox-inducible NPY knockout mouse, Ste Marie et. al. demonstrated that NPY depletion does not alter feeding habits although did transiently effect body weight (41). The conclusion of this study was that due to the likely compensation that occurs in response to NPY deletion, NPY targeting therapy may not have sustainable benefit on food intake. Here we used a similar approach, inducibly overexpressing Insig-1 in adipocytes of adult mice to mimic the sterol-induced sequestration of SREBPs by the Insig-1/SCAP complex. We have demonstrated that mTORC1 is essential for the maintenance of adipocyte lipogenesis when DNL substrates are available. Through this mechanism and ChREBP activity, the
adipocyte can maintain lipogenesis even under conditions that would inhibit lipogenesis in other cell types to guarantee adequate storage of otherwise toxic carbohydrates.
METHODS

Animals - Insig1 was inducibly overexpressed in mature adipocytes using a tetracycline response element (TRE)-driven Insig1 construct. The mouse Insig1 gene was subcloned into the pTRE vector (Clontech Laboratories; 631059) with a rabbit β-globin 3’ UTR. Specificity for the adipocyte in this model was accomplished by using the reverse tetracycline trans activator (rtTA) under the control of the adiponectin (APN) promoter (42). Mice expressing both the TRE-Insig1 and rtTA transgenes no not express Insig1 until doxycycline is present (Figure 1B). Raptor floxed mice were purchased from The Jackson Laboratory (B6.Cg-Rptorim1.1Dmsa/J; No: 013188 ). All cohorts of mice were male and 10-15 wk old.

Mouse Treatments – Insig-1 gene expression was induced with mouse chow, high fat diet or ketogenic diet containing 600 mg/kg doxycycline (Bio Serve; S4107, S7597, S5867). MitoQ (a generous gift from Mike Murphy, Cambridge, UK) was dissolved in PBS, 5% DMSO. Mice were injected IP with a single 5 mg/kg injection of mitoQ at the initiation of dox chow-feeding. Rapamycin (LC Laboratories, R-5000) was dissolved in 100% ethanol at a concentration of 50 mg/kg. Aliquots were made and stored at -80°C. The day of injections a 1 mg/ml rapamycin stock was prepared in a solution containing 10% PEG400, 10% tween80 and ddH2O. Mice were injected with 4 mg/kg every second day. Where indicated mice received a single 250 mg/kg 2-DG (Sigma, D6134) injection at the initiation of dox chow feeding. 2DG was dissolved in PBS. When 2-DG was supplied in the drinking water it was dissolved in ddH2O to 0.5% (w/v) and filter-sterilized. Fructose was dissolved to 20% (w/v) in ddH2O, filter sterilized, and supplied as drinking water for the 12 hr fructose treatment studies.

Metabolic Phenotyping - For the OGTT, mice were fasted for 3 h before to the administration of glucose (1.25 g/kg body weight) by gastric gavage. At the indicated time-points, venous blood samples were collected in capillary tubes from the tail-vein. Glucose levels were measured using an oxidase-peroxidase assay. Insulin concentration was determined using a commerical Insulin Elisa Kit (Crystal Chem, 90095). For the TG clearance procedure, mice were fasted for 15 h, then gavaged with 20% Intra-lipid (15 ul/g body-weight; Sigma-Aldrich, I141).
**Western blot** - Protein was extracted from adipose tissue by homogenization in PBS supplemented with 1 mM EDTA, 20 mM NaF, 2 mM Na$_3$VO$_4$, and protease inhibitor cocktail. 5X RIPA buffer was added to the homogenate for a final concentration of 10 mM Tris-HCl, 2 mM EDTA, 0.3% NP40, 0.3% deoxycholate, 0.1% SDS and 140 mM NaCl pH 7.4. The sample was cleared by centrifugation at 10 000 xg for 5 min. 20-50ug/lane of supernatant protein was separated by SDS-PAGE (ThermoFisher, NP0335BOX) and transferred to nitrocellulose membrane. The blots were then incubated overnight at 4°C with primary antibodies in a 1% BSA TBST blocking solution. The Odyssey Infrared Imager was used to visualized Western blots with Li-cor IRdye secondary antibodies. Please see supplemental table 2 for a list and the source of all primary and secondary antibodies.

**qPCR** – Tissues were homogenized in TRIlzol (Fisher Scientific, 12034977) using the Qiagen TissueLyser II. RNA was isolated per the manufacturer’s protocol. RNA quality and yield were determined by absorbance at 260nm, 280nm and 230nm. cDNA was prepared by reverse transcribing 1 µg of RNA with the iScript cDNA Synthesis Kit (BioRad, 1708890). The results were calculated by the standard threshold cycle method and was used for normalization. Please refer to Table S1 for qPCR primer sequences.

**Nuclear and ER membrane fractionation** – Freshly harvested subcutaneous adipose tissue was homogenized with a motor-driven Teflon/glass homogenizer in 10 ml ice cold buffer (10 mM MOPS, 1 mM EDTA, 210 mM Mannitol, 70 mM Sucrose, 20 mM NaF, 2 mM Na$_3$VO$_4$, and protease inhibitor cocktail, pH 7.4). The nucleus was pelleted by centrifugation at 720 g for 10 min. The pellet was resuspended in 10 ml of the same buffer and filtered through cheesecloth. The cleared sample was centrifuged again at 720 g for 10 min. The nuclear pellet was resuspended in PBS. The supernatant from the first 720 g spin was centrifuged at 30 000 xg to recover ER-enriched cellular membranes. The ER pellet was resuspended in PBS. Following protein determination, fractions were subjected to Western blot analysis for processed SREBP1 (nuclear fraction) and precursor SREBP1 (membrane fraction).

**Histology** - sWAT was excised and fixed in 10% PBS-buffered formalin for 24 h. Tissues were paraffin-embedded and sectioned (5 µm) at the UTSW Molecular Pathology Core. Following hydration, tissues were stained with H&E.
Ex vivo lipolysis assay – subcutaneous adipose tissue was harvested and washed in cold PBS. Small pieces of adipose tissue (~ 50 mg) were equilibrated for 1 hr in 150 ul DMEM containing 2% BSA. Adipose tissue pieces were then transferred into another culture dish containing 200 ul of lipolysis buffer (5 mM HEPES, 125 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$ and 2.6 mM MgSO$_4$, pH 7.4) in the presence or absence of isoproterenol (10 µm; Sigma-Aldrich, I6504). Explants were incubated for 2 hr at 37˚C. Buffer was removed from the explants and assayed for glycerol using the Free Glycerol Determination Kit (Sigma-Aldrich, F6428). Final glycerol values were normalized to total weight (grams) of tissue in the corresponding well.

LC/MS/MS analysis of hexose phosphate TCA cycle intermediates – clamp-frozen tissue sections (100 mgr) were homogenized with a mechanical tissue disruptor in 0.8 mL of 80% Methanol in a screw cap borosilicate glass tube. The homogenizer probe was rinsed with 1.6 mL of 80% MeOH and the methanolic solution was combined with the homogenate. The tissues were kept on ice during the homogenization/quenching process. Immediately afterwards 40 µL of Internal solution was added (7-methyluric acid (2,4,5,6-$^{13}$C$_4$, 99%, 1,3,9-$^{15}$N$_3$, 98%) 25 µgr/mL. Cambridge Isotope Laboratories, Inc., Tewksbury, MA). Samples were thoroughly vortexed and then centrifuged in a benchtop centrifuge at 2,500 g (Sorvall Legend XTR, Thermo Fixher Scientific, Whaltham, MA). Supernatant was transferred to a 5.0 mL polypropylene cell culture tube. Protein pellets were re-extracted with 2 mL of 80% MeOH and the supernatants were combined. Tissue extracts were dried in a speed-vap concentrator. The dried residues were reconstituted in 220 µL of H$_2$O 0.1% Formic Acid for TCA cycle intermediates and H$_2$O 10 mM tributyl amine for hexose phosphates. Reconstituted samples were transferred to GC vials.

TCA intermediates were analyzed by injecting 1 µL of sample into the LC/MS/MS system consisting of a Shimadzu LCMS-8060 triple quadrupole mass spectrometer operating the DUIS ion source in electrospray mode (Shimadzu Scientific Instruments, Columbia, MD coupled to a Nexera X2 UHPLC chromatographer equipped with three pumps, autosampler SIL-30AC and CTO-20AC (Shimadzu Scientific Instruments). Compounds were resolved and analyzed using the instrumental parameters and chromatographic conditions described in the Shimadzu LC/MS/MS method package for cell culture profiling (Shimadzu Scientific Instruments). Hexose phosphate intermediates were analyzed by injecting 5 µl of sample in the same LC/MS/MS system. Compound
were resolved combining ion pair chromatography and differential fragmentation patterns \((43, 44)\). Data were processed using the LabSolutions V 5.82 and LabSolutions Insight V 2.0 program packages (Shimadzu Scientific Instruments).

**Statistics**- All data is presented as mean ± SEM. *p<0.05, ** p<0.01 and ***p<0.001 by two-tailed Student’s \(t\) test, or in the case of systemic assays two-way ANOVA. A \(p\) value < 0.05 was determined to be statistically significant. All qPCR data points were the average of technical duplicates. For all mouse studies, the \(n\) value corresponds to individual mice of a given treatment. Data was analyzed used Prism GraphPad Software.

**Study Approval** - All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern (UTSW) Medical Center (Dallas, TX).

**Author Contributions**
C.C conceptualized the project, designed experiments, analyzed and interpreted data, conducted experiments, with the exception of that listed below, and wrote the manuscript. Y.Z produced and validated the TRE-Insig1 mice as well as conducted chronic high fat diet studies. V.A.P, N.J, and A.L.G assisted in mouse studies and sample processing. R.G conducted the LC/MS/MS analysis of TCA cycle intermediates. D.O, G.L and J.H provided guidance and were consulted for data interpretation. P.E.S was involved in experimental design and the writing of the manuscript.

**Acknowledgements**
We thank UT Southwestern Transgenic Core for their help in the generation of mice. We also thank the UTSW Molecular Pathology Core for imbedding and sectioning samples for histology. The MitoQ compound was a kind gift from Dr. Mike Murphy, University of Cambridge, UK. This study was supported by US National Institutes of Health (NIH) grants R01-DK55758, P01-DK088761, R01-DK099110 and P01-AG051459, as well as by an
unrestricted grant from the Novo Nordisk Research Foundation (P.E.S.). Contributions of G.L and J.H were supported by the NIH (HL-20948). C.C is supported by F32-DK113704.
REFERENCES


**Figure 1:** Overexpression of Insig1 In Adipocytes Results in Suppression of Lipogenesis Followed by Compensation. (A) Mouse model of inducible, adipocyte-specific over-expression of Insig1 (ad-Insig1). Doxycycline (dox) binds the reverse tetracycline trans-activator (rtTA) expressed in adipocytes via the adiponectin promoter. The rtTA is thereby activated to bind the tetracycline response element (TRE) to drive Insig1 expression. (B) qPCR analysis of Insig1 in sWAT or liver tissue from control and ad-Insig1 mice ± dox treatment for 5 d (n=3-4). (C) Insig1 protein expression in the ER membrane fraction of control or ad-Insig1 mouse sWAT treated with dox for 24 hr or 2 wk. (D-F) Control or ad-Insig1 mice were provided a high fat diet (HFD) containing chow for 16 wk during which (D) body weight was measured (n=7). (E) An oral glucose tolerance test was conducted at 16 wk on diet (n=7) and (F) a triglyceride clearance test was performed at the 10 wk timepoint on the same diet (n=8). (G) Lipogenic gene expression in sWAT 12 hr post injection of 2 mg/kg dox (n=8) or (H) 3 wk of dox-containing chow diet (n=5-8). (I) Representative Western blots and densitometry of ACC and FASN in sWAT at the indicated times of dox exposure (n=3-4). Only the gel lanes that are cropped together can be compared. Blots for GAPDH were run separately from ACC and FASN, although consistent biological samples are presented. (J) Control and ad-Insig1 mice were fasted for 16 hr and refed dox-containing chow for 12, 24 hr or 2 wk. sWAT tissue was separated into nuclear- and ER membrane-enriched fractions for processed and precursor SREBP1 respectively. Calreticulin (CALR) was used as a loading control from membrane fractions and Histone 3 (H3) was used as the loading control for the nuclear fractions. Shown is a representative of n=3 Western blots and corresponding densitometry of nuclear-located SREBP1. All data is presented as mean ± SEM. *p<0.05 and ***p<0.001 by two-tailed Student’s t test.
**Figure 2: Manipulation of Adipose DNL Alters Whole-Body Metabolism.**

(A-D) 12 hr post dox injection (2 mg/kg) mice were subjected to an (A) oral glucose tolerance test (n=6) and a (B) triglyceride clearance test (n=9-10). At this 12 hr timepoint, another cohort of mice were used for (C) sWAT and eWAT depot weights (n=8) and (D) total body weight (n=8). (E-L) Mice were provided with chow diet containing dox for 3 d or 3 wk at which time mice were subjected to an (E and I) oral glucose tolerance test (n=5-8) and a (F and J) triglyceride clearance test (n=5-8) or (G and K) sacrificed for white AT weights. (H and L) body weight measurements at the indicated times. (M) qPCR for lipogenic genes in liver of control and ad-Insig1 mice following 3 wk of chow diet supplemented with dox (n=7). All data is presented as mean ± SEM. *p<0.05, ** p<0.01 and ***p<0.001 by two-tailed Student’s t test (bar graphs) or two-way ANOVA (systemic assays).
Figure 3: mTORC1 is Activated in Response to a Block in AT Lipogenesis.

(A) Representative Western blot for the specified proteins in the sWAT of control or ad-Insig1 mice exposed to dox for 12 hr or 2 wk. (B-C) Densitometric quantification of Western blots for (B) pS6Ser240/244 or (C) pAKTThr308 and pAKTSer473 following the indicated durations of dox exposure (n=3-5). (D) Control and ad-Insig1 mice were injected with rapamycin (rapa; 4 mg/kg) every second day for 2 wk while on dox-containing chow. Lipogenic gene expression was determined in sWAT following the 2 wk treatment (n=5). (E) Representative Western blot and densitometry of sWAT FASN and ACC protein levels in control or ad-Insig1 mice ± rapa (n=5-7). (F) sWAT and eWAT weights post 2 wk treatment with both dox and rapa (n=5-6). (G) Triglyceride clearance test performed following 3 wk dox and rapa treatment (n=6-7). (H) Oral glucose tolerance test conducted following 2 wk dox and rapa treatment (n=6). (I) Insulin measurements during oral glucose tolerance (n=6). H&E stain of pancreatic islet (arrow) at 20X magnification. All data is presented as mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 by two-tailed Student’s t test (bar graphs) or two-way ANOVA (systemic assays).
**Figure 4: Acute Suppression of AT DNL Induces Mitochondrial Oxidative Stress, Resulting in mTORC1 Activation.** (A) mRNA expression in sWAT of the citrate transporter (CtC) over the indicated dox treatment duration (n=8-12). (B) Mice were injected with dox (2 mg/kg). At 3.5 hr post injection mice were gavaged with 1:1 glucose fructose. At 4 hr post dox injection sWAT was harvested for determination of glycolytic and TCA cycle intermediate concentrations. (C) Wild-type mice were treated ± 20% fructose in the drinking water for 12 hr. Image displays a representative Western blot and densitometry for pS6Ser240/244 and pAKTThr308 in sWAT (n=5). (D) Protein carbonylation blot densitometry over the indicated time course of dox exposure (n=4). (E) qPCR analysis of antioxidant genes in sWAT 8 hr after an injection of 2 mg/kg dox (n=6). (F and H) Control or ad-Insig1 mice were injected with 5 mg/kg MitoQ or 250 mg/kg 2DG and simultaneously supplied dox-supplemented diet for 24 hr. (F) Representative Western blot and densitometry of pS6Ser240/244 from the sWAT of control or ad-Insig1 treated ± MitoQ or 2DG (n=3-4). (G) AT depots weights with mitoQ treatment. (H) An example Western Blot for ACC and FASN levels in sWAT from control or ad-Insig1 mice treated with MitoQ or 2DG and corresponding densitometry (n=3-4). In this instance FASN was run on a separate gel from ACC and GAPDH although the biological sample presented is consistent. All data is presented as mean ± SEM. *p<0.05, ** p<0.01 and ***p<0.001 by two-tailed Student’s t test.
**Figure 5: Chronic Compensation to DNL Suppression is Dependent on Substrate Availability.**

(A) GLUT5 mRNA expression in control or ad-Insig1 mice at the indicated timepoints on dox diet. (B) Western blot image and corresponding densitometry for GLUT4 and GLUT5 in sWAT of control or ad-Insig1 mice following the indicated duration of dox treatment in the diet (n=3-8). (C) Representative Western blot of n=3 from the sWAT of control or ad-insig1 mice following 2 wk dox treatment with rapamycin (rapa) or vehicle injections. (D) Wild-type mice were treated ± 20% fructose in the drinking water for 12 hr. Displayed is a representative Western blot and densitometry for GLUT4 and GLUT5 in sWAT (n=5). (E and F) Control and ad-Insig1 mice were supplied with a ketogenic diet containing dox for 3 wk, after which (E) sWAT and eWAT weight were measured and (F) DNL gene expression was quantified (n=5-7). (G and H) Mice were provided with 2-DG in the drinking water (0.5%) in addition to the dox diet. Following 2 wk treatment (G) sWAT and eWAT weights were measured and (H) lipogenic enzyme mRNA was quantified (n=5-6). All data is presented as mean ± SEM. *p<0.05, ** p<0.01 and ***p<0.001 by two-tailed Student's t test.
**Figure 6:**
*Model for mTORC1-mediated Compensation of Obstructed AT Lipogenesis.*

Insig1 overexpression results in general suppression of lipogenic gene expression, including that of the citrate transporter (CiC). Diminished efflux of citrate from the mitochondria results in a “back-up” of the TCA cycle and mitochondrial ROS production, as citrate carbons are abruptly diverted into the TCA cycle for ATP production instead of lipogenesis. ROS activates mTORC1, which stimulates both SREBP1 transcription and the processing of SREBP1 into its nuclear/active form. High levels of glycolytic intermediates may also stimulate ChREBP activity. The consequence is restoration of lipogenic gene expression. This mechanism of compensation is transient, but chronic compensation is maintained by greater uptake of fructose via GLUT5. Both acute and chronic compensation is dependent on DNL substrate availability.