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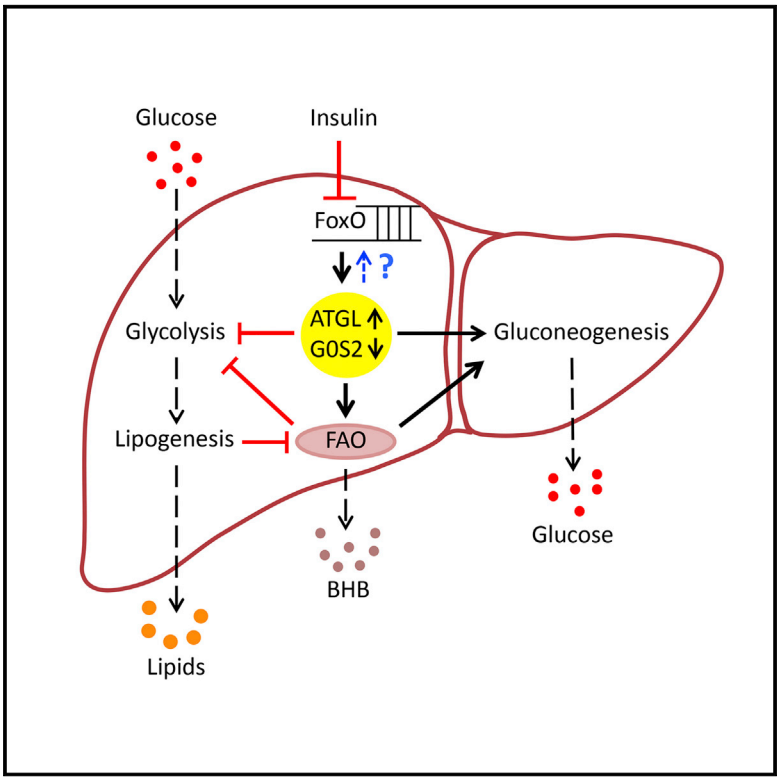
Integrated Regulation of Hepatic Lipid and Glucose Metabolism by Adipose Triacylglycerol Lipase and FoxO Proteins

Graphical Abstract



Highlights

- FoxO1 regulates expression of ATGL lipase and G0S2, its inhibitor, in the liver
- FoxO1 promotes intrahepatic lipolysis and FAO via ATGL-dependent effects
- FoxO1 effects on glucokinase and PEPCK gene expression are ATGL dependent
- ATGL-dependent lipolysis regulates glucose/lipid metabolism downstream from FoxO

Authors

Wenwei Zhang, So Young Bu, Mara T. Mashek, ..., Christopher B. Newgard, Douglas G. Mashek, Terry G. Unterman

Correspondence

dmashek@umn.edu (D.G.M.), unterman@uic.edu (T.G.U.)

In Brief

FoxO proteins are major targets of insulin that regulate hepatic glucose metabolism. Zhang et al. report that FoxO proteins also promote triacylglycerol catabolism through regulation of ATGL and its inhibitor, G0S2, and that ATGL-dependent lipolysis plays an important role in mediating the effects of FoxO on liver glucose and lipid metabolism.

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Integrated Regulation of Hepatic Lipid and Glucose Metabolism by Adipose Triacylglycerol Lipase and FoxO Proteins

Wenwei Zhang,[1,2] So Young Bu,[3,4] Mara T. Mashek,[3,4] InSug O-Sullivan,[1,2] Zakaria Sibai,[1,2] Salmaan A. Khan,[3,4] Olga Ilkayeva,[5,6,7] Christopher B. Newgard,[5,6,7] Douglas G. Mashek,[3,4,*] and Terry G. Unterman[1,2,*]
[1]Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA
[2]Medical Research Service, Jesse Brown VA Medical Center, Chicago, IL 60612, USA
[3]Department of Biochemistry, Molecular Biology and Biophysics
[4]Division of Diabetes, Endocrinology and Metabolism, Department of Medicine
University of Minnesota, Minneapolis, MN 55455, USA
[5]Sarah W. Stedman Nutrition and Metabolism Center
[6]Department of Pharmacology
[7]Department of Medicine
Duke University, Durham, NC 27710, USA
*Correspondence: dmashek@umn.edu (D.G.M.), unterman@uic.edu (T.G.U.)
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SUMMARY

Metabolism is a highly integrated process that is coordinately regulated between tissues and within individual cells. FoxO proteins are major targets of insulin action and contribute to the regulation of gluconeogenesis, glycolysis, and lipogenesis in the liver. However, the mechanisms by which FoxO proteins exert these diverse effects in an integrated fashion remain poorly understood. We report that FoxO proteins also exert important effects on intrahepatic lipolysis and fatty acid oxidation via the regulation of adipose triacylglycerol lipase (ATGL), which mediates the first step in lipolysis, and its inhibitor, the G0/S1 switch 2 gene (G0S2). We also find that ATGL-dependent lipolysis plays a critical role in mediating diverse effects of FoxO proteins in the liver, including effects on gluconeogenic, glycolytic, and lipogenic gene expression and metabolism. These results indicate that intrahepatic lipolysis plays a critical role in mediating and integrating the regulation of glucose and lipid metabolism downstream of FoxO proteins.

INTRODUCTION

FoxO transcription factors are major targets of insulin action. Insulin stimulates the phosphorylation of three residues in FoxO proteins by Akt, corresponding to T24, S256, and 319 in human FoxO1. Phosphorylation at these sites promotes the binding of 14-3-3 proteins, which mask the DNA-binding domain and nearby nuclear localization signals, thereby disrupting the effects of FoxO proteins on gene expression and promoting their sequestration in the cytoplasmic compartment (Brunet et al., 1999; Zhao et al., 2004). In the liver, FoxO proteins contribute to the regulation of multiple metabolic pathways involved in the response to fasting, including gluconeogenesis (GNG), glycolysis, and lipogenesis (Haeusler et al., 2014; Xiong et al., 2013; Zhang et al., 2006, 2012), and suppressing hepatic FoxO1 function is critical for the ability of insulin to regulate hepatic glucose production and maintain glucose homeostasis (Dong et al., 2008; O-Sullivan et al., 2015; Titchenell et al., 2015). However, little is known regarding the role of FoxO proteins in regulating triacylglycerol (TAG) catabolism in the liver.

FoxO proteins promote TAG catabolism in adipose tissue by stimulating the expression of adipose TAG lipase (ATGL) (Chakrabarti and Kandror, 2009), which mediates the first step in lipolysis, after which other lipases, including hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL), promote the removal of additional fatty acids from the glycerol backbone of TAG (Coleman and Mashek, 2011; Zechner et al., 2009). Initially identified in adipose tissue (Zimmermann et al., 2004), ATGL plays an important role in regulating TAG turnover in many tissues. In the liver, overexpressing ATGL decreases TAG content and promotes fatty acid oxidation (FAO) (Ong et al., 2011; Turpin et al., 2011). Conversely, disrupting hepatic ATGL expression promotes steatosis while improving glucose tolerance and insulin sensitivity (Kienesberger et al., 2009; Ong et al., 2013), indicating that regulating intrahepatic TAG catabolism may be important for the ability of insulin to maintain glucose homeostasis. FoxO proteins interact with response elements in the ATGL promoter and stimulate ATGL expression in adipose tissue (Chakrabarti and Kandror, 2009). However, the role of FoxO proteins in regulating hepatic ATGL expression and activity, and the role of ATGL in mediating effects of FoxO proteins on other aspects of metabolism in the liver, are less clear.

ATGL activity is inhibited by interaction with the G0/G1 switch-2 protein (G0S2) (Yang et al., 2010). Consistent with its role as a physiologically relevant inhibitor of ATGL, altering G0S2 expression in the liver is associated with changes in TAG content and

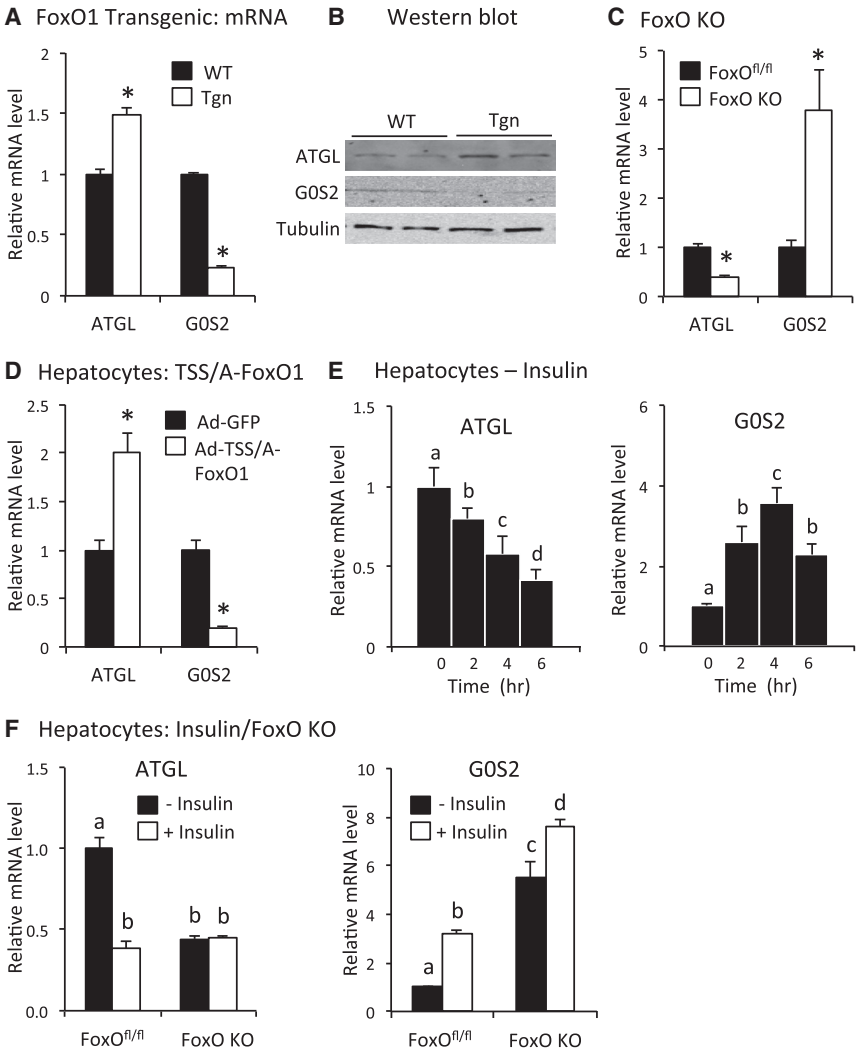

Figure 1. ATGL and G0S2 Gene Expression

(A) FoxO1 Tgn mice. Relative ATGL and G0S2 mRNA levels in liver of WT (solid bar) and FoxO1 Tgn (open bar) mice 6 hr after refeeding (n = 4–6).

(B) Western blot. ATGL, G0S2, and tubulin proteins in liver were measured by western blot in refed WT and Tgn mice.

(C) FoxO KO mice. Relative ATGL and G0S2 mRNA levels in FoxO$^{fl/fl}$ (solid bar) and FoxO KO (open bar) mice (n = 5).

(D) Effects of FoxO1 in hepatoctyes. Relative ATGL and G0S2 mRNA levels in primary hepatocytes from WT mice transfected with adenovirus-expressing (Ad)-TSS/A-FoxO1 (open bar) or GFP (Ad-GFP, solid bar) (n = 4).

(E) Regulation by insulin. ATGL (left) and G0S2 (right) mRNA levels in primary hepatocytes treated with or without 100 nM insulin (n = 3).

(F) FoxO KO hepatocytes. ATGL (left) and G0S2 (right) mRNA levels were measured in hepatocytes isolated from FoxO$^{fl/fl}$ or FoxO KO mice following a 4-hr treatment with (open bar) or without (solid bar) insulin (n = 3).

Statistical significance (*p < 0.05) was determined by Student's t test when only two groups (Tgn versus WT) were compared or by ANOVA when multiple comparisons were made. Bars that are labeled with different letters (a, b, c, or d) differ significantly from each other. Data indicate mean ± SEM.

FAO (Wang et al., 2013; Zhang et al., 2014). Insulin has been reported to stimulate G0S2 expression in adipose tissue, but the mechanism mediating this effect remains unknown (Heckmann et al., 2013). Gene profiling studies that we previously performed in transgenic (Tgn) mice suggested that hepatic expression of G0S2 may be suppressed by FoxO1 (Zhang et al., 2006). Based on these observations, we asked whether FoxO proteins stimulate ATGL and suppress G0S2 expression and whether ATGL-dependent lipolysis contributes to other effects of FoxO proteins in the liver. Our results show that FoxO proteins stimulate ATGL and suppress G0S2 expression in the liver and that ATGL-dependent lipolysis plays an important role in mediating effects of FoxO proteins on both lipid and glucose metabolism in the liver, including effects on glycolysis, lipogenesis, and GNG.

RESULTS

FoxO Proteins Regulate ATGL and G0S2 in Liver

ATGL and G0S2 mRNA and protein levels were measured in Tgn mice expressing TSS/A-FoxO1 in the liver. All three FoxO1 Akt phosphorylation sites (T24, S256, and S319) have been replaced by alanines, so that the function of TSS/A-FoxO1 is not inhibited by insulin action and Akt. As shown in Figures 1A and 1B, hepatic ATGL mRNA and protein levels were increased and G0S2 mRNA and protein levels were decreased in liver-specific TSS/A-FoxO1 Tgn mice, compared to wild-type (WT) littermates. Conversely, hepatic ATGL expression was reduced and G0S2 expression was increased in liver-specific FoxO knockout (KO) mice, in which the expression of FoxO1, FoxO3, and FoxO4 had been disrupted in the liver, compared to floxed controls (FoxO$^{fl/fl}$) (Figure 1C), demonstrating that endogenous FoxO proteins promote ATGL and suppress G0S2 expression in the liver.

FoxO1 stimulated ATGL and suppressed G0S2 expression in primary hepatocytes from WT mice (Figure 1D), showing that FoxO1 promotes ATGL and inhibits G0S2 expression in a cell-autonomous fashion. Conversely, insulin, which inhibits the effects of FoxO proteins on gene expression, reduced ATGL mRNA levels by ~60% and increased G0S2 expression ~-fold in WT hepatocytes (Figure 1E). ATGL expression was decreased in hepatocytes from FoxO KO versus FoxO$^{fl/fl}$ mice, and there was no additional effect of insulin on ATGL mRNA levels in FoxO KO hepatocytes (Figure 1F, left panel). Conversely, G0S2 expression was ~6-fold higher in FoxO KO versus FoxO$^{fl/fl}$ hepatocytes, and the ability of insulin to stimulate G0S2 expression was markedly reduced in hepatocytes from FoxO KO (~36%

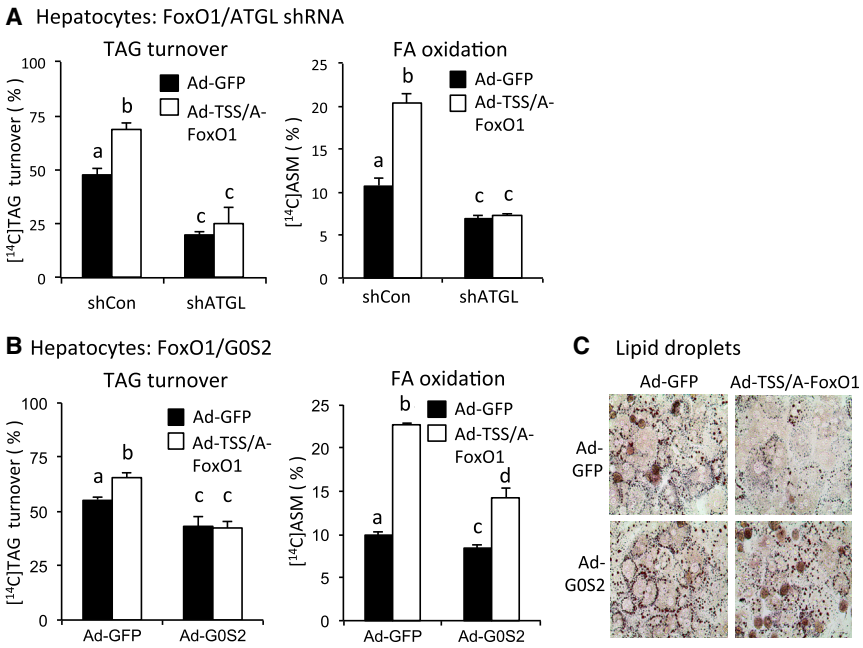

Figure 2. TAG Catabolism in Hepatocytes

(A) ATGL-dependent effects of FoxO1. TAG turnover (left) and FAO (accumulation of ASMs in hepatocyte conditioned medium) (right) were measured in [1-^{14}C]oleate-loaded hepatocytes following transfection of adenovirus-expressing control/scrambled shRNA or ATGL shRNA plus adenovirus-expressing TSS/A-FoxO1 (open bars) or GFP (solid bars) (n = 3).

(B) Co-expression of G0S2. TAG turnover (left) and FAO (right) in hepatocytes transfected with adenovirus ATGL shRNA or control/scrambled shRNA plus adenovirus-expressing TSS/A-FoxO1 (open bars) or GFP (solid bars) (n = 3).

(C) Lipid droplets. Hepatocytes expressing TSS/A-FoxO1 or GFP plus G0S2 or GFP were stained with oil red O to visualize lipid droplets.

Statistical significance ($p < 0.05$) was determined by ANOVA, and bars that are labeled with different letters (a, b, c, or d) differ significantly from each other. Data indicate mean ± SEM.

increase) versus FoxO$^{fl/fl}$ (~3-fold increase) mice (Figure 1F, right panel). These results show that FoxO proteins regulate and mediate the effects of insulin on ATGL and G0S2 expression in the liver.

ATGL-Dependent Effects of FoxO1 on TAG Turnover and FAO in Hepatocytes

Pulse-chase studies showed that FoxO1 stimulated [1-^{14}C] oleate-labeled TAG turnover (Figure 2A, left panel) and the oxidation of hydrolyzed fatty acids (Figure 2A, right panel) in isolated hepatocytes. Knocking down ATGL reduced basal TAG turnover and FAO by 50%–60% and disrupted the effects of TSS/A-FoxO1. Co-expressing G0S2, which inhibits ATGL activity, also reduced basal TAG turnover and FAO, but not to the same extent as disrupting ATGL expression (Figure 2B). Rescuing G0S2 expression also disrupted the effect of FoxO1 on TAG turnover and reduced the effect of FoxO1 on FAO by ~75% (Figure 2B). FoxO1 also promoted the depletion of lipid droplets in hepatocytes, and co-expression of G0S2 also blocked this effect (Figure 2C). These results show that FoxO1 promotes TAG turnover and FAO in liver cells in an ATGL-dependent fashion.

ATGL-Dependent Effects of FoxO1 on TAG Catabolism and FAO In Vivo

Treatment with adenovirus-expressing ATGL-specific short hairpin RNA (shRNA) suppressed ATGL mRNA and protein levels in the liver of FoxO1 Tgn and wild-type mice (Figures 3A and 3B), but not in adipose tissue (Figure S2), confirming that the ATGL knockdown (KD) is liver specific. ATGL KD increased liver TAG content in WT mice (Figure 3C), consistent with previous studies (Ong et al., 2011, 2013). Liver TAG content was low in Tgn versus WT mice and was restored to WT levels by ATGL KD (Figure 3C), indicating that ATGL-dependent lipolysis contributes to effects of FoxO1 on liver TAG content.

Plasma beta-hydroxybutyrate (BHB) levels were increased in briefly (4 hr) fasted Tgn versus WT mice, and knocking down ATGL reversed this effect (Figure 3D), indicating that FoxO1 promotes FAO in the liver in an ATGL-dependent fashion. Circulating levels of non-esterified fatty acids (NEFAs) were reduced in Tgn versus WT mice, and knocking down ATGL reversed this effect (Figure 3E), indicating that increased FAO in Tgn mice was not due to increased availability of circulating NEFAs. Glycerol levels were not altered in Tgn versus WT mice (Figure 3F), suggesting that lipolysis in white adipose tissue (WAT) was not reduced and that decreased NEFAs may reflect increased re-esterification in WAT (Wolfe and Peters, 1987) and/or uptake and utilization by the liver in Tgn versus WT mice.

We also considered whether FAO is increased in Tgn mice 6 hr after refeeding, when endogenous FoxO proteins are inactive but TSS/A-FoxO1 remains active in Tgn mice. Although BHB levels are suppressed in both WT and Tgn refed mice (Zhang et al., 2006), long-chain fatty acylcarnitine levels were increased in the liver of Tgn versus WT mice, and knocking down ATGL disrupted this effect (Figure 3G). This result indicates that FAO also is increased in refed Tgn versus WT mice in an ATGL-dependent fashion and that suppressing FoxO1 and ATGL function is required to fully suppress intrahepatic lipolysis and FAO in the refed state.

We also examined the expression of other genes related to TAG turnover and FAO. The expression of G0S2 was suppressed in refed Tgn versus WT mice and was partially restored to WT levels by ATGL KD in Tgn mice (Figure 3H). This result indicates that FoxO1 suppresses G0S2 expression through both ATGL-dependent and -independent mechanisms. Conversely, expression of HSL, which catabolizes diacylglycerols, was increased in Tgn versus WT mice, and ATGL KD also reversed this effect. These results indicate that, in addition to its direct effect on TAG turnover, ATGL also promotes intrahepatic lipolysis by altering G0S2 and HSL expression downstream from FoxO1.

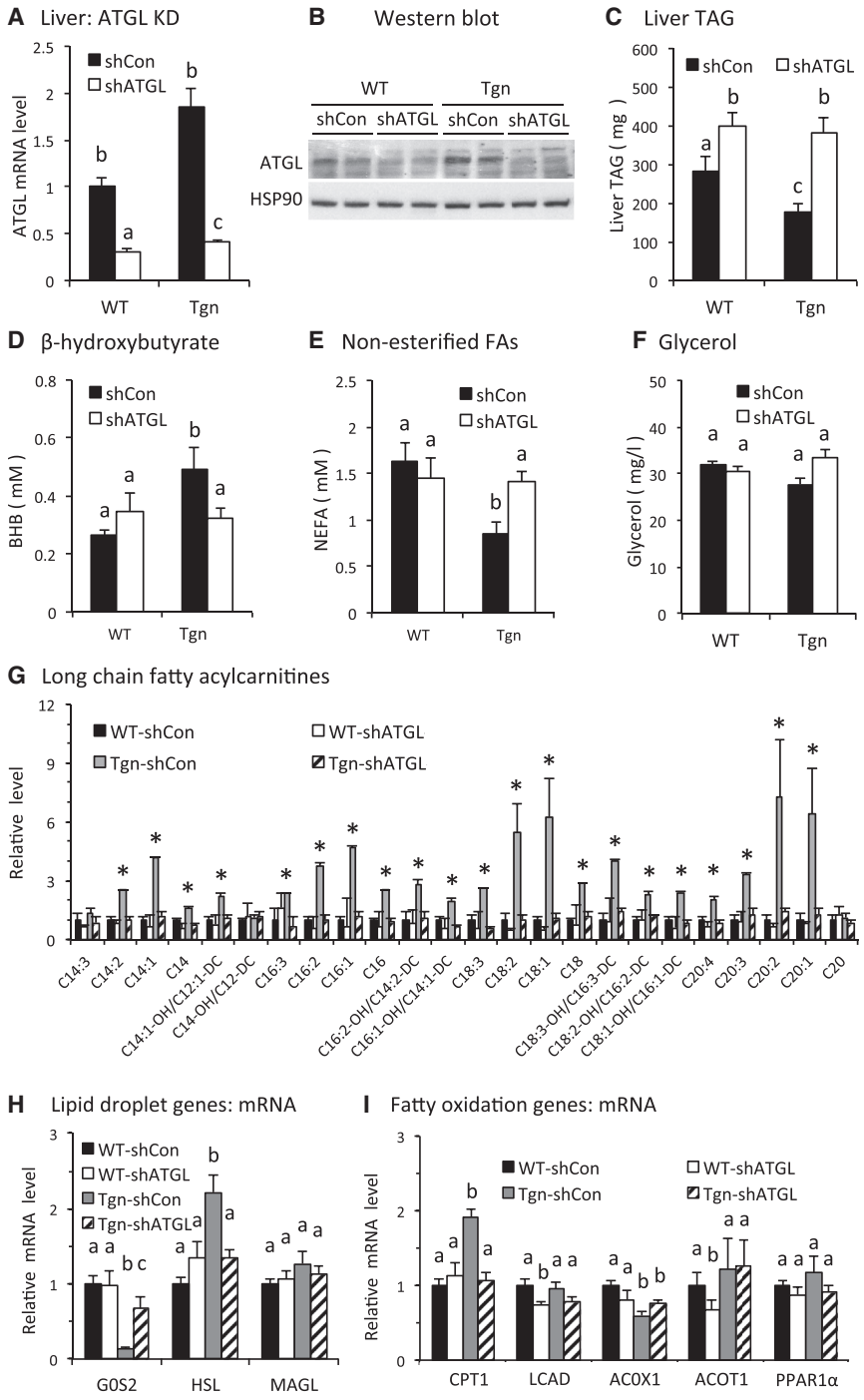

Figure 3. TAG Catabolism in Liver

(A) ATGL KD in vivo. ATGL mRNA levels were measured in liver in WT and Tgn mice 7 days after tail vein injection with adenovirus-expressing control/scrambled shRNA (shCon, solid bars) or ATGL shRNA (shATGL, open bars) (n = 4–5).

(B) Western blot. ATGL protein level in liver from WT and Tgn mice with or without ATGL KD.

(C) Liver TAG. TAG in refed WT and Tgn mice 7 days after ATGL KD (n = 4–5).

(D–F) Plasma levels of β-hydroxybutyrate (D), NEFAs (E), and glycerol (F). Plasma was collected from briefly (4-hr) fasted mice from WT and Tgn mice 5 days after treatment with control shRNA (shCon, solid bar) or ATGL shRNA (shATGL, open bar) adenovirus. Plasma BHB levels in 4-hr-fasted WT and Tgn mice 5 days post-ATGL KD (n = 6–8).

(G) Fatty acylcarnitines. Long-chain fatty acyl carnitines in liver from refed WT and Tgn mice 7 days after treatment with adenovirus-expressing scrambled/control shRNA (shCon) or ATGL shRNA (shATGL, n = 4–5).

(H) Lipid droplet genes. Relative levels of G0S2, HSL, and MAGL mRNA in WT and Tgn mice 7 days post-ATGL shRNA or control adenovirus treatment (n = 4–5).

(I) FAO genes. CPT-1, LCAD, ACOX1, ACOT1, and PPARα mRNA levels 7 days post-treatment with ATGL shRNA or control adenovirus are shown (n = 4–5).

Statistical significance ($p < 0.05$) was determined by ANOVA, and differences between groups are identified by labeling with different letters (a, b, c, or d) or by an asterisk (for fatty acyl carnitines). Data indicate mean ± SEM.

The expression of carnitine palmitoyl transferase 1 (CPT1), which promotes translocation of fatty acyl-CoAs into mitochondria, was increased 2-fold in Tgn versus WT mice, and this effect was reversed by ATGL KD (Figure 3I). Although fatty acid activation of peroxisome proliferator receptor-α (PPARα) can promote the expression of CPT1, the expression of other PPARα-regulated genes involved in promoting FAO was not increased in Tgn versus WT mice (Figure 3I), including very long chain acyl dehydrogenase (LCAD), acyl-CoA oxidase 1 (ACOX1), and acyl-CoA thioesterase 1 (ACOT1), suggesting that other mechanisms may mediate the effects of FoxO1 and ATGL on CPT1 expression.

Together, these results indicate that ATGL promotes FAO in the liver, downstream from FoxO proteins by multiple mechanisms, including direct effects on TAG turnover and effects on gene expression.

ATGL-Dependent and -Independent Effects on Lipid Levels and Lipogenesis

We also examined circulating TAG levels in Tgn and WT mice 6 hr after refeeding, when de novo lipogenesis is stimulated in the liver. Previous studies indicate that FoxO proteins suppress de novo lipogenesis in the liver and circulating TAG levels (Haeusler et al., 2014; Zhang et al., 2006, 2012). As shown in Figure 4A, TAG levels are reduced in Tgn versus WT mice treated with control adenovirus, and ATGL KD increased TAG levels in Tgn mice ~3-fold, indicating that ATGL contributes to the effects of FoxO1 on TAG levels. At the same time, ATGL KD

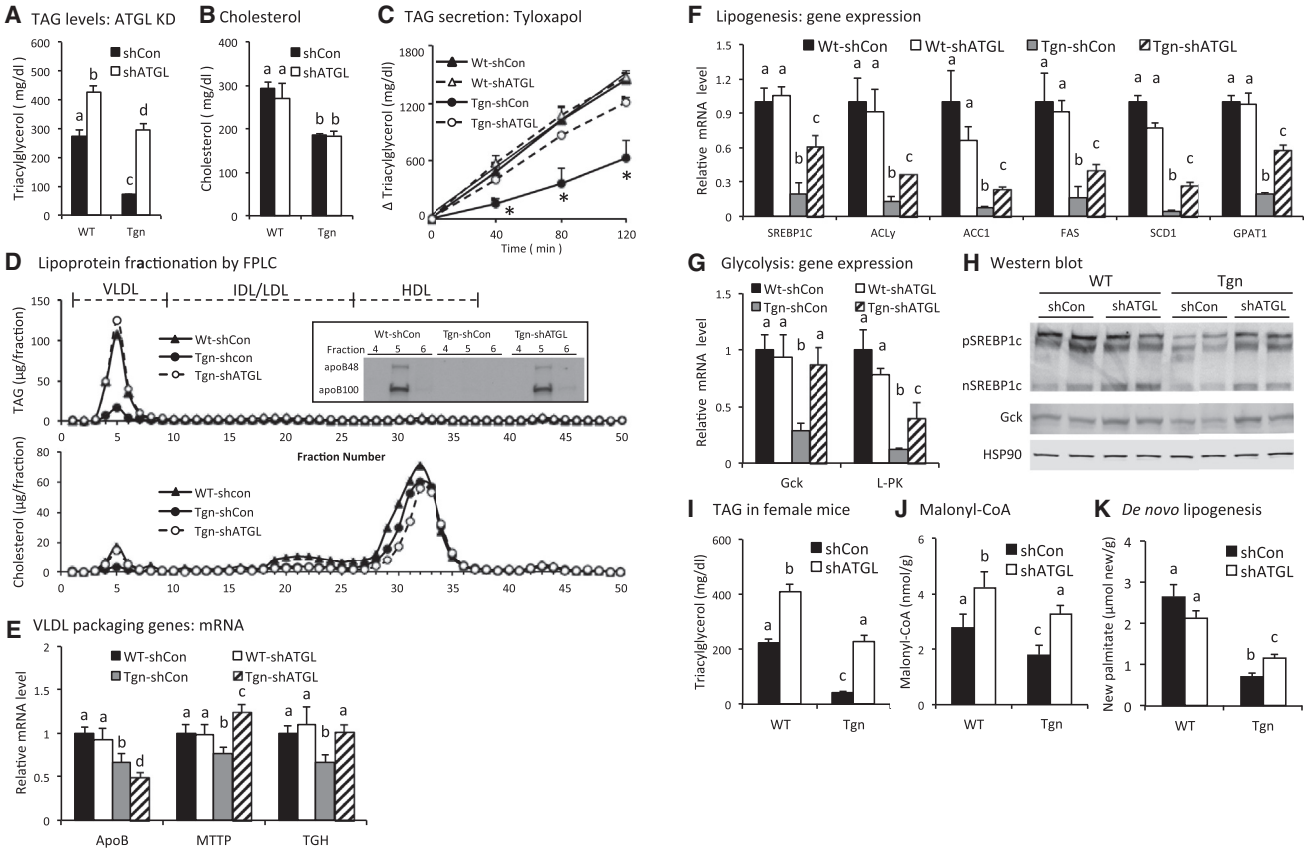

Figure 4. Lipid Levels and Lipogenesis

(A) Serum TAG levels in refed male WT and Tgn mice with or without ATGL KD (n = 4–5). shATGL, adenovirus-expressing ATGL shRNA; shCon, adenovirus-expressing control/scrambled shRNA.

(B) Cholesterol levels in refed mice with or without ATGL KD (n = 4–5).

(C). Hepatic TAG secretion. WT and Tgn mice with or without ATGL KD were treated with Tyloxapol 4 hr after refeeding. Plasma TAG level was determined 0, 40, 80, and 120 min after Tyloxapol treatment, and change in TAG level is shown. (n = 4)

(D) Lipoprotein fractionation. Plasma from refed WT and Tgn mice with or without ATGL KD was fractionated by FPLC and TAG (upper panel), and cholesterol (lower panel) content was determined in FPLC fractions. Average values are shown for two samples from each group. Inset: western blot of apoB in FPLC fractions 4–6 is shown.

(E) VLDL packaging genes. Liver apoB, MTTP, and TGH mRNA levels in refed mice 7 days post-ATGL KD are shown (n = 4–5).

(F) Lipogenic genes. Liver SREBP-1c, ATP citrate lyase (ACLy), acetyl-CoA carboxylase (ACC1), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD1), and glycerol-3-phosphate acyltransferase-1 (GPAT1) mRNA levels in refed mice are shown (n = 4–5).

(G) Glycolytic genes. Liver mRNA levels for Gck and pyruvate kinase in refed mice (n = 4–5).

(H) Western blot. Liver SREBP-1c, Gck, and tubulin protein levels in refed mice (see Figure S4).

(I–K) Lipogenesis. Plasma TAG levels (I) and malonyl-CoA in liver (J) were measured in refed female WT and Tgn mice 7 days after treatment with shATGL or shCon. Deuterated water was administered 2 hr after refeeding, and tissue was harvested 4 hr later for determination of newly synthesized palmitate in liver (K) (n = 4–5).

Statistical significance ($p < 0.05$) was determined by ANOVA, and differences between groups are identified by labeling with different letters (a, b, c, or d), or by an asterisk (for Tyloxapol studies). Data indicate mean ± SEM. See also Figure S4.

also increased TAG levels in WT mice by ~40% (Figure 4A), so that TAG levels were still higher in WT versus Tgn mice after ATGL KD (Figure 4A). Adenoviral expression of G0S2 also improved TAG levels in Tgn mice by ~3-fold and increased TAG levels by ~30% in WT mice (Figure S3A). These results indicate that ATGL-dependent lipolysis contributes to the effects of FoxO on TAG levels and that FoxO suppresses TAG levels by both ATGL-dependent and -independent mechanisms. Cholesterol levels also were low in Tgn versus WT mice but were not affected by ATGL KD (Figure 4B) or G0S2 expression (Figure S3B), indicating that the effect of ATGL was specific for TAGs.

Studies with Tyloxapol, which inhibits the endovascular lipolysis of TAG, showed that the appearance of newly secreted TAG was reduced in Tgn versus WT mice and was restored by ATGL KD (Figure 4C). Fractionation of plasma lipoproteins in refed mice revealed that VLDL TAG content was decreased in Tgn versus WT mice and restored by ATGL KD (Figure 4D, upper panel), and western blotting showed that VLDL apolipoprotein B (apoB) levels also were reduced in Tgn versus WT mice and

restored by ATGL KD (Figure 4D, inset). Since each VLDL particle contains one apoB molecule, this result indicates that changes in TAG levels in Tgn mice reflect ATGL-dependent effects on hepatic secretion of VLDL particles. VLDL (but not HDL) cholesterol content also was reduced in Tgn versus WT mice and restored by knocking down ATGL (Figure 4D, lower panel), consistent with changes in VLDL particle number. These results indicate that suppressing FoxO1 and ATGL function is important in promoting hepatic VLDL production in the postprandial state.

Liver apoB mRNA levels were modestly reduced in Tgn versus WT mice (Figure 4E) but remained low after ATGL KD (Figure 4E), indicating that changes in apoB expression were not responsible for the recovery of VLDL production in Tgn mice treated with ATGL shRNA. In contrast, expression of microsomal TAG transfer protein (MTTP), which promotes the packaging of TAG in VLDL, and triacylglycerol hydrolase (TGH)/carboxylesterase 3, which is required for VLDL secretion (Lian et al., 2012), were reduced in Tgn versus WT mice and restored by ATGL KD. These results indicate that changes in MTTP and TGH expression may contribute to improved VLDL secretion and TAG levels when ATGL is knocked down in Tgn mice.

Previous studies have shown that FoxO proteins can suppress de novo lipogenesis by reducing glycolytic and/or lipogenic gene expression and metabolism in the liver (Haeusler et al., 2014; Zhang et al., 2006). As shown in Figure 4F, the expression of sterol response element binding protein-1c (SREBP-1c), a major regulator of lipogenic gene expression, and several of its downstream target genes (including ATP citrate lyase, acetyl-CoA carboxylase-1, fatty acid synthase, stearoyl-CoA desaturase-1, and glycerol-3 phosphate acyltransferase-1) were suppressed in Tgn mice and partially restored by ATGL KD. Glucokinase (Gck) and pyruvate kinase (L-PK) expression also were suppressed in Tgn versus WT mice and largely restored by ATGL KD (Figure 4G). Western blotting confirmed that SREBP-1c and Gck protein levels also were largely restored to WT levels in Tgn mice by ATGL KD (Figures 4H and S4A). Expression of G0S2 also restored Gck and improved lipogenic gene expression in Tgn mice (Figure S3C), supporting the concept that ATGL-dependent lipolysis contributes to the regulation of glycolytic and lipogenic gene expression by FoxO proteins.

In a separate study, we examined effects on TAG levels and de novo lipogenesis in female mice. TAG levels were decreased in refed female Tgn versus WT mice and were partially restored to WT levels by ATGL KD (Figure 4I), similar to males (discussed earlier). Liver levels of malonyl-CoA levels were decreased in refed Tgn versus WT mice and were increased by ATGL KD (Figure 4J), consistent with increased glycolytic and lipogenic metabolism. Studies with deuterated water showed that newly synthesized palmitate in liver also was reduced in refed Tgn versus WT liver, consistent with previous studies (Zhang et al., 2006), and was improved by knocking down ATGL (Figure 4K). The increase in newly synthesized palmitate may be underestimated in Tgn mice, since hepatic secretion of VLDL also markedly improved ATGL KD in Tgn mice (discussed earlier). These results support the concept that ATGL contributes to the suppression of de novo lipogenesis by FoxO1. At the same time, since ATGL KD does not fully restore levels of TAG, malonyl-CoA, or newly synthesized palmitate, both ATGL-dependent and -independent effects appear to contribute to effects of FoxO1 on TAG levels and de novo lipogenesis.

ATGL-Dependent and -Independent Effects of FoxO1 on Glucose Homeostasis

We also asked whether ATGL contributes to FoxO effects on glucose homeostasis. Glucose tolerance was impaired in 4-hr-fasted Tgn versus WT mice, and ATGL KD restored glucose tolerance in Tgn mice (Figure 5A), indicating that ATGL-dependent lipolysis contributes to the effects of FoxO1 on glucose homeostasis. At the same time, ATGL KD also improved glucose tolerance in WT mice, consistent with previous reports (Ong et al., 2013), so that glucose tolerance remained significantly different in Tgn versus WT mice after ATGL KD (Figure 5A), indicating that ATGL-independent mechanisms also contribute to the effects of FoxO1 on glucose tolerance. Adenoviral expression of G0S2 in the liver of female Tgn and WT mice yielded similar results (Figure 5B), supporting the concept that ATGL-dependent lipolysis contributes to the effects of FoxO1 on glucose homeostasis and that FoxO1 impairs glucose tolerance by both ATGL-dependent and -independent mechanisms.

Pyruvate tolerance was also impaired in Tgn versus WT mice and restored to normal by knocking down ATGL (Figure 5C), indicating that glucose production from pyruvate may be increased in FoxO1 Tgn mice and that ATGL contributes to this effect. Studies in hepatocytes showed that FoxO1 increases glucose production from pyruvate in isolated liver cells and that knocking down ATGL disrupted this effect (Figure 5D), supporting the concept that ATGL contributes to the ability of FoxO1 to promote hepatic glucose production in a cell-autonomous fashion.

Given that ATGL promotes FAO downstream from FoxO1 and FAO promotes GNG in the liver (Lewis et al., 1997; Perry et al., 2015), we also asked whether increased FAO may contribute to the effect of FoxO1 to promote glucose intolerance in Tgn mice. Treatment with a low dose of etomoxir (3 mg/kg), which is sufficient to suppress FAO in the liver (Satapati et al., 2012) and BHB levels in briefly fasted FoxO1 Tgn mice (Figure S5A), restored normal glucose tolerance in FoxO1 Tgn mice (Figure 5E). This result is consistent with the concept that increased FAO in the liver contributes to effects of FoxO1 on glucose tolerance, although we cannot exclude the possibility that effects of etomoxir on other tissues also may contribute to this result.

We also asked whether ATGL contributes to the effects of FoxO proteins on glycolytic and/or gluconeogenic gene expression in briefly fasted mice. Gck mRNA and protein levels are suppressed in FoxO1 Tgn mice and restored by ATGL KD (Figures 5F and S4B). At the same time, ATGL KD increased Gck expression in WT mice so that Gck mRNA levels were still lower in Tgn versus WT mice when ATGL expression is suppressed (Figure 5F). These results indicate that FoxO1 suppresses Gck expression by both ATGL-dependent and -independent mechanisms. Conversely, the expression of several genes that promote GNG, including phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator receptor gamma coactivator-1α (PGC-1α), and pyruvate carboxylase (PC), was increased in Tgn versus WT mice and normalized in an ATGL-dependent fashion (Figure 5F). The expression of glucose-6 phosphatase (G6pase)

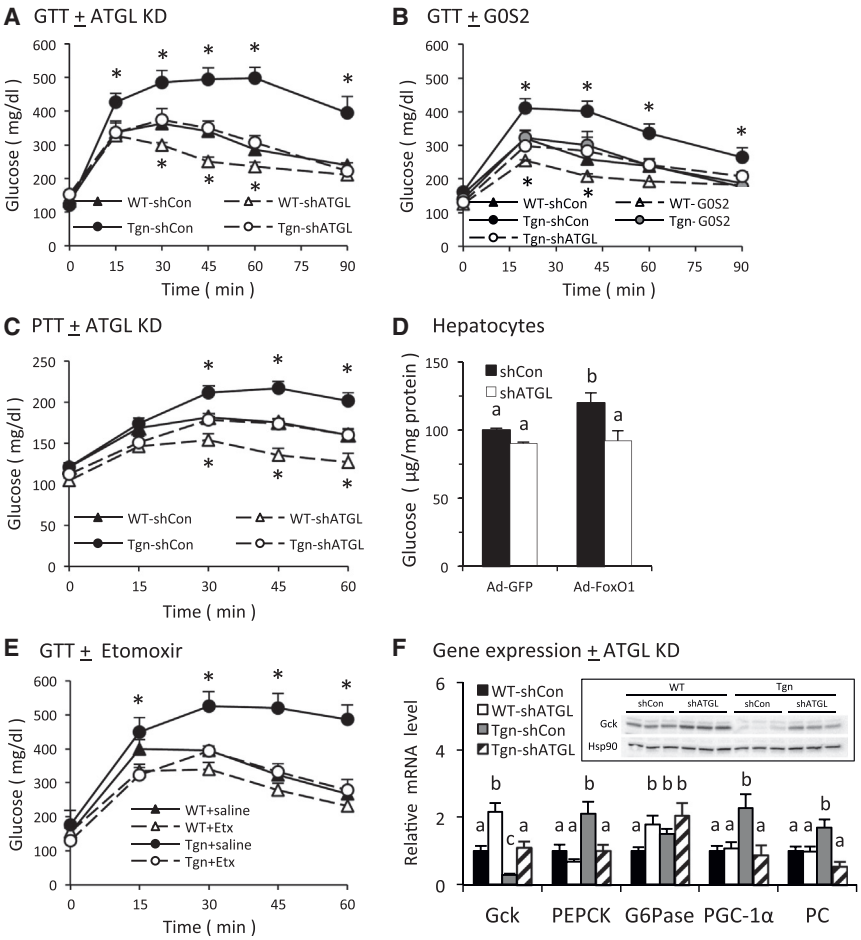

Figure 5. Glucose Tolerance and Glucose Production

(A) Glucose tolerance: ATGL KD. Glucose tolerance tests (GTTs) were performed in 4-hr-fasted WT and Tgn male mice 5 days after treatment with adenovirus-expressing ATGL shRNA (shATGL) or control shRNA (shCon) (n = 4–5).

(B) Glucose tolerance: G0S2 expression. GTTs were performed in 4-hr-fasted WT and Tgn female mice 5 days after treatment with adenovirus-expressing G0S2 or GFP (control) (n = 4–5).

(C) Pyruvate tolerance. Pyruvate tolerance tests (PTTs) were performed in 4-hr-fasted WT and Tgn mice 5 days post-ATGL KD (n = 4–5).

(D) Hepatocytes. Production of glucose from pyruvate and lactate was measured in hepatocytes co-transfected with adenovirus-expressing TSS/A-FoxO1 or GFP, plus adenovirus-expressing ATGL shRNA (open bars) or control shRNA (solid bars) (n = 3–4).

(E) Etomoxir. GTTs were performed in 4-hr-fasted WT and Tgn mice 30 min after treatment with etomoxir or PBS (n = 4–6).

(F) Gene expression. PEPCK, G6Pase, PGC-1α, and PC mRNA levels in liver from refed mice 5 days after treatment with adenovirus-expressing ATGL shRNA or control shRNA (n = 4) (see Figure S4).

Statistical significance ($p < 0.05$) was determined by ANOVA and differences between groups are identified by labeling with different letters (a, b, c, or d), or by an asterisk for differences relative to WT-shCon in GTT and PTT studies. Data indicate mean ± SEM. See also Figure S4.

also was increased in Tgn versus WT mice but was not dependent on ATGL.

Adenoviral expression of G0S2 also restored the expression of Gck and suppressed PEPCK expression in Tgn mice (Figures S3C and S3D), supporting the concept that that ATGL-dependent lipolysis contributes to the effects of FoxO1 on glycolytic and gluconeogenic gene expression. In contrast, inhibition of FAO with etomoxir failed to disrupt effects of FoxO1 on glycolytic (Gck, PK), lipogenic (SREBP-1c, SCD-1), or gluconeogenic (PEPCK) gene expression in Tgn mice (Figure S5E), indicating that FAO is not required for these ATGL-dependent effects of FoxO1 on gene expression and that other mechanisms are involved.

DISCUSSION

The results of this study provide several insights regarding the role of ATGL and its inhibitor, G0S2, in mediating the effects of FoxO proteins on gene expression and metabolism in the liver. Key findings include the following: (1) FoxO proteins regulate and mediate the effects of insulin on ATGL and G0S2 expression in the liver in a cell-autonomous fashion; (2) FoxO proteins promote intrahepatic TAG catabolism and FAO in an ATGL-dependent fashion; (3) ATGL-dependent FAO contributes to the effects of FoxO proteins on glucose homeostasis; and (4) ATGL-dependent lipolysis also contributes to the effects of FoxO proteins on glycolytic, lipogenic, and gluconeogenic gene expression and metabolism. Together, these findings reveal that ATGL-dependent lipolysis plays an important role in mediating the effects of FoxO proteins on multiple aspects of glucose and lipid metabolism in the liver.

We found that FoxO proteins stimulate ATGL expression in the liver and are required for insulin to regulate ATGL in hepatocytes. FoxO1 directly targets the ATGL promoter and promotes ATGL expression in adipose tissue (Chakrabarti and Kandror, 2009). While other pathways also may contribute to the ability of insulin to regulate ATGL in adipose tissue (Chakrabarti et al., 2013), our results indicate that FoxO proteins play a major role in regulating and mediating the effects of insulin on ATGL expression in the liver.

We also found that FoxO proteins suppress the expression of G0S2 and play an important role in mediating the effects of insulin on G0S2 expression in the liver. Insulin stimulates G0S2 expression in adipose tissue (Yang et al., 2010), yet the mechanism mediating this effect has not been identified. Our data indicate that insulin stimulates the expression of G0S2, at least in part, by disrupting the negative effects of FoxO proteins on G0S2 expression in the liver and, possibly, other tissues. Interestingly, knocking down ATGL partially restored the expression of G0S2 to WT levels in FoxO1 Tgn mice, indicating that

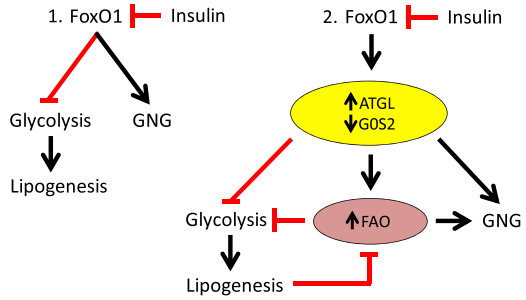

Figure 6. Integrated Regulation of Glucose and Lipid Metabolism by FoxO Proteins and ATGL-Dependent TAG Hydrolysis

(1) Previous studies have shown that FoxO proteins suppress glycolytic/lipogenic metabolism and promote gluconeogenic metabolism in the liver and that insulin disrupts this effect of FoxO proteins.

(2) FoxO proteins promote ATGL and suppress G0S2 expression, as well as promote intrahepatic lipolysis and FAO in an ATGL-dependent fashion. Increased FAO promotes GNG and suppresses glycolytic/lipogenic metabolism downstream from FoxO1 and ATGL. Reduced lipogenesis and production of malonyl-CoA promotes increased FAO (and, thereby, glucose production). ATGL-dependent mechanisms also contribute to the effects of FoxO proteins on reduced glycolytic/lipogenic and increased gluconeogenic gene expression and metabolism. Inhibition of FoxO function by insulin contributes to changes in FAO, glycolysis, lipogenesis, and GNG, due, at least in part, to ATGL-dependent effects.

ATGL-dependent mechanisms contribute to the effect of FoxO proteins on G0S2 expression and that ATGL promotes its own activity by suppressing the expression of its inhibitor, G0S2.

ATGL-dependent lipolysis also may promote the effects of FoxO proteins on FAO in the liver by multiple mechanisms. For example, promoting the expression of HSL, an important diacylglycerol lipase, would enhance intrahepatic lipolysis, and increasing the expression of CPT1, which is required for the translocation of long-chain fatty acyl-CoAs into the mitochondria, would enhance β-oxidation. Suppression of stearoyl CoA desaturase-1 and glycerol-3-phosphate acyltransferase would help to ensure that free fatty acids derived from either extrahepatic sources or intrahepatic lipolysis would be partitioned toward FAO rather than storage as TAGs in lipid droplets. Further, suppressing glycolytic and lipogenic gene expression, and lowering levels of malonyl-CoA—an important inhibitor of CPT1—also would contribute to increased FAO. Together, these effects would contribute to the ability of FoxO1 and ATGL to promote TAG turnover and FAO in the liver.

Our results also indicate that ATGL-dependent lipolysis and FAO play an important role in mediating the effects of FoxO proteins on glucose homeostasis by multiple mechanisms. Recent studies indicate that ATGL influences hepatic glucose metabolism (Brown et al., 2010; Ong et al., 2013; Zhang et al., 2014). We found that FoxO1 promotes hepatic TAG turnover and FAO in an ATGL-dependent manner, and studies with etomoxir showed that FAO contributes to the effects of FoxO1 on glucose homeostasis. FAO promotes GNG by providing ATP and reducing equivalents required for glucose production, as well as acetyl-CoA, which increases the activity of PC (Freedman and Kohn, 1964; Perry et al., 2015; Williamson et al., 1966). Conversely, FAO suppresses glycolysis at multiple levels, including the activity of pyruvate dehydrogenase (Freedman and Kohn, 1964; Garland and Randle, 1964; Hue and Taegtmeyer, 2009). Thus, FoxO proteins may limit glycolysis and the diversion of pyruvate into other pathways and promote hepatic glucose production, at least in part, by promoting FAO.

Our results also indicate that ATGL-dependent lipolysis may play an important role in mediating the effects of FoxO1 on gene expression related to glycolytic/lipogenic and gluconeogenic metabolism, including the suppression of glucokinase and stimulation of PEPCK and PGC-1α mRNA levels. Previous studies indicate that FoxO1 may suppress glucokinase expression by interacting with other *trans*-acting factors, including hepatocyte nuclear factor-4 (Ganjam et al., 2009; Hirota et al., 2003, 2008), and that FoxO1 can promote the expression of PEPCK and PGC-1α expression, at least in part, through direct interaction with *cis*-acting elements located in the PEPCK and PGC-1α promoters (Daitoku et al., 2003; Hall et al., 2000; Yeagley et al., 2001). The observation that the effects of FoxO1 on the expression of these genes are largely reversed by knocking down ATGL suggests the interesting possibility that ATGL may function in a "feed-forward" fashion to promote the effects of FoxO1 on gene expression. Although the form of FoxO1 that is expressed in our Tgn mice (TSS/A-FoxO1) is altered so that it is no longer subject to phosphorylation and inactivation by Akt, its function still may be subject to regulation by other post-translational modifications, as well as other intracellular signaling pathways that are regulated by the lipid droplet (Haemmerle et al., 2011; Khan et al., 2015; Mullins et al., 2014; Tang et al., 2013), and may modulate the function of FoxO proteins through mechanisms that remain to be determined. Additional studies are needed to determine whether ATGL-dependent mechanisms do, indeed, modulate the function of FoxO proteins on hepatic gene expression and to examine the role that FoxO proteins may play in mediating the effects of ATGL on gene expression and metabolism in the liver.

Figure 6 summarizes some of the major findings of this study. Previous studies indicate that FoxO proteins can suppress glycolysis/lipogenesis and promote GNG through direct effects on gene expression (Figure 6, number 1). As shown in Figure 6, number 2, the results of the present study indicate that FoxO proteins also increase ATGL and decrease G0S2 expression and, thereby, enhance ATGL-dependent lipolysis and the availability of fatty acids for FAO in the liver. ATGL also contributes to negative effects of FoxO on glycolytic/lipogenic gene expression and metabolism, including the production of malonyl-CoA, a negative regulator of CPT-1 activity. This suggests that ATGL-dependent effects of FoxO proteins also may promote increased utilization of fatty acids by disinhibiting CPT1-mediated translocation of fatty acyl-CoAs into mitochondria, where β-oxidation occurs. Since FAO is known to promote GNG and suppress glycolysis, ATGL-dependent effects on FAO likely contribute to the effects of FoxO proteins on glucose utilization and production in the liver. Interestingly, ATGL also appears to contribute to effects of TSS/A-FoxO1 on gluconeogenic gene expression, including PEPCK and PGC-1α, which promote increased glucose production, suggesting the intriguing possibility that ATGL-dependent mechanisms may contribute to the regulation

of FoxO activity. At the same time, since insulin suppresses the function of FoxO proteins, our results also suggest that regulation of ATGL-dependent lipolysis may play an important role in mediating the effects of insulin on multiple aspects of metabolism in the liver, including FAO, glycolysis, lipogenesis, and GNG.

In summary, these studies demonstrate that FoxO proteins regulate ATGL and G0S2 expression in the liver and reveal an important role for ATGL-dependent lipolysis in mediating the effects of FoxO proteins on glycolytic, gluconeogenic, and lipogenic gene expression and metabolism in the liver. Since FoxO proteins are major targets of insulin action, these findings also indicate that regulation of intrahepatic lipolysis may be important in mediating the effects of insulin on multiple aspects of glucose and lipid metabolism in the liver and suggest that targeting ATGL-dependent lipolysis and its downstream effectors may provide an effective strategy for improving the treatment of diabetes and hepatic insulin resistance.

EXPERIMENTAL PROCEDURES

Animal Studies

Animal studies were approved by the institutional animal care committees of the Jesse Brown VA Medical Center and the University of Minnesota. Liver-specific Tgn mice expressing a modified form of human FoxO1, in which all three Akt phosphorylation sites (T24, S256, and S319) have been replaced by alanine residues (TSS/A-FoxO1), were previously described (Zhang et al., 2006). FoxO KO mice were made by crossing $FoxO^{fl/fl}$ mice (from Dr. Ron DePinho) with albumin-Cre (Jackson Laboratory) mice, and disruption of liver FoxO1, FoxO3, and FoxO4 was confirmed by real-time qPCR and western blotting (Figures S1A–S1C). WT male FVB/N mice were purchased (Harlan Laboratories). Studies were performed on 8- to 12-week-old male mice, unless otherwise stated. Mice were housed on a 12-hr:12-hr light:dark cycle, with lights off at 18:00. Adenoviral vectors (0.5 to 1 × 10^9 plaque-forming units [pfu] per mouse) were injected by tail vein 5–7 days before studies.

For glucose and pyruvate tolerance tests, 4-hr-fasted mice were treated with glucose (2 g/kg intraperitoneally [i.p.]) or sodium pyruvate (2 g/kg i.p.) in PBS, or with PBS alone, and tail blood glucose levels were measured with a OneTouch Ultra glucose meter (Lifespan) at time (t) = 0, 15, 30, 60, and 90 min. For studies in refed mice, chow was removed at 16:00 and replaced 18 hr later, and mice were sacrificed 6 hr later by decapitation following brief sedation with isoflurane. Snap-frozen liver and serum samples from cervical blood were stored at −80°C. To assess liver TAG secretion, mice were injected by tail vein with Tyloxapol (350 mg/kg, Sigma-Aldrich) 4 hr after refeeding, and serial tail blood samples were collected for analysis of TAG levels. For analysis of de novo lipogenesis, 18-hr-fasted mice were allowed to refeed for 2 hr prior to treatment with deuterated water (25 μl/g) and were sacrificed 4 hr later.

Hepatocytes

Primary hepatocytes were isolated by collagenase perfusion and transfected with adenoviral vectors as previously reported (Bu et al., 2009). For studies of insulin effects, hepatocytes were cultured in M199 media supplemented with 10% FBS, 10 nM insulin, and 10 nM dexamethasone for 4 hr after plating, and then in serum-free medium with or without 100 nM insulin for 4 hr, before cell extracts were collected for analysis.

Pulse-chase studies were performed to measure TAG turnover and FAO of fatty acids in the TAG pool, as previously described (Sapiro et al., 2009). Hepatocytes were exposed to 500 μM [1-^{14}C]oleate for 2 hr (pulse), followed by a 6-hr chase period with media devoid of fatty acids. Radiolabeled TAG was quantified in cell extracts following separation by thin-layer chromatography, and media acid-soluble metabolites (ASMs) were measured as markers of FAO (Sapiro et al., 2009). To visualize lipid droplets, hepatocytes were harvested 24 hr following transfection with adenoviral vectors, rinsed with PBS, fixed with 3.7% formaldehyde, and stained with oil red O.

To measure glucose production, hepatocytes were cultured in glucose-free DMEM without phenol red, supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate (pH 7.4) and 10 nM insulin for 3 hr. Glucose concentration in medium was measured by glucose assay (Sigma) and normalized to total protein from cell lysates.

Biochemical Analysis

Plasma levels of BHB, NEFAs, TAGs, and cholesterol were measured with commercially available kits (Wako Chemicals). Hepatic TAG content was measured following extraction with chloroform-methanol after the method of Bligh and Dyer (1959). Liver acylcarnitines were measured by flow-injection tandem mass spectrometry using a Waters Acquity UPLC System equipped with a TQ (triple quadrupole) detector and a data system controlled by a MassLynx 4.1 operating system (Waters), as previously described (An et al., 2004).

Plasma lipoproteins were analyzed after fractionation by FPLC using two Superose 6 columns linked in tandem, and 0.5-ml fractions were collected for analysis of TAG and cholesterol concentration as previously reported (Bu et al., 2009). Two samples from each treatment group were analyzed, and the average is presented for each group.

Gene Expression and Western Blotting

RNA was isolated using available kits (QIAGEN), and cDNA transcripts were prepared (SuperScript VILO) for real-time qPCR using SYBR Green. mRNA levels were adjusted for variances in L32 ribosomal protein mRNA abundance. Primer sequences for qPCR are shown in Table S1.

For western blotting, tissue lysates were prepared with T-Per (Pierce) supplemented with protease and phosphatase inhibitors (Pierce), and protein concentration was quantified by the Bradford assay (Bio-Rad). Proteins were resolved by denaturing SDS/Laemmli gel electrophoresis with 4%–15% gradient acrylamide gels. Membranes were blocked with 5% milk in PBS with Tween 20 (PBST) and probed with antibodies against ATGL, tubulin, actin, HSP90, FoxO1, or FoxO3 (Cell Signaling); glucokinase or SREBP-1c (Santa Cruz Biotechnology) (1:1,000 dilution); G0S2, provided by Jun Liu (Yang et al., 2010) (1:500 dilution); or apoB (Nauli et al., 2006) (1:4,000 dilution). Results of western blotting were quantified using ImageJ software.

Statistical Analysis

Experiments were performed with three to six mice per group, and mean ± SEM are reported. Statistical significance ($p < 0.05$) of differences between groups was determined by Student's t test when two groups were compared or by ANOVA with post hoc testing (least mean squares) when three or more groups were compared. When multiple groups are compared, groups that differ significantly from each other are labeled in the figures with different letters (a, b, c, or d), so that a is different from b; c is different from a and b; and d is different from a, b, and c.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.03.021.

AUTHOR CONTRIBUTIONS

W.Z., S.Y.B., M.T.M., S.A.K., and O.I. conducted experiments. I.O.-S. derived FoxO KO mice. Z.S. contributed to data analysis. C.B.N. contributed to data analysis and interpretation. D.G.M. and T.G.U. designed studies and wrote the manuscript.

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