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# Hepatic mTORC2 Activates Glycolysis and Lipogenesis through Akt, Glucokinase, and SREBP1c

Asami Hagiwara,[1] Marion Cornu,[1,4] Nadine Cybulski,[1,4] Pazit Polak,[1] Charles Betz,[1] Francesca Trapani,[2] Luigi Terracciano,[2] Markus H. Heim,[3] Markus A. Rüegg,[1] and Michael N. Hall[1,*]
[1]Biozentrum, University of Basel, CH-4056 Basel, Switzerland
[2]Institute of Pathology
[3]Department of Biomedicine
University Hospital, CH-4003 Basel, Switzerland
[4]These authors contributed equally to this work
*Correspondence: m.hall@unibas.ch
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## SUMMARY

**Mammalian target of rapamycin complex 2 (mTORC2) phosphorylates and activates AGC kinase family members, including Akt, SGK1, and PKC, in response to insulin/IGF1. The liver is a key organ in insulin-mediated regulation of metabolism. To assess the role of hepatic mTORC2, we generated liver-specific *rictor* knockout (LiRiKO) mice. Fed LiRiKO mice displayed loss of Akt Ser473 phosphorylation and reduced glucokinase and SREBP1c activity in the liver, leading to constitutive gluconeogenesis, and impaired glycolysis and lipogenesis, suggesting that the mTORC2-deficient liver is unable to sense satiety. These liver-specific defects resulted in systemic hyperglycemia, hyperinsulinemia, and hypolipidemia. Expression of constitutively active Akt2 in mTORC2-deficient hepatocytes restored both glucose flux and lipogenesis, whereas glucokinase overexpression rescued glucose flux but not lipogenesis. Thus, mTORC2 regulates hepatic glucose and lipid metabolism via insulin-induced Akt signaling to control whole-body metabolic homeostasis. These findings have implications for emerging drug therapies that target mTORC2.**

## INTRODUCTION

Target of rapamycin (TOR) is a highly conserved protein kinase that controls cell growth and metabolism in response to nutrients, growth factors, and energy status. TOR exists in two structurally and functionally distinct complexes termed TOR complex 1 (TORC1) and TORC2 (Loewith et al., 2002). Mammalian TORC1 (mTORC1) contains mTOR, raptor, and mLST8 and phosphorylates a variety of substrates to control protein synthesis, ribosome biogenesis, autophagy, and other growth-related processes (Laplante and Sabatini, 2009; Polak and Hall, 2009; Russell et al., 2011; Wullschleger et al., 2006). The two best-characterized mTORC1 substrates are ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP), both of which control protein synthesis. mTORC2 comprises mTOR, rictor, mSin1, mLST8, and PRR5 (also known as protor) and phosphorylates members of the AGC kinase family, including Akt (also known as PKB), SGK1, and PKC, via which mTORC2 controls cell survival, actin cytoskeleton organization, and other processes (Cybulski and Hall, 2009; García-Martínez and Alessi, 2008; Ikenoue et al., 2008; Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004; Yang et al., 2006). By regulating a wide range of anabolic and catabolic processes, the mTOR complexes play a key role in growth, development, metabolism, and aging and are implicated in a variety of pathological states including cancer, obesity, and diabetes (Dazert and Hall, 2011).

Many studies with genetically modified animals indicate mTOR signaling plays a role in whole-animal metabolism and in the development of disease. Full-body knockout of any component of mTORC1 or mTORC2 causes embryonic lethality (Gangloff et al., 2004; Guertin et al., 2006; Jacinto et al., 2006; Murakami et al., 2004; Shiota et al., 2006; Yang et al., 2006). More recent studies have focused on mTOR function specifically in metabolic tissues, in large part due to these tissues being particularly sensitive to the three inputs that control mTOR (nutrients, insulin, and energy status). Conditional knockout of *raptor* (mTORC1) in skeletal muscle results in muscle dystrophy, glucose intolerance, and short lifespan, whereas knockout of *rictor* (mTORC2) in skeletal muscle confers little to no phenotype (Bentzinger et al., 2008; Kumar et al., 2008). Adipose-specific *raptor* knockout mice display increased energy expenditure and resistance to diet-induced obesity (Polak et al., 2008). Adipose-specific *rictor* knockout mice are characterized by increased glucose metabolism and somatic growth due to high circulating levels of insulin and IGF1 (Cybulski et al., 2009; Kumar et al., 2010). In podocytes, mTORC1 plays a role in the development of diabetic nephropathy, whereas mTORC2 appears to have a minor role (Gödel et al., 2011; Inoki et al., 2011). Recent findings suggest that hepatic mTORC1 controls ketogenesis and possibly lipid metabolism (Kenerson et al., 2011; Sengupta et al., 2010; Yecies et al., 2011). Together, the above studies suggest that the two mTOR complexes contribute to whole-body metabolic homeostasis

via distinct roles in different metabolic tissues. However, the role of mTORC2 in the liver remains to be determined.

The liver plays a central role in whole-body glucose and lipid homeostasis (Postic et al., 2004). In the fasted state, the liver maintains blood glucose levels by producing glucose via glycogen breakdown and via gluconeogenesis. In the postprandial state (i.e., satiety—the increased availability of glucose and insulin), the liver ceases to produce glucose and takes up excess circulating glucose to replenish glycogen and triglyceride (TG) stores. Insulin is the major hormone controlling the fasted to postprandial transition (Saltiel and Kahn, 2001). In type 2 diabetes, hepatic insulin resistance leads to altered glucose metabolism and thereby hyperglycemia. Insulin signals through the PI3K-Akt pathway to inhibit gluconeogenesis and activate glycolysis and lipogenesis. Akt inhibits expression of gluconeogenic genes, by inhibiting FoxO (Puigserver et al., 2003), and induces glycolytic and lipogenic genes, by activating sterol regulatory element-binding protein 1c (SREBP1c) and glucokinase (GK). SREBP1c is a transcription factor that promotes expression of a number of lipogenic genes (Horton et al., 2002). GK, the rate-limiting enzyme of glycolysis in the liver, stimulates glycolysis and lipogenesis by enhancing glucose flux, including production of acetyl-CoA for lipid synthesis (Foufelle and Ferré, 2002). Furthermore, GK stimulates glycolysis and lipogenesis at the transcriptional level via the carbohydrate responsive element-binding protein (ChREBP) (Uyeda and Repa, 2006). Thus, the combined action of insulin signaling and glucose flux regulates glucose and lipid metabolism in the liver.

mTORC2 phosphorylates Ser473 in the so-called hydrophobic motif of Akt and thereby activates Akt toward some but not all substrates (Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005; Shiota et al., 2006; Yang et al., 2006; Zinzalla et al., 2011). To elucidate the role of mTORC2 in the liver in vivo, we generated liver-specific *rictor* knockout mice and investigated Akt signaling and glucose and lipid metabolism. We find that the mTORC2-deficient liver is unable to sense the state of satiety. Hepatic mTORC2 activates both Akt signaling and glucose flux to control glucose and lipid metabolism in the liver and thereby overall metabolic homeostasis.

## RESULTS

### mTORC2 Deficiency in the Liver Leads to Hyperglycemia, Hyperinsulinemia, and Hypolipidemia

To investigate the role of mTORC2 in the liver, we generated mice lacking rictor, an essential and specific component of mTORC2, exclusively in the liver (see Experimental Procedures). *Li*ver-specific *ric*tor *k*nock*o*ut (*rictor*$^{fl/fl}$ *Alb-Cre*$^{Tg/0}$) mice (LiRiKO mice) were viable, born at the expected frequency for Mendelian inheritance, and showed normal fertility. In all subsequent experiments, littermates without the *Cre* transgene (*rictor*$^{fl/fl}$) were used as controls. In LiRiKO mice, rictor protein was absent only in the liver (Figure S1A). To evaluate mTORC2 activity, we investigated the phosphorylation status of mTORC2 substrates in the liver of mice treated with insulin. mTORC2 phosphorylates the hydrophobic (Ser473) and turn (Thr450) motifs in Akt (Facchinetti et al., 2008; Ikenoue et al., 2008; Jacinto et al., 2006; Sarbassov et al., 2005; Yang et al., 2006). Akt phosphorylation at these two sites was significantly reduced in LiRiKO liver (Figure 1A). Phosphorylation of Akt Thr308, a site in the catalytic loop phosphorylated by PDK1, was normal. mTORC2 also phosphorylates and thereby controls the stability and activity of PKCα and SGK1, respectively (García-Martínez and Alessi, 2008; Ikenoue et al., 2008). As expected, PKCα protein levels and phosphorylation of the SGK1 substrate NDRG1 were decreased in LiRiKO liver (Figure 1A). These observations confirm that both rictor protein and mTORC2 activity are absent in the liver of LiRiKO mice.

We next examined systemic parameters of LiRiKO mice fed a normal (chow) or high-fat diet (HFD). On a chow diet, LiRiKO mice showed normal growth rates (Figure 1B) and normal body composition (Figure S1B). When fed a HFD for 10 weeks, LiRiKO mice were slightly lighter than controls, but this difference was not statistically significant (Figure S1C). After 20 weeks on a HFD, fat mass was significantly reduced (5%) in LiRiKO mice (Figure S1D). This was likely due to increased lipolysis and mitochondrial oxidation in adipose tissue of LiRiKO mice, as suggested by an increased level of plasma glycerol (Figure S1E) and increased expression of genes involved in lipolysis and mitochondrial oxidation in adipose tissue (Figure S1F). Analysis of plasma parameters revealed that plasma TG and cholesterol (total and HDL cholesterol) levels were significantly lower (Figure 1C), while plasma glucose (Figure 1D) and insulin (Figure 1E) levels were significantly higher, in both fasted and chow-fed LiRiKO mice. The same differences in blood parameters were observed with mice on a HFD (Figures S1G–S1I). The levels of ALT and AST were similar for the two genotypes (Figures S1J and S1K). Thus, deletion of mTORC2 in the liver leads to hyperglycemia, hyperinsulinemia, and hypolipidemia, suggesting that hepatic mTORC2 mediates metabolic homeostasis.

The liver of LiRiKO mice displayed normal gross morphology (data not shown), and normal cell size and histopathology (Figure 1F). However, the weight of the liver of LiRiKO mice was 20% less compared to controls, for mice fed ad libitum either a chow or a HFD (Figures 1G and S1L). This difference was not observed in fasted mice (Figure S1M). Further analysis revealed that glycogen and TG levels in the liver of fed LiRiKO mice were significantly lower compared to controls (Figures 1F, 1H, 1I, and S1N). Thus, loss of hepatic mTORC2 results in a smaller liver with reduced glycogen and TG content in fed mice.

### Deletion of mTORC2 in the Liver Causes Glucose Intolerance

LiRiKO mice developed mild hyperglycemia and hyperinsulinemia at a young age (6 weeks), indicating that impaired glucose homeostasis is an early effect of mTORC2 loss in the liver (Figures S2A and S2B). To evaluate further whole-body glucose homeostasis, we performed a glucose tolerance test (GTT) and an insulin tolerance test (ITT). In the GTT, LiRiKO mice exhibited significantly higher blood glucose levels before and after glucose administration (Figure 2A). Plasma insulin levels were also significantly higher in LiRiKO mice (Figure 2B). The glucose intolerance became more pronounced in older (>8 months) LiRiKO mice (Figure S2C). In the ITT, when the mice were fasted for 6 hr before the experiment, LiRiKO mice showed normal insulin sensitivity (Figure 2C). In contrast, when the mice were fasted overnight, LiRiKO mice showed slightly but significantly decreased glucose clearance (Figure 2D). The fact that lower

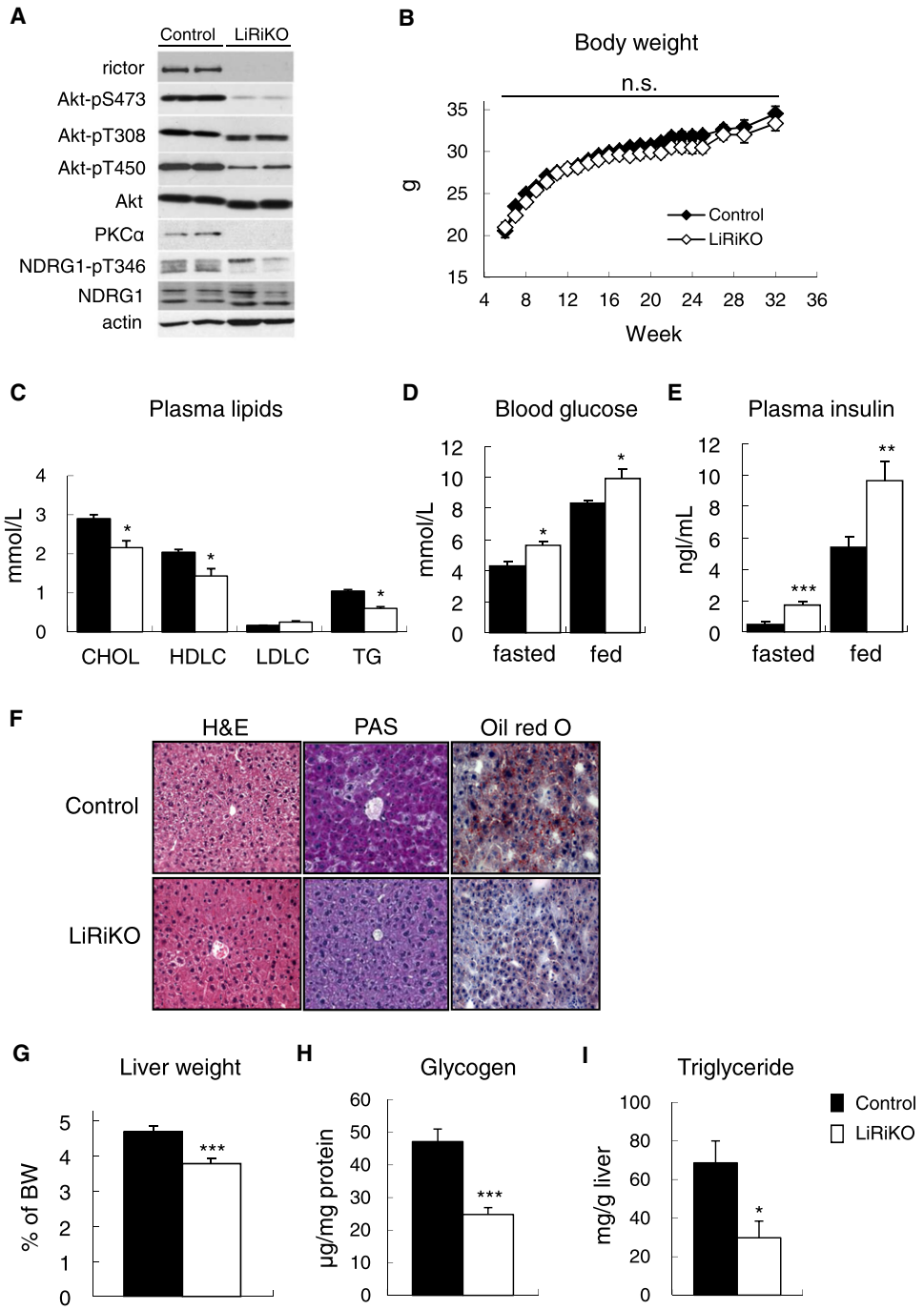


**Figure 1. LiRiKO Mice Develop Hyperglycemia, Hyperinsulinemia, and Hypolipidemia**

(A) Western blots showing loss of rictor expression and mTORC2 activity in the liver of LiRiKO mice. Fasted mice were injected with insulin as described in Experimental Procedures.

(B) Body weight (BW) gain of mice on a chow diet (n = 12 for control, n = 19 for LiRiKO). BW was monitored every week from 6 weeks of age for 26 weeks.

(C) Plasma total cholesterol (CHOL), HDL cholesterol (HDLC), LDL cholesterol (LDLC), and TG levels of ad libitum fed mice (n = 6 per group).

(D and E) Blood glucose (D) and plasma insulin (E) levels of overnight fasted and ad libitum fed mice (n = 8 for control and n = 6 for LiRiKO).

(F) Representative images of H&E, PAS, and oil red O staining of liver sections from ad libitum fed mice. Original magnification, ×40.

(G) Liver weight (normalized to BW) of 10- to 18-week-old mice fed ad libitum (n = 12–13 per group).

(H) Hepatic glycogen content of mice after 4 hr refeeding (n = 7 per group).

(I) Hepatic TG content of mice after 4 hr refeeding (n = 7 per group). Black bars represent control mice and white bars represent LiRiKO mice. Values are expressed as mean ± SEM. * indicates statistical significance from control mice (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

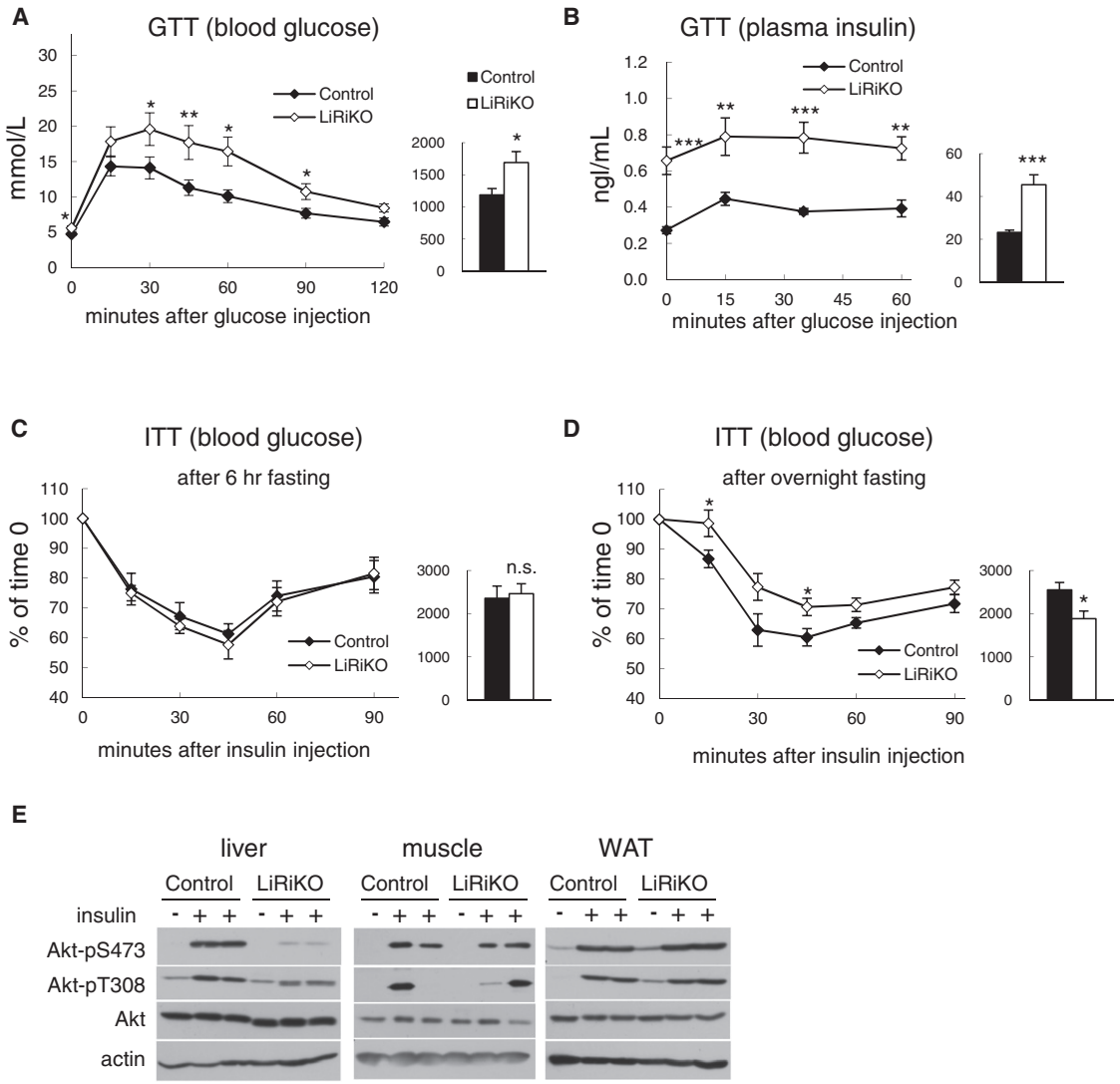


**Figure 2. Deletion of mTORC2 in the Liver Causes Glucose Intolerance**

(A and B) Glucose tolerance test (GTT) in overnight fasted 14-week-old mice. Mice were injected with glucose (2 g/kg) and blood glucose (A) and plasma insulin (B) levels were measured at the indicated times. Bar graphs to the right show the respective area under the curve (AUC) of glucose and insulin (n = 7–8 per group).

(C and D) Insulin tolerance test (ITT) in 6 hr fasted (C) and overnight fasted (D) 16-week-old mice. Mice were injected with insulin (0.5 or 0.25 IU/kg, ip), and blood glucose levels were measured at the indicated times. Results are expressed as percentage of the initial blood glucose levels. Bar graphs to the right show the respective inverse area under the curve (AUC) of glucose (n = 6–7 per group).

Values are expressed as mean ± SEM. * indicates statistical significance from control mice (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(E) Insulin-stimulated phosphorylation of Akt in the liver, skeletal muscle, and epididymal WAT of control and LiRiKO mice. Fourteen-week-old mice were injected with insulin as described in Experimental Procedures.

insulin sensitivity in LiRiKO mice was observed only after overnight fasting, when hepatic gluconeogenesis is more active, suggests that LiRiKO mice have hepatic insulin resistance but normal insulin sensitivity in other tissues. Consistent with this, insulin-induced activation of Akt was normal in both skeletal muscle and adipose tissue in LiRiKO mice (Figure 2E). However, older LiRiKO mice (>8 months) showed impaired glucose clearance even after 6 hr fasting (Figure S2E), suggesting that whole-body insulin sensitivity decreased with age as a consequence of chronic hyperinsulinemia. When fed a HFD, LiRiKO mice displayed glucose intolerance (Figure S2F) and moderate insulin resistance (Figure S2G) already at 14 weeks of age. Thus, LiRiKO mice develop diabetes (glucose intolerance, hyperinsulinemia, and insulin resistance), further suggesting that hepatic mTORC2 controls whole-body metabolic homeostasis.

## Hepatic mTORC2 Is Required for Insulin-Akt Signaling to FoxO1 and GSK3α/β but Not to mTORC1

The above findings suggest that the primary defect of a hepatic mTORC2 deficiency may be insulin resistance in the liver. To investigate this further, we examined insulin signaling, in particular insulin-activated Akt signaling, in the liver. Previous studies have shown that mTORC2 (i.e., Akt Ser473 phosphorylation) appears to be necessary for Akt activity toward some but not

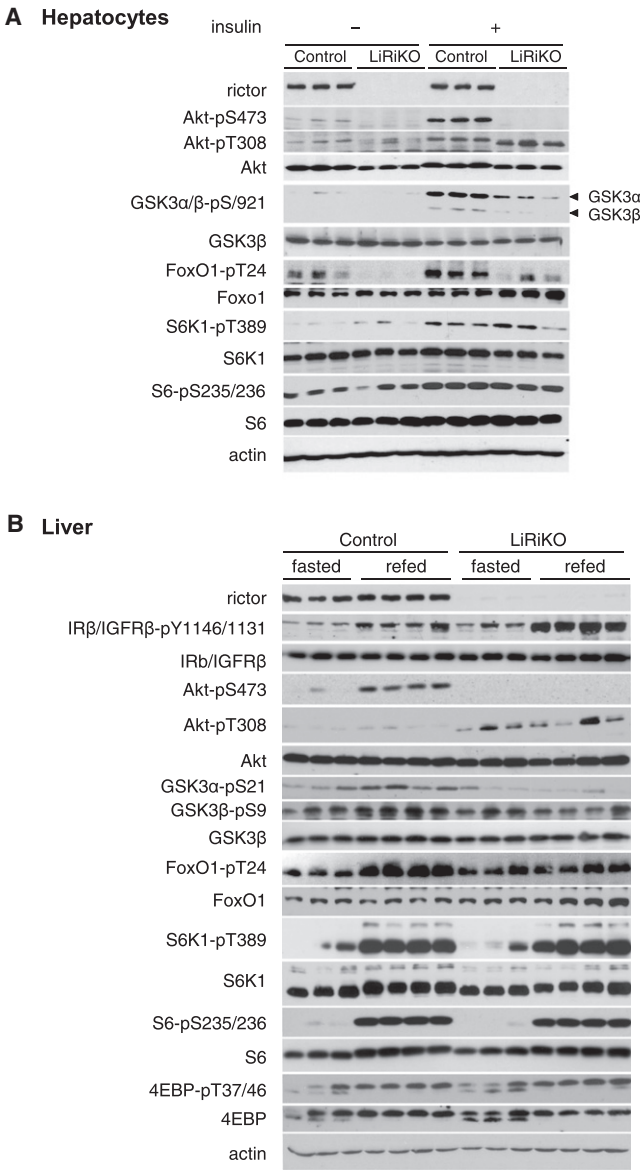


**Figure 3. Hepatic mTORC2 Is Required for Insulin-Akt Signaling to FoxO1 and GSK3α/β but Not to mTORC1**

(A) Western blots of signaling molecules involved in the Akt and mTORC1 pathway in primary hepatocytes stimulated with 50 nM insulin for 30 min.

(B) Western blots of signaling molecules involved in the Akt and mTORC1 pathway in the liver of fasted and refed mice. Fourteen- to sixteen-week-old mice (n = 4–6 per condition) were either fasted or refed a chow diet for 2 hr.

all substrates (Guertin et al., 2006; Jacinto et al., 2006; Polak and Hall, 2006; Sarbassov et al., 2005; Shiota et al., 2006; Yang et al., 2006). We first analyzed the phosphorylation status of Akt and several Akt downstream effectors in insulin-stimulated primary hepatocytes. In control hepatocytes, insulin stimulated phosphorylation of Akt (Ser473 and Thr308), GSK3α/β (Ser21 and Ser9), FoxO1 (Thr24), S6K (Thr389), and S6 (Ser235/236) (Figure 3A). In LiRiKO hepatocytes, insulin failed to stimulate Akt Ser473 phosphorylation whereas Thr308 phosphorylation was normal (Figure 3A), confirming our in vivo findings described above. The Akt substrates GSK3α/β and FoxO1 were significantly hypophosphorylated in insulin-treated LiRiKO hepatocytes (Figure 3A). Importantly, phosphorylation of the Akt downstream effectors S6K and S6 was normal, supporting the previous finding that Ser473 phosphorylation is not necessary for Akt to signal to mTORC1. Next, we examined Akt effectors in the liver of fasted and refed mice. Refeeding stimulated phosphorylation in control and LiRiKO livers in the same manner as observed in insulin-stimulated control and LiRiKO hepatocytes (Figure 3B). The only noteworthy exception in results obtained with hepatocytes versus livers is that Akt Thr308 was hyperphosphorylated in the liver of fasted and refed LiRiKO mice (Figure 3B). This is consistent with the hyperinsulinemia and increased tyrosine phosphorylation of the insulin receptor in LiRiKO mice (Figure 3B). Thus, hepatic mTORC2 is required for insulin-Akt signaling to FoxO1 and GSK3α/β, but not for insulin-Akt signaling to mTORC1. Furthermore, these findings confirm hepatic insulin resistance, albeit partial, in LiRiKO mice.

## Loss of mTORC2 Results in Dysregulated Hepatic Gluconeogenesis and Glycolysis

How might the observed defect in hepatic insulin signaling lead to defects in metabolic homeostasis, e.g., hyperglycemia? As shown above, FoxO1 is hypophosphorylated in the liver of refed LiRiKO mice (Figure 3B). FoxO1 is a transcription factor that functions with the transcriptional coactivator PGC1α to induce gluconeogenic genes such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) in the fasted state. During the fasted to postprandial transition, FoxO1 is phosphorylated by Akt, which results in nuclear exclusion of FoxO1 and inihibition of gluconeogenesis (Matsumoto et al., 2007; Puigserver et al., 2003). Consistent with hypophosphorylation of FoxO1 in the liver of LiRiKO mice, nuclear exclusion of FoxO1 in response to feeding was severely impaired (Figure 4A). Furthermore, visualization of FoxO1 by immunostaining revealed that FoxO1 is preferentially localized in the nucleus in LiRiKO hepatocytes (Figures 4B and S3A). To further investigate the regulation of gluconeogenesis, we examined glucose production and gluconeogenic gene expression. LiRiKO hepatocytes exhibited higher basal glucose production, which was less inhibited by insulin, as compared to controls (Figure 4C). Consistent with these observations, mRNA levels of PEPCK and G6Pase in basal and insulin-treated conditions were significantly higher in LiRiKO hepatocytes (Figure S3B). Higher mRNA levels of gluconeogenesis-related genes were also observed in the liver of refed LiRiKO mice (Figure 4D). Finally, in a pyruvate challenge test, LiRiKO mice showed mildly but significantly higher blood glucose levels compared to controls, further suggesting increased hepatic glucose production capacity (Figure S3C). Thus, gluconeogenesis is constitutively active in the liver of LiRiKO mice, accounting for the observed high levels of blood glucose in both fasted and fed LiRiKO mice.

To further investigate the cause of the hyperglycemia, we examined hepatic glucose uptake and glycolysis. Glucose uptake was significantly reduced in LiRiKO hepatocytes compared to control hepatocytes (Figure 4E). Glucose uptake by hepatocytes is determined by the rate of glycolysis which is mainly dependent on GK activity (Ferre et al., 1996). GK, the first enzyme in the glycolytic pathway, is induced by insulin

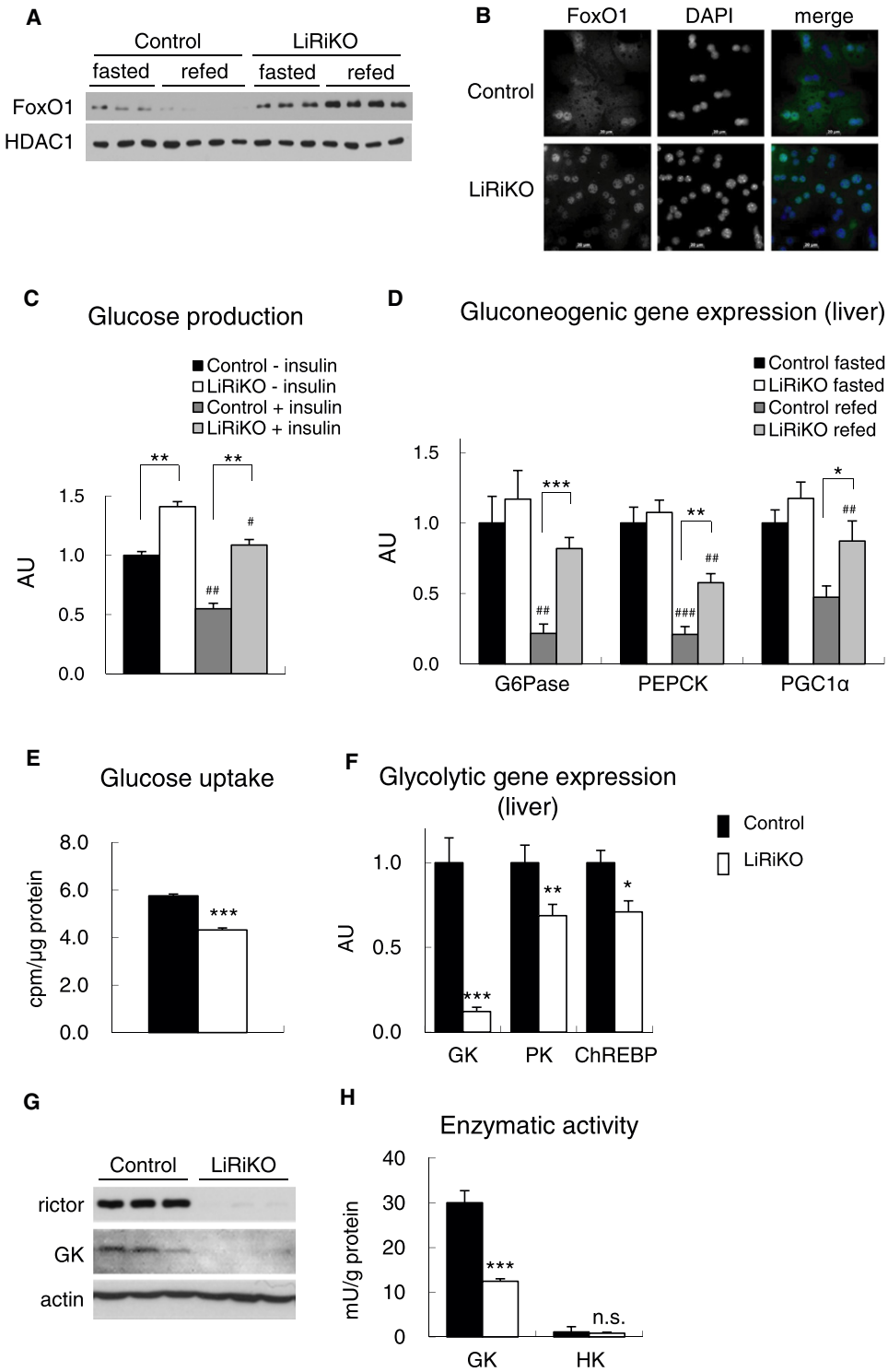


**Figure 4. Loss of mTORC2 Results in Active FoxO1 and Constitutively Elevated Hepatic Gluconeogenesis, Reduced Glucokinase Expression, and Glucose Flux**

(A) FoxO1 protein levels in nuclear fractions from the liver of fasted and refed mice.

(B) Immunofluorescent staining of FoxO1 in primary hepatocytes isolated from control and LiRiKO mice.

(C) Glucose production from primary hepatocytes. Hepatocytes were incubated in glucose production media in the presence or absence of insulin, and glucose release into the media was measured after 12 hr incubation. Results are representative of three independent experiments. Results are normalized to the level of control without insulin.

(D) mRNA levels of G6Pase, PEPCK, and PGC-1α in the liver of control and LiRiKO mice after overnight fasting and after 4 hr of refeeding. Results are normalized to the levels of fasted control mice.

and converts glucose to glucose-6-phosphate (G6P). G6P is metabolized through glycolysis, glycogen synthesis, and via the pentose phosphate shunt. In the liver of LiRiKO mice, GK was markedly decreased, as determined by the levels of GK mRNA, protein, and activity (Figures 4F–4H). This correlated with decreased ChREBP expression and nuclear translocation (Figures 4F and S3D), and low expression of a ChREBP target gene (L-pyruvate kinase) in LiRiKO liver (Figure 4F). Furthermore, insulin-induced glycogen synthesis was also significantly decreased in LiRiKO hepatocytes (Figure S3E). The reduced glycogen synthesis in LiRiKO liver could be explained not only by reduced glucose uptake, but also by lower glycogen synthase (GS) activity, as indicated by higher GS phosphorylation (Figure S3F). This was consistent with the observed hypophosphorylation of GSK3, a kinase responsible for inhibitory phosphorylation of GS, in LiRiKO liver (Figure 3B). In contrast, GLUT2 expression in the liver of LiRiKO mice was not changed (Figures S3G and S3H). Taken together, these results indicate that glucose uptake, glycolysis, and glycogen synthesis are impaired in the liver of LiRiKO mice, possibly due to decreased mTORC2-mediated insulin-induced GK expression. Thus, constitutive gluconeogenesis and defective glycolysis (and glycogen synthesis) together account for the observed hyperglycemia. Furthermore, the above findings suggest that defective mTORC2-Akt signaling (hepatic insulin resistance) accounts for the hyperglycemia in LiRiKO mice.

## Hepatic mTORC2 Is Required for Insulin-Stimulated De Novo Lipid Synthesis

The glycolytic defect described above and the decreased TG levels in the liver of LiRiKO mice suggested that mTORC2 is required for hepatic lipogenesis. Hepatic lipogenesis is induced by insulin via stimulation of lipogenic gene expression. To investigate lipogenesis, we examined both lipogenic gene expression in the liver of refed mice and de novo lipid synthesis in insulin-stimulated primary hepatocytes. mRNA levels of the key lipid synthesis enzymes, including ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), glycerol-3-phosphate acyl transferase (GPAT), and diacylglycerol acyl transferase (DGAT2), were all significantly decreased in the liver of refed LiRiKO mice (Figure 5A). In addition, mRNA expression of SREBP1c and PPAR$\gamma$, two transcription factors that activate lipogenic genes, was significantly reduced in LiRiKO liver (Figure 5A). De novo lipid synthesis was also significantly decreased in insulin-stimulated LiRiKO hepatocytes (Figure 5B). Thus, impaired lipid synthesis in the liver of LiRiKO mice is due, at least in part, to decreased expression of lipogenic genes. Conversely, genes related to fatty acid oxidation, such as acyl-CoA oxidase (ACO), carnitine palmitoyltransferase 1 (CPT1), and PPAR$\alpha$, were increased in fasted LiRiKO mice (Figure S5A). This observation together with a trend of increased plasma ketone bodies ($\beta$-hydroxybutyrate) (Figure S5B) suggests higher fatty acid oxidation in the liver of LiRiKO mice. Moreover, expression of genes encoding fatty acid uptake, including fatty acid-binding protein 1 (FABP1) and CD36, was significantly decreased in the liver of HFD-fed LiRiKO mice, while expression of genes encoding fatty acid export, including ApoB and MTP, was unaffected (Figure S5C). Taken together, these observations suggest that the mTORC2-deficient liver displays reduced TG levels due to reduced lipogenesis, increased fatty acid oxidation, and reduced uptake.

Postprandial expression of lipogenic genes is mediated mainly by insulin-induced activation of SREBP1c (Shimomura et al., 1999). SREBP1c is synthesized as an ER bound precursor. Upon activation by insulin, SREBP1c moves to the Golgi where it is proteolytically processed into a mature form. The cleaved mature form of SREBP1c translocates into the nucleus, where it upregulates target lipogenic genes (Horton et al., 2002). Insulin-mediated activation of SREBP1c is Akt dependent (Yellaturu et al., 2009a). Interestingly, recent cell culture-based studies suggest that Akt stimulates lipogenesis via mTORC1-dependent SREBP1 activation (Düvel et al., 2010; Li et al., 2010; Peterson et al., 2011; Porstmann et al., 2008; Yecies et al., 2011), whereas mainly in vivo studies suggest that Akt activates SREBP1c and lipogenesis via an mTORC1-independent pathway (Wan et al., 2011; Yecies et al., 2011). To investigate further the role of mTORC2(-Akt) signaling in lipogenesis, we examined SREBP1c activation in the liver of refed LiRiKO mice. In particular, we examined the levels of precursor and mature forms of SREBP1c. The mature nuclear form of SREBP1c was significantly decreased in the liver of refed LiRiKO mice, whereas the precursor form was unchanged (Figure 5C), suggesting that the posttranslational activation of SREBP1c is defective in LiRiKO liver. Expression of the SREBP1c target genes ACC and FAS was reduced (Figures 5A and 5C). Thus, mTORC2 is required for SREBP1c activation and lipogenesis in the liver.

## Constitutively Active Akt2 Restores Glucose Flux and Lipogenesis in mTORC2-Deficient Hepatocytes

The above findings suggest that loss of hepatic mTORC2 results in defective Akt activation (Ser473 phosphorylation) which eventually results in impaired metabolic homeostasis. To investigate further whether loss of mTORC2-mediated Akt Ser473 phosphorylation is responsible for the defects in glycolysis and lipogenesis observed in LiRiKO hepatocytes, we determined if a constitutively active Akt is able to suppress these defects. By adenoviral gene transfer, we introduced an Akt2 mutant in which Ser474 (equivalent to Ser473 in Akt1) is mutated to phosphomimetic aspartic acid (Akt2-S474D). This Akt mutant does not require mTORC2 for activation and is thus considered constitutively active. LacZ and wild-type Akt2 (Akt2-WT) were used as controls. Insulin-stimulated phosphorylation of Akt substrates FoxO1 and GSK3$\beta$ was significantly reduced in

(E) 2-deoxyglucose uptake in primary hepatocytes. Results are representative of three independent experiments.
(F) mRNA expression levels of GK, liver-type pyruvate kinase (PK) and ChREBP in the liver of 4 hr refed mice. Results are normalized to the levels of control mice.
(G) Western blot analysis of GK expression in the liver of ad libitum fed mice.
(H) Enzymatic activity of GK and hexokinase in the liver of ad libitum fed mice. Results are representative of three independent experiments. Black and dark gray bars represent control, and white and light gray bars represent LiRiKO (n = 6–7 per group for mice). Values are expressed as mean ± SEM. * indicates statistical significance from control, # indicates statistical significance from respective fasted or untreated control. ($^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,\#\#\#}p < 0.001$).

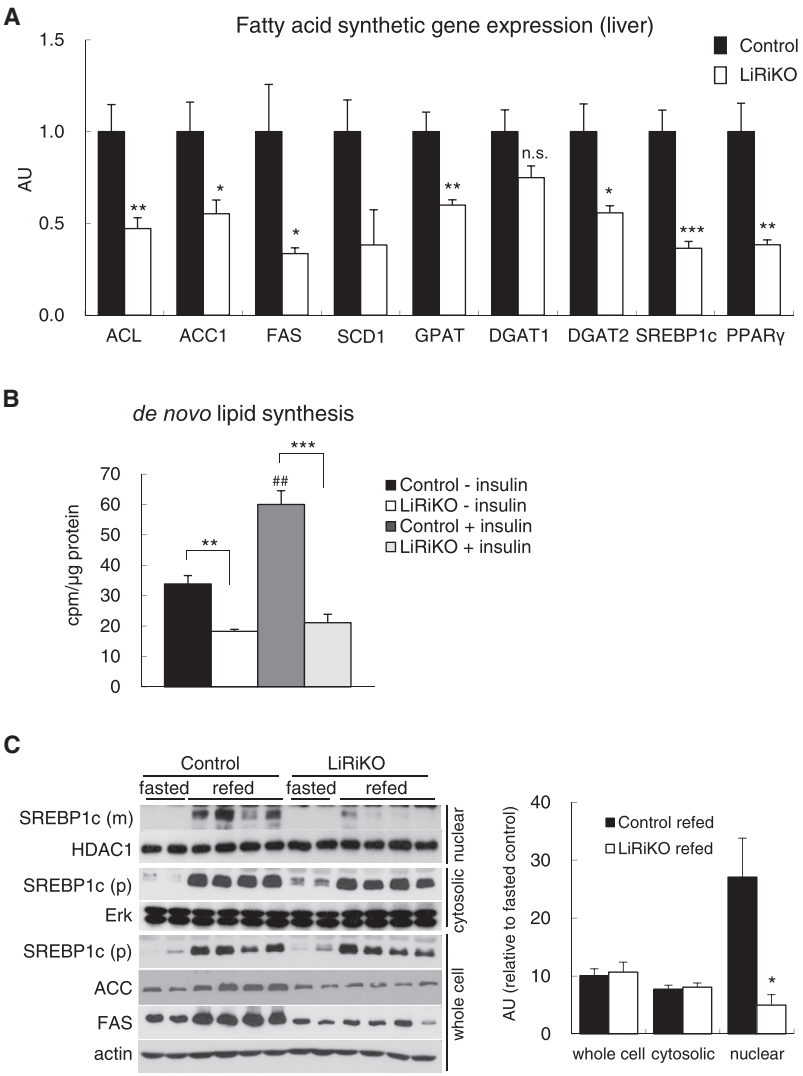


**Figure 5. Hepatic mTORC2 Is Required for Insulin-Stimulated De Novo Lipid Synthesis**

(A) mRNA levels of genes involved in de novo lipid synthesis including ACL, ACC1, FAS, SCD1, GPAT, DGAT1/2, SREBP1c, and PPARγ, in the liver of mice after 4 hr refeeding. Results are normalized to the levels of control mice. Black bars represent control mice and white bars represent LiRiKO mice (n = 6–7 per group).

(B) De novo lipid synthesis in primary hepatocytes. Serum-starved primary hepatocytes were stimulated with 50 nM insulin for 12 hr. $^{14}$C-labeled acetate incorporation into cellular lipids was determined as described in Experimental Procedures. Results are representative of three independent experiments. Black and dark gray bars represent control hepatocytes, and white and light gray bars represent LiRiKO hepatocytes.

(C) SREBP1c processing was analyzed by western blot analysis on nuclear and cytosolic fractions from the liver of mice after 4 hr refeeding. The precursor form of SREBP1c (SREBP1c(p)) was detected in cytosolic fraction and whole cell lysates, and the mature form of SREBP1c (SREBP1c(m)) was detected in nuclear fraction. HDAC1 and Erk were used as marker proteins of nuclear and cytosolic fractions, respectively. Protein levels of SREBP1c target genes (ACC and FAS) were also analyzed using whole-cell lysates. Bar graph to the right represents quantification of band intensities of SREBP1c normalized to its respective internal control. Average value from fasted controls was set to 1. Values are the mean ± SEM, * indicates statistical significance from control, and # indicates statistical significance from respective insulin-untreated control (*, #$p < 0.05$, **, ##$p < 0.01$, ***, ###$p < 0.001$).

lacZ- or Akt2-WT-expressing LiRiKO hepatocytes compared to control hepatocytes (Figure 6A). In contrast, mild overexpression of Akt2-S474D increased insulin-stimulated phosphorylation of FoxO1 and GSK3β in LiRiKO hepatocytes, as in control hepatocytes (Figure 6A). Importantly, restoration of Akt signaling in LiRiKO hepatocytes suppressed the previously observed defects in glucose uptake (Figure 6B) and de novo lipid synthesis (Figure 6C). Furthermore, Akt2-S474D restored expression of GK, SREBP1c, and FAS, in LiRiKO hepatocytes (Figure 6D). Thus, mTORC2-mediated Akt phosphorylation is essential for insulin-induced glycolysis and lipid synthesis in the liver.

### Restoration of Glucose Flux Is Not Sufficient to Rescue De Novo Lipid Synthesis in mTORC2-Deficient Hepatocytes

mTORC2-mediated Akt phosphorylation enhances both insulin signaling and glucose flux, which then contribute to activation of lipogenesis (see Introduction and above). To investigate if the defect in lipogensis observed in LiRiKO hepatocytes is due to a defect in glucose flux, we restored glucose flux by overexpressing GK (Figure 7A) and examined if this was sufficient to suppress the lipogenesis defect. Overexpressed GK enhanced glucose uptake to a similar extent in LiRiKO and control hepatocytes (Figure 7B). Consistent with elevated glucose uptake, expression of genes encoding ChREBP, pyruvate kinase, and lipogenic enzymes (FAS, ACC1/2) was significantly increased in GK-overexpressing LiRiKO and control hepatocytes (Figure 7C). However, the defect in de novo lipid synthesis was not suppressed in LiRiKO hepatocytes (Figure 7D). Whereas de novo lipid synthesis was significantly elevated upon GK overexpression, the increase was similar to that observed in control cells (about 2.6-fold), and LiRiKO hepatocytes still displayed only 30% activity compared to control cells (Figure 7D). Thus, glucose flux, although required, is not sufficient to induce lipogenesis. Overexpressed GK did not suppress the defect in Akt Ser473 phosphorylation (Figure 7A) or rescue expression of SREBP1c and its target gene *SCD1* (Figure 7C), indicating that activation of Akt and SREBP1c is essential for lipogenesis in the liver. Taken together, the above findings suggest that mTORC2 activates Akt, which stimulates glycolysis and lipogenesis via GK and SREBP1c (Figure 7E).

## DISCUSSION

In this study, we demonstrate that deletion of mTORC2 in the liver (LiRiKO mice) leads to enhanced gluconeogenesis and

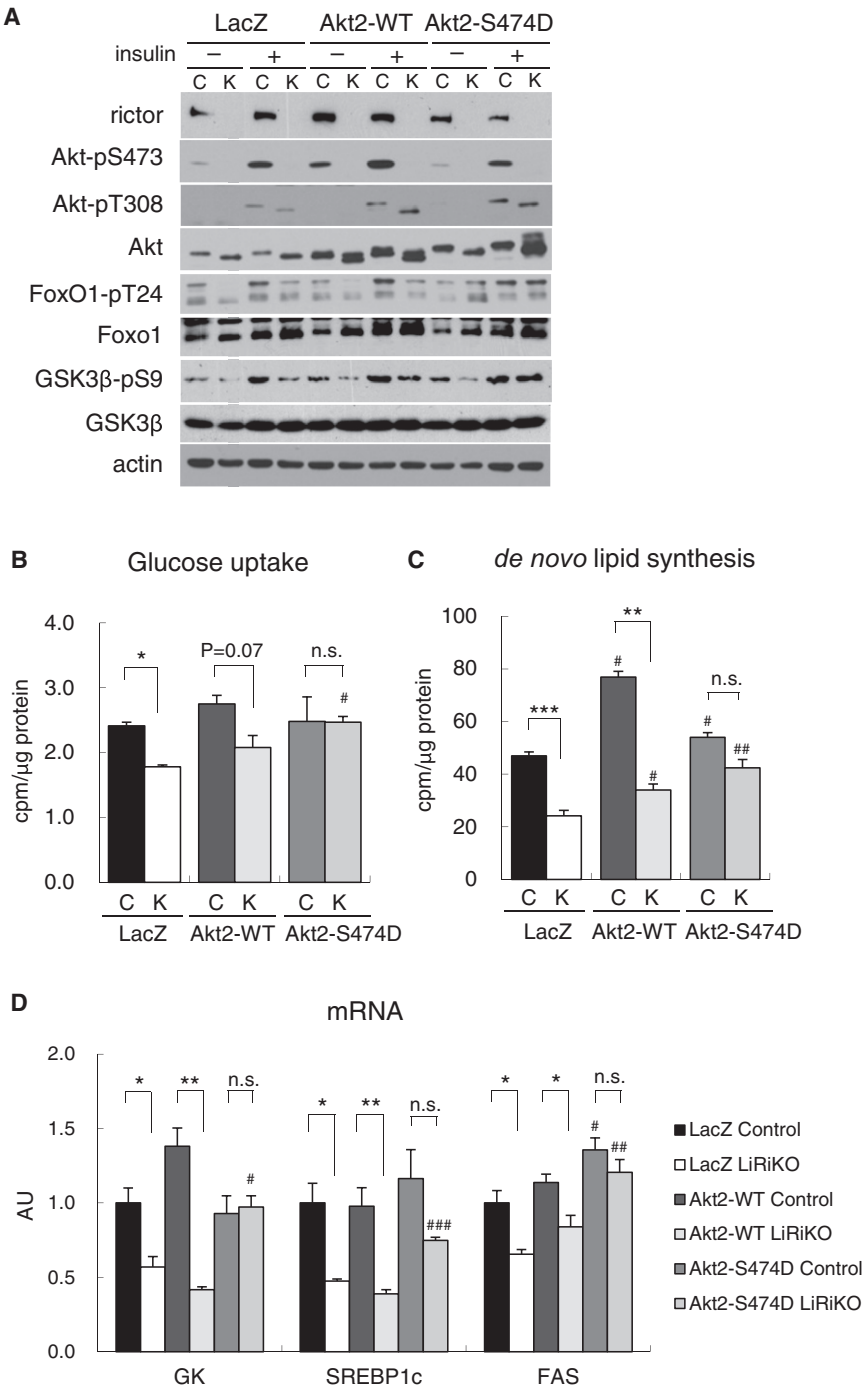


**Figure 6. Expression of Constitutively Active Akt2 Restores Glucose Uptake and Lipogenesis in mTORC2-Deficient Hepatocytes**

(A) Western blots of control (C) and LiRiKO (K) hepatocytes infected with adenoviruses expressing lacZ, Akt2-WT, and Akt2-S474D. Infected cells were serum starved and stimulated with 50 nM insulin for 30 min.

(B) Glucose uptake by hepatocytes infected with adenoviruses as in (A).

(C) De novo lipid synthesis in hepatocytes infected with adenoviruses as in (A).

(D) mRNA expression levels of GK, SREBP1c, and FAS in hepatocytes infected with adenoviruses as in (A). Results are normalized to the levels of lacZ expressing control hepatocytes.

Values are the mean ± SEM. * indicates statistical significance from control in the same condition and # indicates statistical significance from respective lacZ expressing control ($^{*,\ \#}p < 0.05$, $^{**,\ \#\#}p < 0.01$, $^{***,\ \#\#\#}p < 0.001$).

decreased glycolysis, glycogen synthesis, and lipogenesis in the fed state, despite hyperglycemia and hyperinsulinemia. This suggests that the mTORC2-deficient liver is unable to make the fasted to postprandial transition. In other words, the mTORC2-deficient liver cannot sense satiety. The constitutively active gluconeogenesis was due to loss of both Akt Ser473 phosphorylation and FoxO1 inhibition, and decreased glycolysis was due to reduced GK and glucose uptake. These two defects in hepatic glucose metabolism in turn led to systemic hyperglycemia and hyperinsulinemia. The decreased hepatic lipogenesis was due to reduced SREBP1c activation and low glucose flux. Finally, constitutively active Akt restored both glucose uptake and lipogenesis, whereas overexpression of GK (enhanced glucose flux) failed to restore lipogenesis. Our findings suggest that hepatic mTORC2 controls Akt Ser473 phosphorylation, and thereby GK and SREBP1c, to coordinate glucose and lipid metabolism (Figures 7E and S5).

Another important observation in this study is that LiRiKO mice are phenotypically similar (glucose intolerance, hyperglycemia, hyperinsulinemia, hypolipidemia, and smaller liver size with decreased glycogen content) to many other genetically modified mice that have more severe defects in the hepatic insulin-PI3K-Akt signaling pathway. The mice with more severely defective insulin signaling include liver-specific insulin receptor knockout mice (Biddinger et al., 2008; Michael et al., 2000), single and double hepatic IRS1/2 knockout mice (Dong et al., 2006; Kubota et al., 2008), and mice with deletion of regulatory subunits of PI3K in the liver (Miyake et al., 2002; Taniguchi et al., 2006). Since insulin signaling is completely ablated in these mice, a broad spectrum of downstream targets is affected, including both mTORC1 and mTORC2. In contrast, deletion of mTORC2 results in loss of phosphorylation of the hydrophobic motif, including Akt Ser473 phosphorylation, in a subset of AGC kinase family members, without affecting mTORC1 signaling. The loss of Akt Ser473 phosphorylation accounts for constitutively active FoxO1 and GSK3 and for the loss of

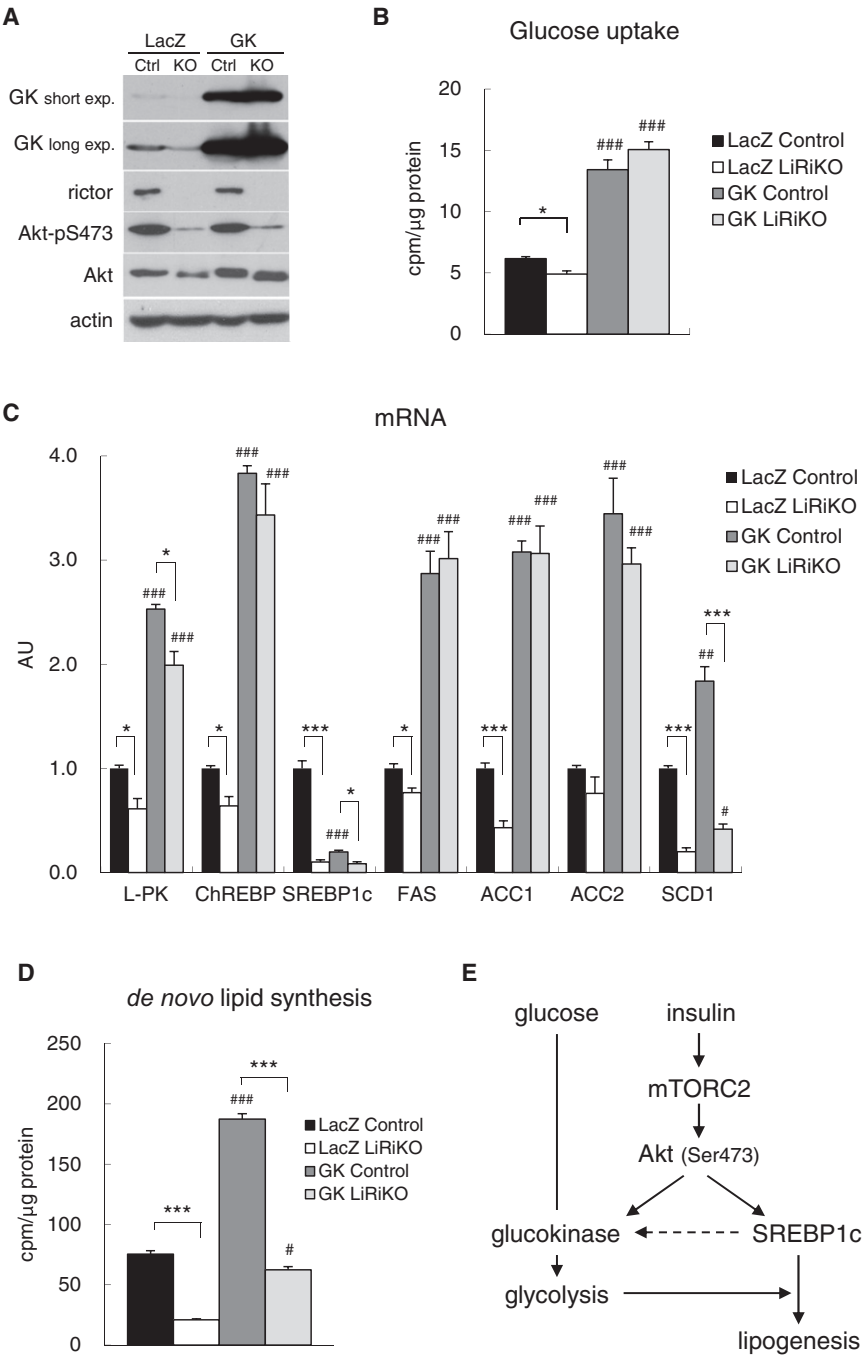


**Figure 7. Restoration of Glucose Flux Is Not Sufficient to Rescue De Novo Lipid Synthesis in mTORC2-Deficient Hepatocytes**

(A) Western blots of hepatocytes infected with adenoviruses expressing lacZ or GK.

(B) Glucose uptake by hepatocytes infected with indicated adenoviruses.

(C) mRNA expression levels of glycolytic and lipogenic genes. Results are normalized to the levels of lacZ-expressing control hepatocytes.

(D) De novo lipid synthesis in hepatocytes infected with indicated adenoviruses. Black and dark gray bars represent control hepatocytes, white and light gray bars represent LiRiKO hepatocytes. Values are expressed as mean ± SEM of three independent experiments. * indicates statistical significance from control in the same condition and # indicates statistical significance from respective lacZ-expressing control (*, #$p < 0.05$, **, ##$p < 0.01$, ***, ###$p < 0.001$).

(E) Model for the role of mTORC2 in the liver.

SREBP1c and GK activities, and thereby appears to be largely responsible for the phenotype of LiRiKO mice. Thus, our findings reveal a particularly important role for mTORC2 and Akt Ser473 phosphorylation in the hepatic insulin pathway in controlling liver and whole-body metabolism.

Akt is a major regulator of insulin-induced lipogenesis in the liver. Hepatic overexpression of constitutively active Akt or deletion of hepatic PTEN increases TG synthesis (Horie et al., 2004; Ono et al., 2003; Stiles et al., 2004). Conversely, liver-specific deletion of Akt2, the major Akt isoform in the liver, protects against diet- or PTEN loss-induced hepatic steatosis (He et al., 2010; Leavens et al., 2009). Akt promotes lipid synthesis primarily through the activation of SREBP1c, a master transcription factor of lipogenic genes. How does Akt activate SREBP1c? Several cell culture-based studies have suggested that Akt activates SREBP1c and lipogenesis via mTORC1 (Düvel et al., 2010; Li et al., 2010; Porstmann et al., 2008; Yecies et al., 2011). Furthermore, a recent study showed that mTORC1 regulates SREBP by controlling nuclear translocation of lipin1, an inhibitor of SREBP activity (Peterson et al., 2011). However, recent animal-based studies using liver-specific TSC1 or Akt2 knockout mice have shown that mTORC1 activation is not sufficient to stimulate SREBP1c without Akt signaling (Kenerson et al., 2011; Wan et al., 2011; Yecies et al., 2011). Yecies et al. presented evidence that Akt activates SREBP1c via inhibition of expression of Insig2, an anchor protein that retains SREBP1c on the ER membrane (Yellaturu et al., 2009b). Another potential mechanism by which Akt activates SREBP1c is via Akt-mediated phosphorylation and inhibition of GSK3. GSK3 phosphorylates mature SREBP1 and thereby promotes its degradation by the ubiquitin-proteasome pathway (Bengoechea-Alonso and Ericsson, 2009; Kim et al., 2004; Sundqvist et al., 2005). Which of the above mechanism(s) of Akt-mediated activation of SREBP1c is defective in LiRiKO mice? Our finding that SREBP1c activation and lipogenenesis are impaired in LiRiKO liver, where mTORC2 is defective but mTORC1 is active, suggest that Akt signals to SREBP1c in the liver at least in part independently of mTORC1. Also, we observed no significant difference in *Insig2a* expression in the

liver of refed LiRiKO mice compared to controls (data not shown). However, we did observe a defect in Akt-mediated GSK3 phosphorylation and decreased expression of mature SREBP1c. Thus, constitutively active GSK3 and enhanced degradation of mature SREBP1c may be the mechanism underlying the observed defect in SREBP1c activation in LiRiKO mice.

Though Akt-activated SREBP1c is considered a primary mediator of insulin-stimulated lipogenesis, GK-activated glucose flux also mediates induction of lipogenesis in the liver (Dentin et al., 2005). In liver-specific GK knockout mice, glycolytic and lipogenic genes are not maximally induced upon refeeding, even though SREBP1c activation is normal (Dentin et al., 2004). Moreover, a high carbohydrate diet induces expression of glycolytic and lipogenic genes even in SREBP1c-deficient mice (Liang et al., 2002). GK-dependent and SREBP1c-independent induction of glycolytic (pyruvate kinase) and lipogenic (FAS, ACC) genes appears to be mediated by the transcription factor ChREBP (Uyeda and Repa, 2006). GK is necessary for both expression and activation of ChREBP. Therefore, GK/glucose flux and SREBP1c act synergistically in the stimulation of lipogenesis. In the liver of LiRiKO mice, lipogenesis was impaired due to a decrease in both GK and SREBP1c activity. The decrease in GK expression in the liver of LiRiKO mice is due to a decrease in Akt signaling (the defect is suppressed by activated Akt). GK gene expression is regulated by a number of Akt-dependent transcriptional factors, including FoxO1, HIF-1$\alpha$/HNF4/p300, SREBP1c, LXR$\alpha$, and PPAR$\gamma$ (Hirota et al., 2008; Kim et al., 2009;Massa et al., 2011; Roth et al., 2004). Indeed, phosphorylation of FoxO1 and expression of SREBP1c and PPAR$\gamma$ were decreased in the liver of LiRiKO mice. Further studies are required to determine which mTORC2-regulated transcription factor(s) is lacking and thus unable to activate GK expression in LiRiKO mice.

We provide evidence that overexpression of GK in LiRiKO hepatocytes restores expression of glycolytic and lipogenic genes in the absence of Akt Ser473 phosphorylation and SREBP1c activation. However, the level of de novo lipid synthesis induced by GK overexpression in LiRiKO hepatocytes was less than that of control hepatocytes overexpressing GK, despite a similar rate of glucose uptake, suggesting that glucose flux is not sufficient to stimulate lipogenesis. Akt signaling and SREBP1c activation are also required for induction of lipogenesis. Thus, mTORC2 is required for the coordinated regulation, by GK and SREBP1c, of insulin-induced glycolysis and lipogenesis.

In summary, the present study demonstrates the functional importance of hepatic mTORC2 in the regulation of glucose and lipid homeostasis. Hepatic mTORC2 inhibition results in decreased glucose metabolism caused by decreased Akt activity (loss of Akt Ser473 phosphorylation) and subsequently reduced SREBP1c and GK activities. These defects in turn lead to hyperglycemia and hyperinsulinemia. These findings should be taken into account when considering clinical use of emerging mTOR inhibitors that target mTORC2 in addition to mTORC1 (Benjamin et al., 2011). Although detailed safety profiles of mTOR kinase inhibitors remain to be seen, inhibition of mTORC2 may contribute to adverse metabolic effects (Markman et al., 2010; Zhang et al., 2011). Our findings suggest that a defect in hepatic mTORC2 signaling could contribute to metabolic disorders.

## EXPERIMENTAL PROCEDURES

A complete version of Experimental Procedures is shown in Supplemental Information.

### Animals

*rictor*$^{fl/fl}$ mice were generated as described previously (Cybulski et al., 2009). Liver-specific rictor knockout mice were generated by crossing *rictor*$^{fl/fl}$ mice with transgenic mice expressing Cre recombinase under the control of the hepatocyte specific *albumin* promoter (C57BL/6-Tg(*Alb-cre*)21Mgn/J; Jackson Laboratory). The resultant *rictor*$^{fl/+}$ *Alb-Cre*$^{Tg/0}$ progeny were crossed with *rictor*$^{fl/fl}$ mice to obtain tissue-specific knockout mice (*rictor*$^{fl/fl}$ *Alb-Cre*$^{Tg/0}$), termed liver-specific rictor knockout (LiRiKO) mice. Littermates without the *Cre* gene were used as wild-type control mice (*rictor*$^{fl/fl}$). All experiments were conducted on male mice between 12 to 17 weeks of age, unless otherwise indicated. All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinaeramt of Kanton Basel-Stadt.

### Glucose, Insulin, and Pyruvate Tolerance Tests

For glucose tolerance tests (GTT), mice were fasted overnight (16 hr) then administrated 2 g/kg of body weight of glucose by intraperitoneal (i.p.) injection. For insulin tolerance tests (ITT), mice were fasted for 6 hr or overnight before i.p. administration of 0.5 or 0.25 unit/kg of body weight of insulin (Actrapid). For pyruvate challenge test, mice were fasted overnight then administrated 2 g/kg of body weight of sodium pyruvate by i.p. injection. Blood glucose concentrations were measured before and after the injection at the indicated time points.

### Adenovirus Generation

We generated adenoviruses encoding wild-type Akt2 and a phosphomimetic Akt2-S474D mutant using the ViraPower Adenoviral Gateway Expression System (Invitrogen). In brief, wild-type human Akt2 and a phosphomimetic Akt2-S474D mutant, in which Ser at residue 474 is substituted by Asp, were subcloned by PCR from pFastBac-PKB$\beta$ and pFastBac- PKB$\beta$-S474D clones (clones were kind gifts of Brian A. Hemmings, FMI, Switzerland) and cloned into pDONR™221 vectors. Entry clones and the pAd/CMV-DEST vector were recombined to generate expression clones (pAd/CMV-Akt2-WT and pAd/CMV-Akt-S474D). Each expression clone was transfected into HEK293A cells to produce adenoviruses. After several amplifications by infecting HEK293A cells, we determined the titer of each adenovirus. AxCA-GK and AxCA-lacZ adenoviruses were a kind gift of Wataru Ogawa (Kobe University, Japan).

### Primary Hepatocyte Isolation and Cultures

Hepatocytes were isolated from 8- to 12-week-old male mice using the standard two-step collagenase perfusion protocol. Adenoviral infection experiments were performed 3 hr after plating at an moi of 1 for Akt2 restoration and moi of 5 for GK restoration. Six hours after infection, cells were washed with PBS and incubated overnight with DMEM (5 mM glucose) containing 100 nM dexamethasone and 1 nM insulin. Experiments were performed the next day. For insulin stimulation, hepatocytes were cultured in high glucose DMEM (25 mM glucose) with 10 or 50 nM insulin for the indicated times.

### Western Blotting

Whole-protein extraction from tissue was performed as described (Polak et al., 2008). Nuclear and cytosolic fractions were prepared using the NE-PER nuclear protein extraction kit (Pierce). Lysates from primary hepatocytes were prepared using lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1% Triton, protease inhibitor, and phosphatase inhibitor. Forty micrograms of protein were loaded for SDS-PAGE and analyzed with the indicated antibodies. Immunoblots were performed using the antibodies against the following proteins: rictor, Akt, Akt (pThr308), Akt (pSer473), GSK3$\alpha$/$\beta$, GSK3$\alpha$/$\beta$ (pSer9/21), FoxO1, FoxO1/3a (pThr24/32), IR$\beta$/IGFR$\beta$, IR$\beta$/IGFR$\beta$ (pTyr1146/1131), S6K1, S6K1 (pThr389), S6, S6 (pSer235/236), 4EBP, 4EBP (pThr37/46), ACC, FAS, Erk, and HDAC (all from Cell Signaling), glucokinase and SREBP1c (Santa Cruz), ChREBP (Novus

Biologicals), Glut2 (a kind gift from Bernard Thorens, University of Lausanne, Switzerland), and actin (Chemicon).

### RNA Isolation and Quantitative PCR

RNA isolation and cDNA synthesis were performed as described (Polak et al., 2008). qPCR was performed using the power SYBR green mix (Applied Biosystems) and quantitated using Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems). Duplicate runs of each sample were normalized to 16S rRNA (liver) or cyclophilin (hepatocytes) to determine relative expression levels. The sequences for the primer pairs used in this study are listed in Table S1.

### Glucose Production Assay

Glucose production assay was performed following Yoon et al. (2001) with modifications. Briefly, primary hepatocytes were cultured in DMEM (5 mM glucose) containing 100 nM dexamethasone in the presence or absence of 50 nM insulin for 16 hr. Cells were washed three times with glucose-free DMEM without phenol red and then incubated with glucose production solution containing 2 mM sodium pyruvate, 20 mM lactate, 100 nM dexamethasone, and 1 mM 8-bromo-cAMP with or without 100 nM insulin. After 12 hr of incubation, glucose concentration in the media was measured using a glucose oxidase kit (Sigma), normalized to the protein amount of the cells. The assay was done in triplicate and values were expressed as arbitrary units relative to control hepatocytes without insulin.

### Measurement of Glucose Uptake

Glucose uptake by primary hepatocytes was analyzed following Yang et al. (2002) with modifications. Briefly, after 12 hr serum starvation, cells were cultured in DMEM containing 25 mM glucose, 100 nM dexamethasone, and 10 nM (in the Akt2 restoration experiment) or 50 nM (in the GK restoration experiment) insulin for 12 hr. To measure the rate of glucose uptake, cells were washed three times with PBS and then incubated for 3 hr in DMEM containing 1 μCi/ml 2-Deoxy-2[1,2-$^{3}$H]glucose (PerkinElmer Life Sciences). The cells were washed three times with ice-cold PBS and solubilized in 1% SDS. The radioactivity of an aliquot was determined in a scintillation counter. Each assay was done in triplicate, and results were expressed as cpm in the cell lysate/mg of protein.

### De Novo Lipid Synthesis Measurement

For de novo lipogenesis analysis, primary hepatocytes were precultured in the same way as for the glucose uptake experiment. Then cells were incubated in culture medium containing 0.5 mM sodium acetate and 0.8 μCi/ml [1-$^{14}$C] acetic acid (Amersham) for 2 hr. After washing with PBS, the cells were lyzed in 0.5 M KOH. The lysates were neutralized with 6% $Na_2SO_4$, then the lipids were extracted using sequential extraction with chloroform:methanol (2:1) and chloroform:methanol:$H_2O$ (10:10:3). Radioactivity in lipid-containing phase was measured using scintillation counter. Each assay was done in triplicate, and results were expressed as cpm in the original cell lysate/mg of protein.

### Statistical analysis

Student's unpaired t test was used to determine differences among two groups. Significance was judged when p value is less than 0.05. Error bars in figures represent standard error of the mean (SEM).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2012.03.015.

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