Activation of Liver mTORC1 Protects Against NASH via Dual Regulation of VLDL-TAG Secretion and *De Novo* Lipogenesis

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**SUMMARY**

Hepatic mechanistic target of rapamycin complex 1 activity is elevated in mouse models of insulin-resistant, fatty liver conditions but is decreased in mouse models of nonalcoholic steatohepatitis. Genetic activation of mechanistic target of rapamycin complex 1 in hepatocytes enhances lipid export from the liver while suppressing lipid synthesis to protect against nonalcoholic steatohepatitis.

**BACKGROUND & AIMS:** Dysregulation of liver lipid metabolism is associated with the development and progression of nonalcoholic fatty liver disease, a spectrum of liver diseases including nonalcoholic steatohepatitis (NASH). In the liver, insulin controls lipid homeostasis by increasing triglyceride (TAG) synthesis, suppressing fatty acid oxidation, and enhancing TAG export via very low-density lipoprotein. Downstream of insulin signaling, the mechanistic target of rapamycin complex 1 (mTORC1), is a key regulator of lipid metabolism. Here, we define the role of hepatic mTORC1 activity in mouse models of NASH and investigate the mTORC1-dependent mechanisms responsible for protection against liver damage in NASH.

**METHODS:** Utilizing 2 rodent NASH-promoting diets, we demonstrate that hepatic mTORC1 activity was reduced in mice with NASH, whereas under conditions of insulin resistance and benign fatty liver, mTORC1 activity was elevated. To test the beneficial effects of hepatic mTORC1 activation in mouse models of NASH, we employed an acute, liver-specific knockout model of TSC1 (L-TSC-KO), a negative regulator of mTORC1.

**RESULTS:** L-TSC-KO mice are protected from and have improved markers of NASH including reduced steatosis, decreased circulating transaminases, and reduced expression of inflammation and fibrosis genes. Mechanistically, protection from hepatic inflammation and fibrosis by constitutive mTORC1 activity occurred via promotion of the phosphatidylcholine synthesizing enzyme, CCTα, and enhanced very low-density lipoprotein-triglyceride export. Additionally, activation of mTORC1 protected from hepatic steatosis via negative feedback of the mTORC2-AKT-FOXO-SREBP1c lipogenesis axis.

**CONCLUSIONS:** Collectively, this study identifies a protective role for liver mTORC1 signaling in the initiation and progression of NASH in mice via dual control of lipid export and synthesis. (Cell Mol Gastroenterol Hepatol 2022;13:1625–1647; https://doi.org/10.1016/j.jcmgh.2022.02.015)
Nonalcoholic fatty liver disease (NAFLD) is now recognized as a leading cause of liver disease worldwide. NAFLD is a spectrum of diseases that comprise benign fatty liver and nonalcoholic steatohepatitis (NASH), which correlate strongly with metabolic diseases such as obesity, insulin resistance, and type 2 diabetes. The hallmarks of NAFLD include increased lipid accumulation in the liver, known as hepatic steatosis or nonalcoholic fatty liver (NAFL). In some cases, individuals with NAFL can develop NASH, where steatosis is accompanied by inflammation, fibrosis, and cell death, which may ultimately lead to cirrhosis and liver failure. Importantly, progression of NASH is associated with increased incidence of hepatocellular carcinoma and severe illnesses from viral infections such as COVID-19.

Weight reduction, including lifestyle interventions and bariatric surgery, show promise in improving NASH pathology. Despite this, there are currently no United States Food and Drug Administration-approved medical treatments for NASH. Therefore, detailed molecular investigation of the metabolic drivers of NAFL and NASH may uncover new therapeutic opportunities. Current research is focused on inactivation of inflammatory and lipogenic metabolic pathways. Many ongoing clinical trials focusing on inflammation include drugs targeting chemokine receptors, thyroid hormone receptors, and farnesoid X receptors. For example, the farnesoid X receptor agonist, obeticholic acid, completed a phase III clinical trial and modestly improved fibrosis in patients with NASH, but did not gain United States Food and Drug Administration approval due to potential safety risks and modest efficacy. As a result, there remains a significant unmet need for the elucidation of the molecular drivers of NAFLD.

In addition to targeting inflammation and fibrosis modulators, the potential of antagonizing key enzymes involved in regulating hepatic lipid metabolism is of significant therapeutic interest. In particular, increased hepatic de novo lipogenesis (DNL) is a distinguishing feature of patients with NAFLD. The liver is a key metabolic organ regulating lipid homeostasis by coordinating lipid synthesis, breakdown, uptake, and export. In response to feeding, insulin signals to the liver to increase DNL, suppress fatty acid oxidation, and promote triglyceride (TAG) esterification to increase lipid secretion. Many lipid metabolism pathways are altered in the development and progression of NAFLD. Blockade of fatty acid synthetase (FASN), acetyl-CoA carboxylase (ACC), or diacylglycerol acyltransferase 2 (DGAT2), key enzymes involved in lipid synthesis, showed positive effects on reducing hepatic lipid burden and fibrosis in clinical trials of NASH. Furthermore, agonists targeting peroxisome proliferator-activated receptors (PPAR), key regulators of lipid oxidation, improved many hallmarks of NASH without worsening of fibrosis. However, these drugs are in ongoing clinical trials, and recent data revealed acetyl-CoA carboxylase inhibition leads to hypertriglyceridemia; thus, it is unclear whether inhibiting DNL or increasing oxidation alone will be sufficient to improve NASH.

In the liver, insulin and other growth factors activate many downstream signaling pathways to regulate lipid homeostasis. Of note, the mechanistic target of rapamycin complex 1 (mTORC1) is a critical node in the regulation of hepatic lipid homeostasis, receiving input from hormones, growth factors, and nutrients. During periods of prolonged fasting, inhibition of mTORC1 is necessary for the induction of hepatic ketogenesis. Postprandially, protein kinase B (PKB or AKT) is activated by insulin, which inhibits the tuberous sclerosis complex (TSC), leading to the activation of mTORC1. Activation of mTORC1 signaling is required, but not sufficient, for the activation of DNL, in part via regulation of the master regulator of lipogenic gene expression, sterol regulatory element binding protein 1 (SREBP1). Paradoxically, constitutive activation of mTORC1 does not promote lipid synthesis, rather, it leads to the suppression of lipogenic transcription, due to a negative feedback regulation of the mTORC2-AKT axis. Lastly, insulin acts through many pathways to regulate hepatic lipid export via very-low-density-lipoproteins (VLDL). Although insulin is canonically known to reduce VLDL-TAG and apolipoprotein B secretion in isolated hepatocytes, hepatic insulin signaling is required for VLDL-TAG export in vivo. Mechanistically, our laboratory recently demonstrated that the mTORC1-dependent control of VLDL-TAG secretion, through its promotion of phosphatidylcholine (PC) synthesis, is critical for both hepatic and circulating TAG homeostasis. Thus, liver mTORC1 signaling plays an essential role in regulating both hepatic and systemic lipid metabolism.

Despite the immense effort to understand how insulin signaling controls liver lipid homeostasis, there remains a dearth of knowledge about how these signaling pathways contribute to the initiation and development of NAFL and NASH. Specifically, the direct role of mTORC1 in NAFL and NASH livers remains elusive. Current data suggest liver mTORC1 activity is elevated in mouse models of insulin-resistance and benign fatty liver, which correlate with increased DNL and VLDL-TAG secretion in NAFL.
However, the role of mTORC1 in NASH is less understood. In animal models, in vivo inhibition of mTORC1, through deletion of hepatic Raptor protein, or through long-term rapamycin treatment, induces inflammation, fibrosis, and liver damage in mice. Clinical studies using mTORC1 inhibitors did not improve overall survival of patients with advanced liver damage. Furthermore, clinical studies in humans have identified a reduction in VLDL synthesis and secretion in pathology-confirmed NASH. Therefore, we hypothesize that mTORC1 has a biphasic role in the development and progression of NAFLD. In this study, we test the hypothesis that reduced liver mTORC1 signaling is a key transition point for the progression of benign fatty liver to steatohepatitis.

Results

mTORC1 Activity is Differentially Regulated During the Initiation and Progression of NASH in Mice

Previous studies demonstrated an enhancement of mTORC1 activity during obesogenic, insulin-resistant, and benign steatotic conditions, such as in diet-induced obese and genetically obese rodents. However, less is known about mTORC1 activity through the progression to bona fide NASH in rodents. To evaluate the role of mTORC1 through NASH initiation and progression, we utilized genetic and diet-induced mouse models of steatosis and steatohepatitis. In the insulin-resistant ob/ob mouse model, pronounced accumulation of lipid droplets was observed without indication of fibrosis (Figure 1A). As previously reported, phosphorylation of AKT and S6 were significantly increased upon fasting, suggesting increased insulin signaling via the AKT-mTORC1 axis under these conditions of insulin-resistance and benign fatty liver with no evidence of fibrosis (Figure 1B).

To directly ascertain the role of mTORC1 signaling in NASH livers, we fed mice 2 distinct NASH-promoting diets. First, a cohort of mice were challenged with a high-fat, low-methionine, choline-deficient diet (45% kcal fat, 0.1% methionine, no added choline), referred to here as "LMCD-HFD." LMCD-HFD-fed mice displayed prominent hepatic steatosis and collagen deposition following 4 weeks of feeding, compared with age-matched mice fed a control L-amino acid diet (10 kcal% fat with methionine and choline), as previously reported. Notably, substantial rise in inflammation and fibrotic gene expression was observed in response to LMCD-HFD-feeding (Figure 1D), correlating with increase in circulating transaminases, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), indicators of liver damage (Figure 1E). The induction of liver damage, fibrosis, and inflammation paralleled a reduction of both VLDL-TAG secretion (Figure 1F) and hepatic mTORC1 activity, as quantified by a decrease in phosphorylated to total ratios of the mTORC1 targets, S6, and 4EBP1 proteins following a 6-hour fast (Figure 1G). Surprisingly, proximal signaling via AKT was not suppressed under these conditions. Rather, phosphorylation was modestly enhanced, indicating mTORC1 is reduced independent of any changes in proximal AKT activity in this NASH model (Figure 1G).

Next, we assessed mTORC1 activity in a dietary NASH model that does not rely on nutrient limitations. The Gubra-modified AMLN diet (40% kcal fat [mostly palm oil], 20% kcal fructose, and 2% cholesterol, Research Diets), referred to here as "GAN diet," induces NASH upon 30 weeks of feeding. Following 30 weeks of diet administration, mouse livers displayed histological induction of fibrosis and steatosis (Figure 1H). Similar to the LMCD-HFD cohort, GAN diet-fed mice had reduced rates of VLDL-TAG secretion (Figure 1I), correlating with a reduction pS6/total S6 ratio indicating reduced mTORC1 activity (Figure 1J and K). Collectively, these data suggest that mTORC1 is differentially regulated during the initiation and development of NAFLD, such that in simple steatosis and insulin-resistance, hyperinsulinemia leads to increased liver mTORC1 activity. However, under NASH-like conditions, hepatic mTORC1 activity is reduced despite normal or enhanced proximal AKT signaling.

Acute mTORC1 Activation Prevents and Improves Steatosis, Inflammation, and Fibrosis in Mice on NASH Diet

To counteract the decrease in mTORC1 signaling in NASH livers, we utilized a mouse model of acute deletion of TSC1. The TSC complex negatively regulates mTORC1 activation via AKT; therefore, mice lacking TSC1 have constitutive mTORC1 activity independent of proximal AKT signaling. Briefly, Tsc1loxP/loxP mice were injected with adeno-associated virus serotype 8, driven by a thyroxine binding globulin (AAV8-TBG) promoter containing either -Cre (L-TSC-KO) or -GFP (Control) 2 weeks prior to a 4-week LMCD-HFD feeding to test whether constitutive mTORC1 activity protects against diet-induced NASH (Figure 2A). Phosphorylation of S6 to total S6 was enhanced in L-TSC-KO mouse livers, and TSC1 protein was undetectable, confirming deletion of TSC1 and in vivo activation of liver mTORC1 (Figure 2B). Four weeks after LMCD-HFD feeding, L-TSC-KO mice displayed reduced expression of several markers of fibrosis (Col1a1, Acta2, Pdgfb, Tgfβ1, Timp1) and inflammation (Il1b, Ccl2, Tnf, Saa1, Adgre1) (Figure 2C). Consistently, L-TSC-KO mice displayed lower plasma ALT and AST activity, suggesting an attenuation of liver damage in L-TSC-KO mice (Figure 2D). Gene expression changes correlated with a histological decrease in collagen deposition as documented by Sirius red staining (Figure 2E). Histological assessment documented an improvement in fibrosis and steatosis staging between L-TSC-KO and control mice (Figure 2F and G). Histological changes were accompanied by a significant decrease in TAG accumulation in the livers of L-TSC-KO mice (Figure 2H).

Previous work in models of both acute and chronic mTORC1 activation exhibit an anti-steatotic effect under fasting and obesogenic challenges, such as HFD feeding. This phenomenon was attributed to a decrease in DNL and a parallel increase in fatty acid oxidation. More recently, our laboratory identified a
dominant role for mTORC1 in the promotion of VLDL-TAG secretion and control of hepatic lipid accumulation. Therefore, to test the specific role of VLDL-TAG secretion in our L-TSC-KO NASH model, we injected fasted L-TSC-KO mice with poloxamer-407 (p407) detergent, a compound that blocks lipoprotein lipases, and measured TAG secretion as the accumulation of TAG in the blood over time. Constitutive mTORC1 activation increased TAG secretion...
in our NASH-fed model (Figure 2J), in agreement with previous observations in L-TSC-KO mice fed a chow diet.\textsuperscript{14} We also observed a parallel suppression of key hepatic lipogenic genes (Srebp1c, Fasn, and Acaca) under conditions of constitutive liver mTORC1 activity (Figure 2J). These changes in hepatic lipid metabolism in L-TSC-KO mice did not correlate with any alterations in body weight of the mice (Figure 2K), nor were there differences in glucose and insulin sensitivity and circulating fasting TAG (Figure 3A–E). Additionally, constitutive mTORC1 activity is known to block prolonged fasting-induced β-oxidation.\textsuperscript{23} However, we did not find differences in fatty acid oxidation-associated genes (Mcad, Ppara, and Cpt1a) (Figure 3F) or circulating ketones (Figure 3G) between 6-hour-fasted LMCD-HFD-fed L-TSC-KO mice and control mice. Additionally, we did not detect an increase in apolipoprotein gene expression in L-TSC-KO mice, suggesting VLDL-TAG secretion is enhanced independent of changes in apolipoprotein expression (Figure 3H). Based on the known role of mTORC1 in regulating autophagy, we measured gene expression of Lc3b and p62 and did not observe a difference between control and TSC-deficient livers (Figure 3J). This finding was consistent with similar protein levels of p62 in both cohorts of mice (Figure 3J). Collectively, these data suggest in vivo mTORC1 activation protects against diet-induced NASH through both enhancement of TAG secretion and suppression of DNL, independent of changes in body weight, insulin sensitivity, AKT signaling, or fatty acid oxidation.

Next, we tested whether acute mTORC1 activation is sufficient to reverse fibrosis and inflammation in livers with pre-established NASH. Here, we challenged Tsc1\textsuperscript{lox/lox} to the LMCD-HFD for 4 weeks prior to injection of AAV8-TBG-Cre (L-TSC-KO) or -GFP (Control). Following AAV injection, mice continued LMCD-HFD feeding for an additional 4 weeks (Figure 4A). Functional activation of mTORC1 in L-TSC-KO mice was confirmed by increased phosphorylation of S6 and loss of TSC1 protein (Figure 4B). Activation of mTORC1 was followed by a decrease in fibrotic (Col1a1, Acta2, Pdgfb, and Tgfb1) and inflammatory (Tnfa and Adgre1) gene markers (Figure 4C) and decreased plasma ALT activity, with no significant change in AST (Figure 4D). Following the establishment of NASH, acute deletion of hepatic TSC1 led to a significant improvement in liver collagen deposition as documented by Sirius red staining and fibrosis staging (Figure 4E and F). Consistent with the NASH prevention L-TSC-KO model, improvements of fibrosis were accompanied by a reduction in hepatic steatosis (Figure 4E and G). Similar to the prevention study, the improvements in fibrosis and steatosis were not observed in the L-TSC-KO model, increases in TAG secretion and suppression of DNL (Srebp1c and Fasn) (Figure 4H and I). These improvements in hepatic fibrosis and TAG accumulation occurred independent of changes in body weight (Figure 4J). In summary, these data reveal that acute in vivo activation of hepatic mTORC1 lowered hepatic TAG accumulation by enhancing VLDL-TAG secretion and suppressing DNL, leading to improved hallmarks of NASH in mice.

To expand our dietary models of NASH in mice, we also employed the NASH-GAN diet to evaluate the effect of mTORC1 activity on NASH resolution. Briefly, Tsc1\textsuperscript{lox/lox} were fed the NASH-GAN diet for 30 weeks, injected with AAV, and sacrificed 6 weeks following AAV administration (Figure 5A). Gene expression measurements confirmed successful knockout of Tsc1 in the liver (Figure 5B). Similar to the results obtained in the LMCD-HFD model, mTORC1 activation did not affect body weight of mice 6 weeks after AAV injection (Figure 5C). Hepatic mTORC1 activation improved histological collagen deposition (Figure 5D and E), with similar trends at the gene expression level (Figure 5G). Further, steatosis was improved in L-TSC-KO livers (Figure 5F), which correlated with reduced lipogenic gene expression (Figure 5H). Altogether, these data indicate that in vivo activation of mTORC1 protects from the development and progression of NASH in mice.

mTORC1 Controls Total Hepatic Lipid Content Through Its Regulation of VLDL-TAG Secretion and Lipid Synthesis

To mechanistically define the anti-steatotic, anti-inflammatory, and anti-fibrotic effects resulting from hepatic Tsc1 deletion, we first evaluated the requirement of mTORC1-regulated VLDL-TAG secretion on hepatic steatosis and inflammation in chow-fed mice. VLDL synthesis and secretion are reduced in biopsy-confirmed patients with NASH compared with patients with non-fibrotic NAFL, highlighting

Figure 1. (See previous page). mTORC1 activity is reduced in murine NASH models. A–B, Ten-week-old ob/ob mice were sacrificed following a 6-hour fast. A, Hematoxylin and eosin (H&E) staining, and picrowe (Sirius red) staining from paraffin-embedded, formalin fixed liver sections. B, Immunoblot of liver lysates, p-AKT (Ser473), total AKT2, p-S6 (Ser240/244), total S6, and HSP90. C–D, Eight- to 12-week-old C57BL6/J mice were fed LMCD-HFD (L-amino acid diet with 45 kcal% fat with 0.1% methionine and no added choline) or control diet (L-amino acid diet with 10 kcal% fat with methionine and choline) for 30 days and fasted 6 hours before sacrifice. C, H&E staining and sirius red staining from paraffin-embedded, formalin-fixed liver sections. D, Gene expression of fibrosis and inflammation genes, n = 6. E, Plasma ALT and AST, n = 4. F, Triglyceride secretion determined in 4-hour fasted animals by blocking uptake via i.p. injection of poloxamer 407, n = 3 per group. G, Immunoblot of liver lysates, p-AKT (Ser473), total AKT2, p-S6 (Ser240/244), total S6, p-4E BP1 (Thr37/46), total 4E BP1, and HSP90. H–K, Eight- to 12-week-old C57BL6/J mice were fed GAN diet (40 kcal% fat (mostly palm oil), 20 kcal% fructose and 2% cholesterol) or normal chow diet for 30 weeks. H, H&E staining and pico red (Sirius red) staining from paraffin-embedded, formalin-fixed liver sections in 6-hour fasted mice. I, Triglyceride secretion determined in 4-hour fasted animals by blocking uptake via i.p. injection of poloxamer 407, n = 4–5. J, Immunoblot of liver lysates, p-S6 (Ser240/244), total S6, and HSP90 from 6-hour fasted animals. K, Quantification of immunoblot expression as a phosphorylated protein proportional to total protein, relative to control mice samples. *P < .05; ***P < .001; ****P < .0001 vs control using Student t test, or 2-way analysis of variance for (F and I). Data shown as mean ± standard error of the mean.
the importance of VLDL in maintaining liver lipid homeostasis.\textsuperscript{38} VLDL production and its subsequent secretion of TAG from the liver is controlled, in part, by the biosynthesis of phosphatidylcholine (PC), the main phospholipid coating lipoproteins.\textsuperscript{48} Numerous studies outline the importance of PC in the development of NASH, depicting that PC is

Figure 2. Acute mTORC1 activation prevents steatosis, inflammation, and fibrosis in mice on NASH diet. A, Experimental design schematic: 6- to 10-week-old Tsc1\textsuperscript{loxP/loxP} animals were injected with either AAV-GFP (Control) or AAV-Cre (L-TSC-KO) 2 weeks prior to a 4-week feeding of LMCD-HFD (L-amino acid diet with 45 kcal\% fat with 0.1\% methionine and no added choline, Research Diets #A06071309). B, Representative immunoblots of liver lysates, TSC1, p-S6 (Ser240/244), and total S6. C, Gene expression of fibrosis and inflammation genes, n = 5–6. D, Plasma ALT and AST, n = 5–10. E, Formalin-fixed liver sections stained with hematoxylin and eosin (H&E) (general morphology) and Sirius red (collagen), representative of n = 5–6 mice. F, Fibrosis scoring from 0 (none) to 3 (bridging fibrosis) quantified by blinded investigator, n = 5–6. G, Steatosis scoring ranked from 0 (<5\% steatosis) to 3 (>66\% steatosis) by blinded investigator, n = 5–6. For scoring, images from 3 random fields per mouse liver section are evaluated. H, Hepatic TAG, n = 5–6. I, Triglyceride secretion determined in 4-hour fasted animals by blocking uptake via i.p. injection of poloxamer 407, n = 5 per group. J, Gene expression of lipogenic genes, n = 5–6. K, Final body weight. Data shown as mean ± standard error of the mean. *P < .05; **P < .01; ***P < .001; ****P < .0001 vs control using Student t test, or 2-way analysis of variance (I) and \chi^2 test (F and G).
decreased in biopsy-confirmed patients with NASH compared with healthy controls. In the liver, PC is regulated by its rate-limiting enzyme, CTP:Phosphocholine cytidylytransferase alpha (CCTα). Congenital deletion of liver CCTα protein in chow-fed mice results in hepatic steatosis due to reduced PC synthesis and impaired VLDL-TAG secretion, with no effect on liver TAG synthesis. Human mutations associated with enhanced hepatic PC content are protected from NASH, further suggesting the importance of PC synthesis in the pathophysiology of NAFLD. Recently, our lab demonstrated that mTORC1 is required for CCTα activity and VLDL-TAG secretion. Consistent with this notion, levels of CCTα protein were reduced under constitutive mTORC1-inhibition, such as the deletion of Raptor protein (L-Raptor-KO) (Figure 6A). In contrast, constitutive activation of liver mTORC1 (L-TSC-KO) elevated levels of CCTα protein in both fasted and postprandial states (Figure 6A). Additionally, L-TSC-KO mice displayed an increase in CCTα protein in their diet-induced NASH prevention model (Figure 2A; Figure 6B). These changes in protein levels in L-TSC-KO were not associated with increased Pcyt1α expression, the gene encoding CCTα protein, suggesting mTORC1 positively regulates CCTα posttranscriptionally (Figure 6C). The increase in CCTα protein expression led to a functional increase in PC and phosphatidylethanolamine (PE) (Figure 6D), which supports the enhanced TAG secretion observed in L-TSC-KO mice on NASH (Figure 2F; Fig. 4H) and L-TSC-KO mice on chow diet, as previously observed. Overall, these key findings suggest that mTORC1 activation increases CCTα protein and hepatic PC levels leading to a functional increase in VLDL-TAG export.

To determine the epistatic relationship between CCTα and mTORC1 in the regulation of VLDL-TAG secretion under normal chow conditions, we crossed Pcyt1αloxp/loxp mice with Tscloxp/loxp mice to generate a mouse model lacking both CCTα and TSC1 in the liver (L-CCTαTSC-double knockout [DKO]). Two weeks following AAV administration, we confirmed deletion of hepatic CCTα and TSC1 via immunoblot, which revealed loss of TSC1 and CCTα proteins and successful activation of mTORC1 through the phosphorylation of S6 (Figure 6F). As expected, loss of CCTα in the liver (L-CCTα-KO) completely blocked VLDL-TAG secretion in chow-fed mice (Figure 6G). Likewise, concomitant loss of CCTα and TSC1 reduced VLDL-TAG secretion to a similar extent to that of L-CCTα-KO (Figure 6G). Both L-CCTα-KO and L-CCTαTSC-DKO mice displayed increased fibrotic gene expression (Col1α1 and Timp1), along with similar changes in inflammatory gene expression, apart from Saa1, a surprising finding for chow-fed conditions (Figure 6H). In parallel, L-CCTα-KO exhibited increased levels of hepatic TAG as compared with control mice, whereas L-TSC-KO mice presented with decreased hepatic TAG (Figure 6F). L-CCTαTSC-DKO displayed significant upregulation of hepatic TAG levels. However, this effect did not fully recapitulate the hepatic TAG levels observed in L-CCTα-KO alone, which suggests an additional role for mTORC1 in regulating total hepatic lipid content (Figure 6F).

In support of this notion, lipogenic gene expression (Srebp1c and Fasn) in L-CCTαTSC-DKO livers were downregulated to similar levels of L-TSC-KO when compared with both control littermates and L-CCTα-KO mice that lack constitutive mTORC1 activation (Figure 6J). Collectively, these data reveal that mTORC1 modulates total hepatic lipid content partially through its control of VLDL-TAG secretion, but the regulation of additional lipid synthesis pathways are involved. Nevertheless, these data support the critical importance of PC and VLDL-TAG export for the onset of liver fibrosis and inflammation.

**Deletion of FOXO1 in Livers With Constitutive mTORC1 Activity Restores DNL and Liver TAGs But Does Not Induce Fibrosis or Inflammation**

mTORC1 activity is required for SREBP1c-driven DNL in response to insulin stimulation. However, many groups have elucidated that activation of mTORC1 alone is not sufficient to induce lipogenesis (Figure 7A). In support of those previous studies, we observed decreased expression of key lipogenic genes (Srebp1c and Fasn) in our L-TSC-KO NASH and chow-fed models (Figures 2F; 4I; 5H; and 6F). Additionally, suppression of lipogenic gene expression in chow-fed L-TSC-KO mice correlated with a reduction in total hepatic TAG content in L-TSC-KO mice compared with controls (Figure 6F). The down-regulation of SREBP1c and its targets in L-TSC-KO mice is attributed in part to a negative feedback-inhibition of mTORC2-AKT (Figure 7A).

AKT is both required and sufficient for lipogenesis; however, the mechanisms of AKT-dependent SREBP induction remain debated. Yecies et al suggest that AKT inhibition promotes Insig2α expression, a negative regulator of SREBPs that blocks SREBP cleavage and activation. However, AKT signals through multiple pathways to regulate lipid synthesis, one of which is the phosphorylation and inhibition of the forkheadbox protein 0 (FOXO) transcription factors. Simultaneous deletion of FOXO1 and TSC1 in AKT-deficient mouse livers completely restored lipogenesis in vivo, independent of increased Insig2α expression, suggesting that the AKT-FOXO1 signaling axis may additionally regulate DNL. In our LMCD-HFD-fed models, we observed decreased levels of AKT phosphorylation following mTORC1 activation (Figure 7B). Moreover, we detected increased insulin-like growth factor binding protein-1 (IGFBP-1) protein levels, a direct target of FOXO, suggesting an increase in FOXO activity in L-TSC-KO liver (Figure 7B).

To determine if upregulation of FOXO1 was driving the anti-lipogenic effects of L-TSC-KO under normal chow conditions, Foxo1loxp/loxp/Tsc1loxp/loxp mice were injected with AAV8-TBG-Cre (L-FOXOTSC-DKO), or -GFP (Control) 2 weeks prior to sacrifice. Deletion of hepatic Foxo1 and Tsc1 were confirmed by gene expression analysis (Figure 7C). Loss of FOXO1 under conditions of constitutive mTORC1 activation (L-FOXOTSC-DKO) induced the mRNA expression of Srebp1c, despite significantly high levels of Insig2α expression (Figure 7D). The changes in lipogenic gene program correlated with a trend in increase in total liver lipid content (Figure 7E) and de novo palmitate synthesis.
Figure 3. mTORC1 activation does not affect glucose and insulin sensitivity, fatty acid oxidation, and autophagy. A–E, Six- to 10-week-old Tsc1loxP/loxP animals were injected with either AAV-GFP (Control) or AAV-Cre (L-TSC-KO) 2 weeks prior to a 4-week feeding of LMCD-HFD. A, Blood glucose measured ad libitum. B, Plasma insulin measured ad libitum. C, Intraperitoneal glucose tolerance test (2 g/kg) on overnight fasted mice, n = 4–5. D, Insulin levels before and after 15 minutes following glucose injection. E, Circulating TAG in 6-hour fasted mice. F, Gene expression of fatty acid oxidation-related genes. G, Circulating β-hydroxybutyrate in 6-hour fasted mice. H, Gene expression of apolipoprotein genes. I, Gene expression of autophagy-related genes. J, Immunoblot of p62 from liver lysates. Quantification normalized to loading control (HSP90) and presented as relative to control (lanes 1-4). ***P < .001 vs control using Student t test. Data shown as mean ± standard error of the mean.
VLDL-TAG export are also required to induce hepatic
lipolysis in NAFLD but that defects in PC biosynthesis and
expression of lipogenic genes, n
0.01; ***

(Figure 7F) in L-FOXOTSC-DKO mice. Furthermore, we
observed an induction of Gck (Figure 7D), the gene encoding
glucokinase, the primary hexokinase and glucose sensor in
the liver that is repressed by FOXO proteins and stimulated
by SREBP1c.

The restoration of lipogenic gene expression correlated with increased hepatic TAG content
(Figure 7G). However, the increase in lipogenic gene expression and hepatic TAG content following inhibition of
FOXO1 alone was not sufficient to induce fibrosis and inflammation (Figure 7H). These data support a hypothesis
that DNL significantly contributes to hepatic TAG accumulation in NAFLD but that defects in PC biosynthesis and
VLDL-TAG export are also required to induce hepatic fibrosis and inflammation.

Exogenous SREBP1c Expression Rescues DNL in Mice With Constitutive Liver mTORC1 Activity

To assess the direct contribution of DNL in our L-TSC-KO models without affecting additional FOXO1-dependent
mechanisms, we injected chow-fed mice with a liver-specific AAV containing the nuclear form of SREBP1c
(AAV8-TBG-nSREBP1c). SREBPs are membrane-bound transcription factors that are activated via cleavage by SREBP cleavage activating protein, leading to its subsequent translation to the nucleus.

Notably, expression of AAV8-TBG-nSREBP1c significantly increased total liver lipid and de novo lipogenesis in mice challenged to a lipogenic diet (70% kcal carbohydrate, 10% kcal fat) (Figure 8A and B). Next, we co-administered the AAV8-TBG-nSREBP1c into Tsc1fl/fl mice in combination with either AAV-GFP (+nSREBP1c) or AAV-Cre (L-TSC-KO + nSREBP1c). Exogenous nSREBP1c successfully promoted expression of key lipogenic genes, Srebp1c, Fasn, and Acaca, to supraphysiologic levels in control and L-TSC-KO livers (Figure 8C). Analogously, nSREBP1c overexpression led to an increase in hepatic TAG content in both +nSREBP1c and L-TSC-KO + nSREBP1c cohorts (Figure 8D). Consistent with our L-FOXOTSC-DKO model, re-establishment of the DNL program in Tsc-deficient mice did not induce fibrosis and inflammation gene markers (Figure 8E). Lastly, we observed no additive effects of nSREBP1c on TAG secretion in L-TSC-KO mice (Figure 8F), indicating mTORC1 activation controls TAG secretion via a parallel pathway. Therefore, consistent with FOXO1 ablation, nSREBP1c expression alone restored the lipogenic gene profile and total hepatic TAG content without any indication of increased hepatic fibrosis or inflammation.

Inhibition of VLDL-TAG Secretion Promotes Fibrosis in NASH-fed Mice Under Constitutive mTORC1 Activation

Based on our finding that inhibition of CCTa promoted fibrosis and inflammation in chow-fed mice and restoring
DNL did not affect fibrotic gene expression, we hypothesized that DNL significantly contributes to hepatic TAG accumulation in NAFLD but that defects in PC biosynthesis and VLDL-TAG export are critical for the induction of hepatic fibrosis and inflammation in NASH. Therefore, we tested whether restoration of DNL or inhibition of VLDL-TAG secretion under constitutive mTORC1 activity would prevent the onset of fibrosis in rodents fed LMCD-HFD NASH-promoting diet. To do so, Pcyt1afl/floxpTsc1fl/floxp and Foxo1fl/floxpTsc1fl/floxp and mice were subjected to the LMCD-HFD for 4 weeks following injection of either AAV-Cre (L-FOXOTSC-DKO or L-CCTaTSC-DKO, respectively) or GFP -(Control) (Figure 9A). Excision of these genes in the liver were confirmed by decreased mRNA expression (Figure 9B). Remarkably, we found L-CCTaTSC-DKO mice on NASH diet had increased incidence and staging of fibrosis; however, they were still somewhat protected from steatosis compared with littermate controls on NASH diet (Figure 9C–E). These findings correlated with decreased VLDL-TAG secretion in L-CCTaTSC-DKO (Figure 9F). Next, we tested the effects of inhibiting FOXO1 in mice with constitutive mTORC1 activation to restore the lipogenic gene program (L-FOXOTSC-DKO) (Figure 9A). Surprisingly, we found no difference in fibrosis but increased levels of steatosis in L-FOXOTSC-DKO mice compared with NASH diet-fed controls (Figure 9G–I). Increased steatosis was driven by a complete restoration of the DNL gene expression (Srebp1c, Fasn, Acaca) (Figure 9J) and de novo palmitate synthesis (Figure 9K). In sum, we observed that acute mTORC1 activation prevented the development of NASH in mice, and inhibition of VLDL-TAG secretion in these mice accelerates fibrosis, but restoring DNL alone does not affect fibrosis progression.

Discussion

Data presented in this manuscript indicate that mTORC1 activity is reduced in murine NASH livers and that activation

(Figure 4). (See previous page). Acute mTORC1 activation improves steatosis, inflammation, and fibrosis in mice on
NASH diet. A, Experimental design schematic: 6- to 10-week-old Tsc1fl/floxp mice were fed LMCD-HFD (L-amino acid diet
with 45 kcal% fat with 0.1% methionine and no added choline, Research Diets #A06071309) for 4 weeks, then injected with
either AAV-GFP (Control) or AAV-Cre (L-TSC-KO) and continued diet regimen for 4 more weeks. B, Representative immu-
noblots of liver lysates, TSC1, p-S6 (Ser240/244), and total S6. C, Gene expression of fibrosis and inflammation genes, n = 5
per group. D, Plasma ALT and AST, n = 6–7. E, Formalin-fixed liver sections stained with hematoxylin and eosin (H&E) (general
morphology) and Sirius red (collagen), representative of n = 5–6 mice. F, Fibrosis scoring from 0 (none) to 3 (bridging fibrosis)
quantified by blinded investigator, n = 5–6. G, Steatosis scoring ranked from 0 (<5% steatosis) to 3 (>66% steatosis) by
blinded investigator, n = 5–6. For scoring, images from 3 random fields per mouse liver section are evaluated. H, Triglyceride
secretion determined in 4-hour fasted animals by blocking uptake via i.p. injection of poloxamer 407, n = 5 per group. I, Gene
expression of lipogenic genes, n = 5. J, Final body weight. Data shown as mean ± standard error of the mean. *P < .05; **P < .01; ***P < .001; ****P < .0001 vs control using Student t test, except 2-way analysis of variance (H), and χ² test (F and G).
Figure 5. Acute mTORC1 activation reverses steatosis, inflammation, and fibrosis in GAN diet-fed mice. A, GAN diet intervention schematic: 6- to 10-week-old Tsc1loxp/loxp animals were fed a GAN diet (40 kcal% fat [mostly palm oil], 20 kcal% fructose, and 2% cholesterol, Research Diets #D09100310) for 30 weeks prior to AAV-GFP (Control) or AAV-Cre (L-TSC-KO) injection. Animals continued GAN diet feeding for 6 weeks after AAV injection. B, Tsc1 gene expression. C, Final body weight. D, Formalin-fixed liver sections stained with hematoxylin and eosin (H&E) (general morphology) and Sirius red (collagen), representative of n = 5–6 mice. E, Fibrosis scoring from 0 (none) to 3 (bridging fibrosis) quantified by blinded investigator, n = 4–5. F, Steatosis scoring ranked from 0 (<5% steatosis) to 3 (>66% steatosis) by blinded investigator, n = 4–5. For scoring, images from 3 random fields per mouse liver section are evaluated. G, Gene expression of inflammation and fibrosis genes. H, Gene expression of lipogenic genes. *P < .05; ****P < .0001 vs control using student t test (B, C, G, H) and χ² test (E, F). Data shown as mean ± standard error of the mean.
of hepatic mTORC1 protects against and reverses NASH in mice through dual promotion of VLDL-TAG secretion and suppression of DNL. This key finding is in agreement with previous studies that observed increased AKT and mTORC1 activation in models of ob/ob (Figure 1A) and diet-induced obesity, in which enhanced DNL is a key feature of patients with early-stage NAFLD.\textsuperscript{13-15,35,39,40} These data are consistent with studies that observed enhanced liver damage and cell death upon chronic mTORC1 inhibition in mice and align with published studies that demonstrate mTORC1 activity is reduced in fibrotic regions of the liver in mice with NASH.\textsuperscript{65,67} Taken together, we postulate that mTORC1 activity has a differential role in the initiation and progression of NAFLD such that its activity is enhanced under insulin-resistant, steatotic conditions, but reduced as NAFLD progresses with increases in fibrosis and inflammation. In this study, we provide new evidence that mTORC1 activity is reduced in 2 models of murine NASH and that activation of mTORC1 under these conditions may be hepatoprotective, but TAG export may be a critical regulator of the onset and progression of fibrosis.

Mechanistically, we identify 2 key pathways responsible for the protection from NASH by mTORC1. First, we demonstrate that constitutive mTORC1 activation suppresses lipogenic gene expression. As previously described, enhanced DNL is a key feature of NAFLD, making the lipogenic pathway a desirable target for the treatment of NAFLD as it protects from steatosis and thus the development of fibrosis and inflammation. Central to this is the mTORC1 signaling pathway and the subsequent activation of several downstream factors required for induction of SREBP-mediated lipogenesis.\textsuperscript{65,66} However, mTORC1 activity alone is not sufficient for the induction of DNL, as reported by several groups.\textsuperscript{28,30,32} Accordingly, models recapitulating constitutive mTORC1 activation observed suppression of hepatic DNL and lipogenic gene expression.\textsuperscript{28,31,32} This phenomenon is attributed to a negative feedback inhibition of the mTORC2-AKT axis (Figure 7A).\textsuperscript{20,67,68} mTORC2 phosphorlylates and activates AKT at Ser473.\textsuperscript{69} Along these lines, we detected decreased levels of AKT activity (Figure 7B), which we attribute to a mTORC1-mediated negative feedback regulation loop. These observations are consistent with impaired AKT activation in congenital models of TSC-deficiency, reversed by short-term treatment with mTORC1 inhibitor, rapamycin.\textsuperscript{31} As such, AKT is both required and sufficient for the induction of lipogenesis.\textsuperscript{29,56} However, the mechanisms of AKT-dependent lipogenesis are still debated.

Studies by the Manning group show that AKT inhibits Insig2a in primary hepatocytes, the gene encoding INSIG2, an inhibitory protein of SREBP, independent of changes to mTORC1 activity.\textsuperscript{25} However, work from our laboratory suggests that inhibition of FOXO1 in AKT-deficient livers is sufficient to restore SREBP activity and lipogenesis \textit{in vivo}, independent of changes in INSIG2.\textsuperscript{30} Moreover, deletion of FOXO1,3,4 in murine livers induces glucokinase and SREBP expression, which, in turn, stimulates DNL\textsuperscript{57,70} Therefore, we postulate that inhibition of FOXO1 is required for the AKT-dependent regulation of DNL induction, as we detected a similar restoration of Srebp1c and Gck following the deletion of FOXO1 in L-TSC-KO livers. In our chow-fed L-FOXOTSC-DKO model, hepatic Srebp1c expression is rescued despite an induction of Insig2a. Nonetheless, we observed a similar impairment of AKT activation and DNL induction in L-TSC-KO mice under both chow and NASH-fed conditions. Decreased AKT activity correlated with a suppression of lipogenic gene panel and a decrease of hepatic TAG in L-TSC-KO mice. Interestingly, \textit{in vivo} experiments that restored lipogenic gene expression and hepatic TAG content were not sufficient to induce inflammation nor drive fibrosis. Altogether, we show that mTORC1 has a biphasic role throughout NAFLD progression. In early stages of NAFLD, mTORC1 activity is elevated, correlating with increase in lipid accumulation. Thus, we predict that inhibition of mTORC1 activity in early NAFL will be beneficial in the protection against the enhanced DNL program, whereas inhibition of mTORC1 activity in progressed NASH may be undesirable.

The molecular pathways involved in NAFLD pathogenesis are heavily debated. Our data support previous studies that indicate TAG export and clearance from the liver are crucial regulators of NASH initiation and progression.

\textbf{Figure 6. (See previous page).} mTORC1 controls total hepatic lipid content through its regulation of VLDL-TAG secretion and lipogenic genes. A. Eight- to 10-week-old Rptor$^{loxp/loxp}$ and Tsc1$^{loxp/loxp}$ animals were injected with either AAV-GFP (Control) or AAV-Cre (L-Raptor-KO) or L-TSC-KO) 2 weeks prior to sacrifice. Representative immunoblots of liver lysates from 16-hour fasted mice (Chow: –) followed by a 4-hour refeed (Chow: +). CCA$	extsuperscript{a}$, p-S6 (Ser240/244), HS90. CCA$	extsuperscript{a}$ protein quantification normalized to loading control (HS90) and presented as relative to control fasted condition (lane 1). B, (Figure 2A) Six- to 10-week-old Tsc1$^{loxp/loxp}$ animals injected with either AAV-GFP (Control) or AAV-Cre (L-TSC-KO) 2 weeks prior to a 4-week feeding of LMCSD-HFD. A, Immunoblot of livers from NASH prevention mice. C, Gene expression of Pcyt1a from chow-fed mice described in (A) n = 4-6. D, PC and phosphatidylethanolamine (PE) measured by high performance liquid chromatography from NASH mice described in (B). E-H, Eight- to 10-week-old Tsc1$^{loxp/loxp}$ animals were injected with either AAV-GFP (Control) or AAV-Cre (L-TSC-KO) and Pcyt1a$^{loxp/loxp}$, Tsc1$^{loxp/loxp}$, and L-TSC-KO animals were injected with either AAV-GFP (Control) or AAV-Cre (L-CCTaTSC-DKO or L-CCTa-KO, respectively) 2 weeks prior to sacrifice. All animals remained on normal chow diet regimen. E, Triglyceride secretion determined in 4-hour fasted animals by blocking uptake via i.p. injection of poloxamer-407, n = 3-5. F, Representative immunoblots of liver lysates from 6-hour-fasted mice, n = 3-4. CCA$	extsuperscript{a}$, TSC1, p-S6 (Ser240/244). HSP90. G, Triglyceride secretion, n = 7; L-CCTa-KO, n = 3. H, Gene expression of inflammation and fibrosis genes, n = 7 per group; L-CCTa-KO, n = 4. I, Hepatic triglycerides of 6-hour fasted mice, n = 6-13. J, Gene expression of lipogenic genes, n = 6-11. Data shown as mean ± standard error of the mean. *P < .05; **P < .01; ***P < .001; ****P < .0001 vs control or as indicated using Student $t$ test (C, D), 2-way analysis of variance (E, G, H, J), or 1-way analysis of variance (I).
Figure 7. Deletion of FOXO1 in livers with constitutive mTORC1 activity restores DNL and liver TAGs but does not induce fibrosis or inflammation. A, Schematic: insulin stimulates the activation of AKT/PKB. AKT phosphorylates and inhibits the TSC complex, leading to the activation of mTORC1. mTORC1, which is recognized by its Raptor protein component, is required and sufficient for de novo lipogenesis and PC biosynthesis. AKT is required for induction of DNL through an mTORC1-independent pathway. However, mTORC1 negatively inhibits AKT activity, and thus activation of mTORC1 leads to a substantial decrease in AKT phosphorylation and activation. Nutrients, growth factors, amino acids, and other stimulants may activate mTORC1 independent of the insulin/AKT/TSC pathway. B, Immunoblot of livers of p-AKT (Ser473) from NASH prevention mice (Figure 2A): 6- to 10-week-old Tsc1lox/lox animals injected with either AAV-GFP (Control) or AAV-Cre (L-TSC-KO) 2 weeks prior to a 4-week feeding of LMCD-HFD. C–H, Eight- to 10-week-old Foxo1lox/loxTsc1lox/lox and Foxo1lox/lox animals were injected with either AAV-GFP (Control) or AAV-Cre (L-FOXOTSC-DKO or L-FOXO1-KO, respectively) for 2 weeks prior to sacrifice. C, Gene expression of Foxo1 and Tsc1, n = 4–5. D, Gene expression of lipogenic genes, n = 4–5. E, Total hepatic palmitate following 4-hour refeed. F, Palmitate synthesis following 4-hour refeed. G, Hepatic triglycerides of 6-hour fasted mice, n = 5–10. H, Gene expression of inflammation and fibrosis markers, n = 5–6. Data shown as mean ± SEM. *P < .05; **P < .01; ***P < .001; ****P < .0001 vs control; ###P < .001; ####P < .0001 L-FOXOTSC-DKO vs L-FOXO1-KO using Student t test (E, F), 1-way analysis of variance (G), or 2-way analysis of variance (C, D, H).
Clinical studies in humans found VLDL-TAG synthesis and secretion are reduced in patients with fibrotic NASH livers compared with patients with fatty liver, implicating the role of VLDL in the progression to fibrosis.38 PC is the main phospholipid coating lipoproteins and is a critical determinant of VLDL-TAG export from the liver.48,71 Patients that received total parenteral nutrition excluding choline developed steatosis, which was reversed with choline supplementation, indicating the importance of choline and PC in maintaining hepatic lipid homeostasis.72 Wild-type mice challenged to a high-fat diet (HFD) developed steatosis; however, mice that lack hepatic CCTα develop NASH within 1 week of HFD feeding, attributed to the decrease in hepatic PC ratios and blunting of TAG export.48 Along these lines, several groups have mapped the lipid profiles of NAFLD in clinical studies and found that hepatic PC content is negatively correlated with increased severity of NAFLD.49,51,54,72-76 Early rodent studies measuring PC and another major phospholipid, phosphatidylethanolamine (PE), identified a correlation between liver damage and decreased PC to PE ratio, implicating PC and PE in the progression of NAFLD.79 In this present study, we detect increased PC and PE in L-TSC-KO mice that are protected from NASH.

In human studies, carriers of the 1148M loss-of-function mutation in patatin-like phospholipase domain–containing protein 3 (PNPLA3) have an increased risk of developing NAFLD, which correlates with decreased PC synthesis and increased hepatic TAG content.75,77 Furthermore, variants that are protective against NASH have increased hepatic PC content. First, carriers of the protein-truncating variant (rs72613567T>TA) in the hepatic lipid droplet protein hydroxysteroid 17-β dehydrogenase 13 protein, are enriched with liver PC and are protected from fibrosis.54,55 Second, a coding variant (rs2642438G>A) in mitochondrial amidoxime reducing component 1 (MARCl1) gene associates with decreased severity of NAFLD, correlating with increased plasma TAG and hepatic PC content.73,74 Additionally, an E167K variant in the TM6SF2 gene correlates with abnormalities in ApoB trafficking and VLDL secretion, leading to an increased risk of NAFLD and progression to NASH.76,78,79 Lastly, mutations in MTTP (rs2306986), a protein required for VLDL assembly, was associated with higher susceptibility of NAFLD.80,81 Collectively, these studies support the hypothesis that defects in hepatic PC content and VLDL-TAG secretion significantly contribute to NASH progression. In our study, we demonstrate that suppression of VLDL-TAG secretion, and not solely the induction of the lipogenic programming, was sufficient to induce markers of fibrosis and inflammation in chow-fed and NASH diet conditions. Taken together, hepatic TAG export may play a key role in the induction of inflammation and fibrosis. Future lipidomic studies will profile both hepatic and circulating lipids to define how hepatic mTORC1 controls specific species and classes of TAGs and phospholipids.

Although our findings shed light on the role of mTORC1 in NASH and we provide evidence that VLDL-TAG secretion is critical for the promotion of fibrosis, the primary cause of death of patients with NASH is cardiovascular disease82; therefore, the cardiovascular impacts of mTORC1 activation in mice should be further evaluated before determining the therapeutic efficacy. Furthermore, in this study, we utilize mouse models with constitutive liver mTORC1 activation through deletion of TSC1 in an acute and inducible setting. Although long-term deletion of TSC is associated with tumorigenesis,83 and directly inhibiting the TSC complex in human patients is not therapeutically attractive, our study provides several key mechanistic insights into the role of mTORC1 signaling responsible for driving the initiation and progression of NASH in mice. Moreover, our data are consistent with the hypothesis that insulin signaling via AKT is intact throughout the NAFL to NASH transition, consistent with elevated hepatic lipid synthesis. In early stages of simple steatosis, mTORC1 is elevated to support lipid synthesis and export. In more advanced liver disease such as NASH, mTORC1 activity is reduced, which may lead to decreased phosphatidylcholine synthesis and VLDL-TAG export, thereby promoting inflammation and fibrosis. Future studies will aim to identify novel ways to selectively modify mTORC1 activity to mimic its downstream protective effects in NASH, potentially through its role in regulating PC biosynthesis, VLDL-TAG secretion, and suppressive effects on lipogenesis. In addition, the activity status of liver mTORC1 in human NAFL and NASH needs to be determined. In summary, this study provides novel insights for the role of liver mTORC1 signaling in the pathogenesis of NAFLD.

Methods

Animal Experiments

Rptorlox/lox, Tsc1lox/lox, Pcyt1αlox/lox, and Foxo1lox/lox mice were backcrossed to the C57BL/6 background,24,53,84 housed and bred under specific pathogen-free conditions in facilities at the University of Pennsylvania. Twelve-week-old B6-Lepob homoygotes (referred to here as ob/ob) were purchased from Jackson Laboratories (#00632), and B6-Lepob heterozygotes were used as controls (ob/+). For acute excision of liver-specific genes, mice were injected with adeno-associated virus (Vector Core, University of Pennsylvania) containing a liver-specific TBG promoter serotype 8 (AAV8-TBG) carrying Cre recombinase, at a dosage of 1.0 × 1011 genome copies at 6 to 10 weeks of age and maintained on respective feeding regimens (Figure 2A; 4A; 5A; and 9A). (AAV8-TBG-nSREBP1c virus was a kind gift from Dr Mitchell Lazar (University of Pennsylvania). All mice were fed chow diet (Labdiet, #5010) unless specified otherwise. Mice fed the LMCD-HFD were given L-Amino Acid diet with 45 kcal% fat with 0.1% methionine and no added choline (Research Diets, #A06071309). Mice fed control diets were given L-Amino Acid diet with 10 kcal% fat with methionine and choline (Research Diets, A06071314). Mice fed the GAN diet were fed rodent diet with 40 kcal% fat (mostly palm oil), 20 kcal% fructose, and 2% cholesterol (Research Diets, #D09100310). Mice fed high-carbohydrate diets were fed rodent diet with 10 kcal% fat (Sucrose Diet) (Research Diets, #D124508B). All animal diets were irradiated. Control animals consist of pools of the appropriate floxed mice for each experiment (Rptorlox/
loxp, Tsc1^{loxp/loxp}, Pcyt1a^{loxp/loxp}, Pcyt1a^{loxp/loxp}Tsc1^{loxp/loxp}, Foxo1^{loxp/loxp}, and Foxo1^{loxp/loxp}Tsc1^{loxp/loxp} that were injected with AAV8-TBG-GFP. Mice that were co-injected with AAV8-TBG-nSREBP1c and AAV8-TBG-Cre received 1.0 x 10^{11} genome copies of respective virus for a total of 2.0 x 10^{11} genome copies in a singular injection. Consistently, control mice were injected with 2.0 x 10^{11} genome copies of AAV8-TBG-GFP. All experiments were performed in male mice.

In Vivo Triglyceride Secretion

Triglyceride secretion was measured in vivo by intraperitoneal (i.p.) injection of 1 mg/kg poloxamer-407 in sterile-saline following a 4-hour fast, as previously described. Accumulation of triglycerides in the serum were measured with Infinity Triglycerides (Thermo Scientific, TR22421).

**Metabolic Measurements**

For experiments involving fasting, mice were deprived of food overnight for 16 hours. For refeeding experiments, mice were overnight fasted and refed normal chow for 4 hours before blood and liver samples were collected. Plasma insulin measurements (Ultra-Sensitive Rat Insulin ELISA kit, Crystal Chem, Inc) were performed on plasma collected from mice ad libitum or 6-hour fasted. Plasma AST and ALT were measured through IDEXX VetTEST Chemistry.
Analyzer. For phospholipid and TAG quantifications, liver homogenate equivalent to 1 mg protein was extracted in the presence of a phosphatidyldimethylethanolamine internal standard using a modified Folch method. Phosphatidylcholine, phosphatidylethanolamine, TAG, free cholesterol, and cholesterol-ester were measured by high performance
Glucose Tolerance Test
Overnight fasted mice were injected with 2 g/kg of glucose solution intra-peritoneally. Blood was collected and blood glucose was measured at 0, 15, 30, 60, and 120 minutes after glucose injection using OneTouch Ultra (LifeScan) glucose strips.

Immunoblotting
Protein lysates were prepared from frozen livers or in a modified RIPA buffer with Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich) and Complete Protease Inhibitor Cocktail (Roche), as described previously. Samples were separated on 4% to 15% Mini-PROTEAN TGX pre-cast gels. The following primary antibodies were used for immunoblotting: p-Akt (CST #4060), Akt2 (CST #2964), p-S6 (CST #4874), Blots were developed using LI-COR Odyssey Imaging System and quantified relative to total protein controls using Image Studio Lite (LI-COR).

mRNA Isolation and Real-Time Polymerase Chain Reaction (PCR)
Total RNA was isolated from frozen livers using the RNeasy Plus kit (Qiagen). Complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase, and the expression of the genes of interest was quantified by real-time PCR using the SYBR Green dye-based assay via ΔΔCT method, relative to TATA-box-binding protein.

Histology
Livers were fixed in 10% buffered formalin overnight, dehydrated in ethanol, paraffin-embedded, and sectioned. Sections were stained with hematoxylin and eosin or picro red stain 0.1% direct red 80 (#365548, Sigma) plus 0.1% fast green FCF (ab146267, Abcam). Slides were scored by a masked investigator for steatosis and stage of fibrosis. Steatosis, referred to as amount of surface area involved by steatosis as evaluated on low- to medium-power examination, as previously described, on a scale of 0 (<5%), 1 (5%–33%), 2 (>33%–66%), and 3 (>66%). Fibrosis stage were evaluated as previously described, on a scale of 0 (None), 1 (perisinusoidal or periportal), 1A (mild, zone 3, perisinusoidal defined as “delicate” fibrosis), 1B (moderate, zone 3, perisinusoidal defined as “dense” fibrosis), 1C (portal/periportal with portal and/or periportal fibrosis without accompanying pericellular/perisinusoidal fibrosis), 2 (perisinusoidal and portal/periportal), 3 (bridging fibrosis), and 4 (cirrhosis).

De Novo Lipogenesis Measurement
The amount of newly made hepatic lipid was determined as previously described. Briefly, mice were fed high-carbohydrate diet (Research Diets #D12450B) for 2 days and fasted overnight. Following the overnight fast, mice were fed for 3 hours then injected i.p. with D2O and allowed access to food for a following 3 hours. Plasma and liver samples were collected 6 hours post-feeding. Palmitate was analyzed using GC/MS, and the absolute amount of newly made palmitate was assumed equivalent to the DNL rate.

Statistical Analysis
Statistical analysis was performed using 1-way analysis of variance when more than 2 groups were compared, 2-way analysis of variance when 2 or more conditions were analyzed followed by Sidak’s multiple comparisons test (2 groups) or Tukey multiple comparisons test (>2 groups), and unpaired 2-tailed Student t test when 2 groups were being assayed. All data were presented as mean ± standard error of the mean. A single asterisk indicates P value < .05, double asterisks indicate P value < .01, triple asterisks indicate P value < .001, and four asterisks indicate P value < .0001 vs control.

Study Approval
The institutional animal care and use committees of the University of Pennsylvania approved all the animal studies, which adhered to the National Institutes of Health guidelines for the care and use of laboratory animals.

Figure 9. (See previous page). Inhibition of VLDL-TAG secretion promotes fibrosis in NASH-fed mice under constitutive mTORC1 activation. A, Experimental design schematic: 6- to 10-week-old Foxo1lox/lox/Tsc1lox/lox and Pcyt1alox/lox/Tsc1lox/lox Pcyt1alox/lox animals were fed LCMC-HFD (L-amino acid diet with 45 kcal% fat with 0.1% methionine and no added choline, Research Diets #A06071309) for 4 weeks, then injected with either AAV-GFP (Control) or AAV-Cre (L-FOXOTSC-DKO or L-CCTaTSC-DKO, respectively) and continued diet regimen for 4 more weeks. B, Gene expression of Tsc1 and Pcyt1a or Foxo1 n = 4–5. C, Formalin-fixed liver sections stained with hematoxylin and eosin (H&E) (general morphology) and Sirius red (collagen), representative of n = 3–5 mice. D, Fibrosis scoring from 0 (none) to 3 (bridging fibrosis) quantified by blinded investigator, n = 3–4. E, Steatosis scoring ranked from 0 (<5% steatosis) to 3 (>66% steatosis) by blinded investigator, n = 3–4. For scoring, images from 3 random fields per mouse liver sections were evaluated. F, Triglyceride secretion determined in 4-hour fasted animals by blocking uptake via i.p. injection of poloxamer 407, n = 3–5. G, Formalin-fixed liver sections stained with H&E (general morphology) and Sirius red (collagen), representative of n = 4 mice. H, Fibrosis scoring, n = 4 mice. I, Steatosis scoring, n = 4 mice. J, Gene expression of lipogenic genes, n = 3–4. K, Palmitate synthesis following 4-hour refeed. Data shown as mean ± SEM. *P < .05; **P < .01; ***P < .001; ****P < .0001 vs control using Student t test (B, J, K), except 2-way analysis of variance (H), and χ² test (D, E, H, I).
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