Hepatocyte Rap1a contributes to obesity- and statin-associated hyperglycemia

Graphical abstract

Highlights

- The hepatocyte small GTPase Rap1a suppresses gluconeogenesis via the Akt-FoxO1 pathway
- Restoring hepatic Rap1a activity improves obesity-associated glucose intolerance
- Rap1a is inhibited by the geranylgeranylation inhibitor statin in hepatocytes and liver
- Geranylgeraniol treatment improves statin-induced glucose intolerance

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In brief

Wang et al. show that activation of hepatic Rap1a suppresses gluconeogenic gene expression and improves glucose intolerance via Akt-mediated FoxO1 inhibition. Statins lower intracellular isoprenoid levels and inhibit Rap1a activation, which contributes to their hyperglycemic effect. These findings identify a role of hepatic Rap1a in obesity- and statin-associated glucose homeostasis.
Hepatocyte Rap1a contributes to obesity- and statin-associated hyperglycemia

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SUMMARY

Excessive hepatic glucose production contributes to the development of hyperglycemia and is a key feature of type 2 diabetes. Here, we report that activation of hepatocyte Rap1a suppresses gluconeogenic gene expression and glucose production, whereas Rap1a silencing stimulates them. Rap1a activation is suppressed in obese mouse liver, and restoring its activity improves glucose intolerance. As Rap1a’s membrane localization and activation depends on its geranylgeranylation, which is inhibited by statins, we show that statin-treated hepatocytes and the human liver have lower active-Rap1a levels. Similar to Rap1a inhibition, statins stimulate hepatic gluconeogenesis and increase fasting blood glucose in obese mice. Geranylgeraniol treatment, which acts as the precursor for geranylgeranyl isoprenoids, restores Rap1a activity and improves statin-mediated glucose intolerance. Mechanistically, Rap1a activation induces actin polymerization, which suppresses gluconeogenesis by Akt-mediated FoxO1 inhibition. Thus, Rap1a regulates hepatic glucose homeostasis, and blocking its activity, via lowering geranylgeranyl isoprenoids, contributes to statin-induced glucose intolerance.

INTRODUCTION

A major cause of hyperglycemia in type 2 diabetes (T2D) is enhanced hepatic glucose production (HGP), which results from an increase in gluconeogenesis (Lin and Accili, 2011; Magnusson et al., 1992). Several factors, including the availability of substrates and an imbalance between glucagon and insulin action, contribute to this process (Petersen et al., 2017). Glucagon plays a major role in hepatic gluconeogenesis, in part by increasing cyclic AMP (cAMP) and intracellular calcium levels, which results in increased expression of two key gluconeogenic enzymes, phosphoenol pyruvate carboxykinase (PCK1) and glucose 6 phosphatase (G6PC) (Ramnanan et al., 2011). A number of transcription factors and coactivators including forkhead box O1 (FoxO1) and cAMP response element-binding protein (CREB) activate and stimulate the transcription of these gluconeogenic genes in T2D; however, little is known about the endogenous regulatory mechanisms that prevent aberrant activation of this pathway.

One of the major complications of T2D is to increase the risk for developing cardiovascular disease, and the majority of T2D patients use cholesterol-lowering statin drugs (Benjamin et al., 2018). By inhibiting HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway, statins decrease hepatic cholesterol synthesis and increase low-density lipoprotein (LDL) receptor (LDLR) levels, which results in enhanced LDL clearance from the plasma (Baigent et al., 2005; Goldstein and Brown, 2015). Although they are generally considered to be safe and well-tolerated, recent evidence from meta-analyses of major statin trials showed that statin therapy is associated with a ~10%–11% increased risk for new-onset T2D (Casula et al., 2017; Ridker et al., 2008; Sattar et al., 2010). The risk is dose-dependent and increased by close to 46% in older patients and patients with pre-existing risk factors for T2D (Cederberg et al., 2015; Crandall et al., 2017; Preiss et al., 2011). These results prompted US Food and Drug Administration (FDA) to approve labeling changes in statins to include a warning about the possibility of increased blood sugar (FDA, 2012). Interestingly, loss-of-function mutations in HMGCR, which encodes HMG-CoA reductase, are also associated with increased incidence of new-onset T2D, suggesting that the diabetogenic effect of statins is “on-target” (Ference et al., 2016; Swerdlow et al., 2015). Despite these findings, the cellular and molecular mechanisms that link statins to increased T2D risk are largely unknown. Besides lowering plasma LDL levels, statins have additional actions, which involve inhibition of the mevalonate pathway. Mevalonate is the precursor for the generation of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP) isoprenoids that are required for the prenylation and activation of a subset of proteins, including the small GTPase, Ras-related protein 1A (Rap1a).

Rap1a belongs to the Ras superfamily of GTPases and cycles between an inactive GDP-bound form and an active GTP-bound form (Gloeirich and Bos, 2011; Kitayama et al., 1989; Wittchen et al., 2011). Post-translational covalent attachment of geranylgeranyl isoprenoid chain to Rap1a results in its membrane
A (HCs) **Rapgef4**

B (HCs)

C (HCs)

D (HCs)

E (HCs)

F (HCs)

G (DIO Rap1a<sup>fl/fl</sup> liver)

H (DIO Rap1a<sup>fl/fl</sup> liver)

I (DIO Rap1a<sup>fl/fl</sup>)

J (DIO Rap1a<sup>fl/fl</sup> liver)

K (DIO Rap1a<sup>fl/fl</sup>)

L (DIO Rap1a<sup>fl/fl</sup>)

(legend on next page)
A recent GWAS study demonstrated that variants in gluconeogenesis and increases HGP. Inhibition of Epac2 or Rap1a stimulates glucose intolerance. These findings identify Rap1a as an important mediator of obesity- and statin-induced glucose intolerance.

In the present study, we explored the role of Rap1a in liver glucose metabolism and show that hepatic Rap1a suppresses gluconeogenesis and glucose production via regulating actin polymerization and Akt-mediated FoxO1 inhibition. Hepatic Rap1a activity is significantly lower in obese mice, and overexpression of a constitutively active variant form of Rap1a in the liver lowers blood glucose and improves glucose intolerance. Given that geranylgeranylation is required for Rap1a to become fully activated, we found that Rap1a activity is also decreased in statin-treated primary hepatocytes and human liver samples from statin users. Accordingly, statin treatment stimulates glucose production in vitro and further increases fasting blood glucose and worsens glucose intolerance in obese mice. Adding back geranylgeranyl isoprenoids restores hepatic Rap1a activity and improves hyperglycemia. These findings identify Rap1a as an important mediator of obesity- and statin-induced glucose intolerance.

RESULTS

**Inhibition of Epac2 or Rap1a stimulates gluconeogenesis and increases HGP**

A recent GWAS study demonstrated that variants in RAPGEF4, which encodes EPAC2, are associated with fasting blood glucose in East Asians; however, the functional significance of this finding is not known (Hwang et al., 2015). To test whether Epac2 and its downstream effector, Rap1a, contribute to hepatic glucose metabolism, we tested their effects on the expression of gluconeogenic genes, G6pc and Pck1. Upon stimulation of freshly isolated primary mouse hepatocytes with forskolin, a glucagon mimetic and potent adenylate cyclase activator, and dexamethasone, which downregulates CAMP-phosphodiesterase (Manganelli and Vaughan, 1972), we observed that the expression of G6pc and Pck1 was further increased in hepatocytes treated with siRNAs against Epac2 or Rap1a (Figures 1A, 1C, and 1D). Accordingly, glucagon treatment resulted in an increase in G6pc and Pck1 mRNA levels in Rap1a-deficient hepatocytes versus control (Figure 1F). A similar data were obtained in a mouse hepatocyte cell line, AML-12 cells (Figures S1A and S1B).

Consistent with an increase in the expression of gluconeogenic genes, we observed a further increase in glucose release in cells lacking Epac2 or Rap1a after forskolin and dexamethasone stimulation (Figures 1B and 1E). To assess the functional role of Rap1a in hepatic glucose metabolism in vivo, we deleted liver Rap1a by injecting diet-induced obese (DIO) Rap1afl/fl mice with adeno-associated virus-8 encoding Cre recombinase driven by the hepatocyte-specific thyroxine-binding globulin promoter (AAV8-TBG-Cre), and AAV8-TBG-Gfp-treated Rap1afl/fl mice served as controls. The TGBO-Cre treatment successfully silenced Rap1a in the liver (Figure 1G) without altering body weight (Figure 1H). In line with the in vitro data, DIO mice that lack hepatocyte Rap1a had higher fasting blood glucose levels (Figure 1I) and hepatic Pck1 expression (Figure 1J) without a change in plasma insulin levels (Figure 1K). Consistent with these results, hepatocyte Rap1a-deficient mice also showed greater blood glucose excursions than control mice upon glucose challenge (Figure 1L). We next evaluated the effect of activated hepatic Rap1a by treating primary hepatocytes with a plasmid encoding a constitutively active Rap1a mutant (CA-Rap1a). CA-Rap1a possesses an amino acid substitution, Q63E, which results in constitutive GTP-binding, so the mutated form of Rap1a is always active and unable to be downregulated by Rap1GAP (Spolitu et al., 2019; Wittchen et al., 2011). Compared with control plasmid-treated cells, CA-Rap1a-overexpressing cells had lower G6pc and Pck1 mRNA expression and showed 50% decrease in glucose production upon forskolin and dexamethasone treatment (Figures S1C and S1D). These results suggested that activation of Rap1a suppresses gluconeogenesis.

To further evaluate the physiologic relevance of Rap1a-mediated gluconeogenesis suppression, we checked hepatic Rap1a activity during the transition from a fed to fasting state in WT mice. Consistent with an increase in hepatic gluconeogenic gene expression, we found that the GTP-bound, active-Rap1a levels in the liver were lower after 6 h of fasting (Figure S2A), which still remained low after 14 and 24 h of fasting (Figures S2B and S2C). Fasting, however, did not increase the levels of hepatic phosphorylated CREB as reported before (Stern et al., 2019). These data support the hypothesis that Rap1a negatively regulates gluconeogenesis, and the major function of Rap1a in hepatic glucose metabolism is to inhibit the aberrant activation of HGP.

**Activation of Rap1a is suppressed in obese mice liver, and restoring Rap1a activity lowers blood glucose and improves glucose intolerance in obese mice**

Because Rap1a activation lowers gluconeogenesis and glucose production (Figures S1C and S1D), we next asked if Rap1a is...
inhibited in mouse models of T2D and insulin resistance where gluconeogenesis and HGP are elevated. In genetically obese and diabetic db/db mice liver, we found that the GTP-bound, active Rap1α levels were significantly decreased compared with heterozygous db/+ controls (Figure 2A). Similar data were obtained in the livers of DIO mice versus their low-fat-fed controls (Figure 2B). We then restored hepatic Rap1α activity by treating db/db mice with AAV8-TBG-CA-Rap1α, which specifically expresses the constitutively active Rap1α in hepatocytes. Despite similar body weight (Figure 2C), we observed that over-expressing TBG-CA-Rap1α in db/db mouse liver lowered fasting blood glucose (Figure 2D), improved glucose intolerance (Figure 2E), and lowered hepatic gluconeogenic gene expression (Figure 2F), without affecting plasma insulin levels (Figure 2G). Similar results were obtained when DIO mice were treated with adeno-CA-Rap1α (Figures 2H–2L). These combined data provide evidence that Rap1α regulates gluconeogenesis and HGP and contributes to glucose homeostasis in vivo.

**Statins inhibit Rap1α in isolated hepatocytes and the human liver**

Attachment of GGPP to Rap1α results in its membrane localization and Epac2-mediated GTP loading, which supports the importance of the mevalonate pathway in Rap1α’s activation (Goody et al., 2017; Jaskiewicz et al., 2018). Interestingly, a previous report showed that statin treatment of skeletal muscle cells in vitro lowers Rap1α isoprenylation, which was suggested as a plausible mechanism to explain statin-mediated myotoxicity (Jaskiewicz et al., 2018). Because the liver is the primary organ responsible for the metabolism and action of statins, we hypothesized that one mechanism by which statins increase the risk for new-onset T2D is their ability to lower GGPP, inhibit Rap1α, and aberrantly activate HGP. As a first step in investigating this hypothesis, we determined whether statin treatment of hepatocytes inhibits Rap1α’s prenylation and membrane localization. We found that treatment of primary mouse hepatocytes with two potent statins in clinical use, simvastatin or rosuvastatin, significantly lowered membrane Rap1α protein, without decreasing the total cellular Rap1α levels (Figures 3A and 3B). Further, the decrease in membrane localization of Rap1α was associated with inhibition of its activity, as rosuvastatin treatment lowered GTP-bound, active Rap1α levels (Figure 3C). To study the relevance to humans, we obtained human liver specimens from the Liver Tissue Cell Distribution System sponsored by the National Institutes of Health. Strikingly, we observed that the GTP-bound, active Rap1α levels were significantly decreased in the livers of human patients on statin therapy compared with their disease-, age-, and sex-matched controls who are not using statins (Figure 3D). We obtained similar results from liver samples of another set of patients that are matched for age, sex, and body mass index (Figure 3E). Thus, statin treatment inhibits Rap1α activity in mouse hepatocytes and the human liver.

**Statins increase gluconeogenesis in WT but not in Rap1α-deficient hepatocytes**

Given that statins lower Rap1α activity and Rap1α inhibition increases HGP, we then sought to investigate whether an increase in hepatic gluconeogenesis is involved in the diabetogenic effects of statins. We observed that treatment of primary mouse hepatocytes with different statins, including, simvastatin, rosuvastatin, or fluvastatin, increased G6pc and Pck1 mRNAs (Figures 4A, S3A, S3C, and S3D). Consistent with an increase in gluconeogenic gene expression, glucose production was also increased upon statin treatment (Figures 4B and S3B). Moreover, rosuvastatin treatment of metabolism-qualified primary human hepatocytes resulted in an increase in gluconeogenic gene expression, which provides information about the human relevance of our murine hepatocyte studies (Figures 4C and 4D). To understand whether inhibition of Rap1α is one of the major mechanisms by which statins induce gluconeogenic gene expression, we incubated control or si-Rap1α-treated hepatocytes with rosuvastatin. Confirming our above results, both silencing Rap1α or rosuvastatin treatment alone increased G6pc mRNA (Figure 4E). Importantly, rosuvastatin’s ability to increase G6pc and Pck1 mRNAs was abolished in Rap1α-silenced hepatocytes (Figures 4E and 4F). These results support the idea that inhibition of Rap1α acts in the same pathway as statins and is a major mechanism by which statins increase gluconeogenesis. To show the relevance of statin treatment on glucose metabolism regulation in vivo, we treated DIO mice with a high-fat diet containing 0.02% simvastatin (w/w) for 12 weeks and observed that both groups gained similar weight during the treatment (Figure 4G). As expected, statin treatment resulted in an increase in the mRNA expression of Srebp2 target genes, including Hmgcs and Hmgcr in the liver (Figure S3E). Similar to hepatic Rap1α silencing, we found that statin-treated obese mice had higher fasting blood glucose, increased gluconeogenic gene expression in the liver, and they were more glucose intolerant compared with control mice, without alterations in plasma insulin levels (Figures 4H–4L). Statins lower plasma LDL via increasing LDLR protein levels and paradoxically increase the expression of PCSK9 (Goldstein and
To understand the involvement of LDLR and PCSK9 in statin-mediated regulation of hepatocyte glucose metabolism, we treated primary hepatocytes isolated from \( \text{Ldlr}^{-/-} \) and \( \text{Pcsk9}^{-/-} \) mice with rosuvastatin. Similar to WT cells, we observed an increase in \( \text{G6pc} \) mRNA levels and glucose production in LDLR- and PCSK9-deficient hepatocytes treated with rosuvastatin compared with vehicle (Figures S3F–S3I). Thus, statins stimulate gluconeogenesis and increase glucose production, and this regulation is independent of LDLR and PCSK9.

GGPP supplementation restores Rap1a activity and improves statin-induced glucose intolerance in obese mice

Inhibition of HMG-CoA reductase by statins suppresses GGPP isoprenoid synthesis, which is required for Rap1a to become activated (Jaskiewicz et al., 2018). Because statin treatment or Rap1a inhibition increases gluconeogenesis, we reasoned that a decrease in intracellular GGPP levels is involved in statin-mediated gluconeogenic gene induction. To test this, we put back intracellular GGPP in statin-treated hepatocytes by incubating them with exogenous GGPP. We found that this treatment brought back plasma membrane-localized Rap1a to vehicle-treated levels, suggesting that Rap1a prenylation was restored (Figure 5A). Consistent with the restoration of Rap1a’s membrane localization, GGPP treatment abrogated the increase in \( \text{G6pc} \) and \( \text{Pck1} \) mRNA levels conferred by statin treatment in isolated hepatocytes (Figure 5B). Of note, GGPP treatment did not inhibit simvastatin-induced \( \text{Hmgcr} \) and \( \text{Hmgcs} \) mRNAs (Figure S4A), suggesting that induction of Srebp2 by statins was intact and unaffected by GGPP restoration. We next sought to determine if GGPP supplementation in statin-treated obese mice could lower HGP and improve glucose intolerance. Accordingly, we fed DIO mice with a high-fat diet containing 0.02% simvastatin (w/w) for 12 weeks. Mice were then administered with GGPP precursor, geranylgeraniol (GGOH, 100 mg/kg), or vehicle control by daily gavage while still receiving the statin-containing diet (Gibbs et al., 1999). After 3 weeks of treatment, we observed higher hepatic active-Rap1a levels (Figure 5B), lower fasting blood glucose (Figure 5C), improved glucose intolerance (Figure 5D), and decreased hepatic gluconeogenic gene expression (Figure 5E), without a change in body weight or plasma insulin levels (Figures 5F and 5G) in the GGOH-supplemented group compared with statin treatment alone. Of note,
we did not observe changes in blood glucose, hepatic gluconeogenic gene expression, body weight, or plasma insulin levels in DIO mice treated with GGOH alone (Figures S4C–S4G).

Given that one of the major effects of statins is to decrease cholesterol biosynthesis, we next asked whether lowering of intracellular cholesterol participates in the gluconeogenic effects of statins. For this purpose, we restored intracellular cholesterol levels in statin-treated hepatocytes by incubating them with cholesterol-enriched phospholipid liposomes (Lipo-Chol) (Wang et al., 2020). As expected, increased expression of cholesterol synthesis genes, including Hmgcr and Hmgcs, upon statin treatment was lowered back to control levels in cells treated with Lipo-Chol and statin together (Figures S5A and SSB). However, restoration of intracellular cholesterol was not able to reverse statin-induced G6pc and Pck1 mRNAs (Figures S5C and SSD). Notably, Lipo-Chol treatment did not affect membrane localization of Rap1a (Figure S5E). These results support the hypothesis that statins inhibit Rap1a activity and increase HGP via lowering GGPP levels, which is independent of a change in intracellular cholesterol content.

Geranylgeranyl transferase-1 inhibition mimics statins and increases hepatocyte gluconeogenesis

Our results thus far suggest that statins inhibit activation of Rap1a and increase HGP, and restoring GGPP levels brings back Rap1a activity and lowers glucose production. Geranylgeranyl transferase 1 (GGT1) covalently attaches GGPP to Rap1a to form a stable thioether bond (Jaskiewicz et al., 2018). This post-translational modification increases Rap1a’s hydrophobicity, which results in Rap1a’s plasma membrane localization (Ghomashchi et al., 1995). To understand the contribution of GGT1 to hepatic glucose metabolism, we used a specific GGT1 inhibitor or silenced Pggt1b, the gene encoding GGT1, in primary hepatocytes. Similar to statin treatment or Rap1a silencing, inhibition or silencing of GGT1 increased gluconeogenic gene expression and glucose production in both primary mouse and human hepatocytes (Figures 5H and S6A–S6D). Consistent with a role for GGT1 in gluconeogenesis in vivo, treatment of DIO mice with hepatocyte-specific AAV8 encoding short hairpin RNA (shRNA) construct targeting Pggt1b resulted in lowering of hepatic Pggt1b mRNA expression by ~50% and increased fasting blood glucose levels in obese mice, without altering body weight or plasma insulin (Figures S5I–L). It is noteworthy to mention that farnesyl transferase inhibitor, FTI-277, did not affect G6pc or Pck1 mRNA levels in primary mouse hepatocytes (Figure S6E). Collectively, these data suggest that inhibition of GGPP synthesis and Rap1a activity by statins increases gluconeogenesis, which is mimicked by GGT1 inhibition.

Rap1a inhibition or statin treatment increases gluconeogenesis via regulating FoxO1 activity

In an attempt to understand the molecular mechanism(s) by which Rap1a regulates HGP, we first determined whether Rap1a deficiency in primary hepatocytes affects intracellular cAMP levels. We found that both basal and forskolin- and dexamethasone-treated intracellular cAMP levels were similar in Rap1a-deficient or CA-Rap1a-overexpressing cells compared with controls (Figures S7A and S7B). Protein kinase A (PKA) is well known for its ability to stimulate gluconeogenesis via increasing the nuclear localization and activity of CREB and CRTC2 transcription factors (Chrivita et al., 1993; Wang et al., 2012b). We next considered the possibility that Rap1a deficiency may stimulate gluconeogenesis via activating PKA. However, we observed that the levels of both p-CREB and p-PKA substrates, as measures of CREB and PKA activities, respectively, were not increased in Rap1a-silenced hepatocytes versus control (Figure S7C). In support of this finding, mRNA levels of one of the downstream effectors for both CRTC2 and CREB pathways, peroxisome proliferator-activated receptor-γ coactivator-1a (Pgc1a) (Herzig et al., 2001), were similar in WT and Rap1a-deficient hepatocytes (Figure S7D). Interestingly, Pgc1a mRNA levels were decreased, not increased, in cells treated with simvastatin or rosuvastatin compared with controls (Figure S7E). These results support the idea that Rap1a regulates gluconeogenesis without affecting PKA activity.

Forkhead box O1 (FoxO1) is a major gluconeogenic transcription factor that induces the transcription of G6pc and Pck1 (Pajvani and Acilli, 2015). To investigate whether statins or Rap1a inhibition enhance gluconeogenesis via regulating FoxO1, we first measured the mRNA levels of another well-known FoxO1 target gene, Igfbp1 (Matsumoto et al., 2006). We observed that Rap1a deficiency, statin treatment, or GGT1II treatment increased hepatic Igfbp1 mRNA levels (Figures 6A, 6B, and S7D). Glucokinase (encoded by Gck) is critical for hepatic glucose utilization and is negatively regulated by FoxOs (Haesler et al., 2014; Langlet et al., 2017). In support of the role of Rap1a in regulating FoxO1, we found lower Gck mRNA levels in Rap1a-deficient DIO liver compared with control (Figure 6C). Likewise, Gck mRNA was significantly lower in Rap1a−/− hepatocytes in response to a combination of forskolin, dexamethasone, and insulin treatment, which has been shown to induce Gck (Langlet et al., 2017) (Figure S7H). Conversely, CA-Rap1a overexpression increased forskolin + dexamethasone + insulin-induced Gck mRNA in WT hepatocytes (Figure S7I). We then addressed whether Rap1a affects FoxO1’s transcriptional activity using reporter assays. For this purpose, we transfected WT or Rap1a−/− hepatocytes with control or
FoxO1-Gfp plasmids (Figures 6D and 6E, lower blots). We observed that the increase in Igfbp1 and G6pc promoter activities upon FoxO1 overexpression was further upregulated in Rap1a-deficient hepatocytes (Figures 6D and 6E). We found similar results upon forskolin and dexamethasone treatment, suggesting that FoxO1’s transcriptional activity is increased in Rap1a-deficient cells (Figure S7J). To test the role of FoxO1 in Rap1a- and statin-mediated gluconeogenesis regulation, we silenced Rap1a in FoxO1,3,4-deficient hepatocytes. As above, Rap1a silenced hepatocytes expressed higher levels of G6pc and Igfbp1 mRNAs than control hepatocytes, and in response to FoxO1,3,4 deficiency, this increment was abrogated, which supports the involvement of FoxO proteins in Rap1a-mediated gluconeogenic gene regulation (Figure 6F). Because nuclear localization of FoxO1 is required for its functional activity, we next measured nuclear FoxO1 levels in Rap1a-deficient or statin-treated hepatocytes. We observed that both Rap1a silencing and statin treatment increased nuclear FoxO1 without altering its total cellular levels (Figures 6G and 6H). One of the major upstream regulators of FoxO1’s subcellular localization is insulin-receptor-signaling-activated serine/threonine kinase, Akt. Activation of Akt phosphorylates FoxO1, which results in FoxO1’s cytoplasmic retention (Brunet et al., 1999). We then sought to determine if phosphorylation of FoxO1 or Akt was altered upon Rap1a deficiency. We found that Rap1a-silenced hepatocytes had significantly lower phospho-Akt and phospho-FoxO1 levels upon insulin stimulation or under basal conditions (Figures 6I and 6J). Similarly, rosvastatin treatment of hepatocytes impaired insulin-induced Akt and FoxO1 phosphorylation (Figures 6K–6M). These results support the idea that statins or Rap1a inhibition reduce phosphorylation of Akt, which results in nuclear localization of FoxO1 and stimulation of gluconeogenesis.

DISCUSSION

The combination of insulin resistance together with unopposed glucagon action results in increased HGP, which is largely responsible for the fasting hyperglycemia observed in obese patients with T2D (Gastaldelli et al., 2000; Unger and Cherrington, 2012). The most commonly used anti-diabetes therapy, metformin, is well-known for its ability to decrease hepatic gluconeogenesis, and other candidates that reduce HGP are being investigated as anti-glycemic agents (Kazda et al., 2016; Lee et al., 2021; Sharabi et al., 2017; Vella et al., 2019). Our results here identify the small GTPase Rap1a as a potential target that regulates HGP and glucose homeostasis. Mechanistically, we show that Rap1a stimulates actin polymerization, which regulates hepatoctyes by staining F-actin filaments with Alexa Fluor 555-conjugated phalloloid in scrambled control or si-Rap1a–treated cells. We observed that si-Rap1a-treated hepatocytes had disrupted cytoskeletal structures as evidenced by short, fragmented F-actin filaments that coalesce to form cytoskeletal clumps (Figure 7A, see white arrows). This fragmented actin phenotype was similar to the actin phenotype of WT cells treated with the potent actin polymerization inhibitor cytochalasin D (Figure 7A), suggesting that Rap1a silencing results in dysregulation of actin cytoskeleton in hepatocytes. We next asked whether Rap1a inhibition induces gluconeogenesis via blocking actin polymerization. Consistent with this idea, we found that treatment of hepatocytes with different concentrations of cytochalasin D increased G6pc and Pck1 mRNAs and resulted in a small but significant increase in glucose production (Figures 7B and 7C). We observed similar results when actin polymerization was inhibited using another actin polymerization inhibitor, latrunculin A (not shown). Conversely, treatment of primary hepatocytes with the actin filament polymerizing and stabilizing agent, jasplakinolide, lowered gluconeogenic gene expression and glucose production (Figures 7D and 7E). Similar to Rap1a inhibition, cytochalasin D was unable to induce G6pc and Igfbp1 mRNAs in FoxO1,3,4-deficient hepatocytes (Figure 7F). Most importantly, nuclear FoxO1 levels were increased, whereas insulin-stimulated phospho-Akt levels were decreased in cytochalasin D–treated hepatocytes (Figures 7G and 7H). Taken together, these data support the idea that hepatocyte Rap1a is involved in actin cytoskeleton dynamics, which contributes to the regulation of gluconeogenesis via Akt-mediated FoxO1 activation.

**Rap1a induces actin polymerization, and actin remodeling contributes to gluconeogenesis**

We next sought to investigate possible mechanisms linking Rap1a activation to Akt-mediated FoxO1 regulation. Previous work in other cell types has demonstrated that Rap1a promotes filamentous actin (F-actin) polymerization (Mun and Jeon, 2012; Wang et al., 2017). As disruption of actin polymerization and cytoskeleton remodeling inhibits insulin-induced Akt activation (Lee et al., 2013; Peyrollier et al., 2000; Wang et al., 2012a), we considered the hypothesis that Rap1a may regulate gluconeogenesis through its effects on actin organization. We first examined whether Rap1a plays a role in actin polymerization in hepatocytes.
FoxO1’s nuclear localization and activity. The role of FoxO1 in Rap1a action is supported by the finding that the stimulatory effects of Rap1a inhibition or actin depolymerization on gluconeogenesis are abrogated in FoxO1,3,4-deficient hepatocytes. With regard to FoxO1 regulation, we provide data that phosphorylation and activation of Akt, the major kinase that inhibits FoxO1 activity, are suppressed upon Rap1a deficiency. Of note, previous studies have implicated actin network and Rap1 in Akt activation through their effects on phosphoinositide 3-kinase (PI3K) activity, the upstream Akt regulator (Cahill et al., 2016; Eyster et al., 2005; Kortholt et al., 2010). Because of the importance of insulin receptor-Akt pathway in the pathogenesis of many obesity-associated metabolic dysfunctions, future investigations will be necessary to determine the molecular mechanisms linking Rap1a to Akt activation in hepatocytes.

In addition to obesity-induced hyperglycemia, our results show that inhibition of Rap1a activity or GGPP synthesis mediates elevated HGP in statin-treated hepatocytes and mice, which could be one of the underlying mechanisms linking statins to new-onset T2D. Interestingly, a recent work from another group independently reported that restoring geranylgeranyl isoprenoids improves statin-associated glucose intolerance in mice (Wang et al., 2022). In vitro findings from Wang et al. suggested that geranylgeranylation of another GTPase, Rab14, contributes to hepatic insulin signaling; however, the importance of Rab14 in statin-associated glucose intolerance was not studied. Given that rosuvastatin’s ability to increase gluconeogenic genes was abolished in Rap1a deficient hepatocytes (Figures 4E and 4F), our results suggest that Rap1a inhibition is one of the major underlying mechanisms by which statins increase gluconeogenesis.

As all statins have high selectivity for the liver, largely because of efficient first-pass uptake (Schachter, 2005), the liver likely plays a major role in statin-induced glucose intolerance. However, statins may also contribute to glucose intolerance through their effects on other insulin-sensitive tissues and the pancreas. For example, statin treatment was shown to inhibit insulin secretion in a pancreatic β cell line (Salunkhe et al., 2016). Interestingly, a recent study in human subjects treated with atorvastatin for 10 weeks reported an increase in steady-state plasma glucose without a decline in insulin secretion (Abbasi et al., 2021), yet the effect of long-term statin-use on β cell function remains to be investigated. It is important to note that activation of Epac2 and Rap1 in β cells is suggested to be beneficial in glucose homeostasis, as Epac2-Rap1 activation increases incretin-mediated insulin secretion (Takahashi et al., 2015). Regarding other possible non-hepatic effects, previous work showed impairment in insulin signaling in cultured adipocytes and myotubes upon statin treatment (Henriksbo et al., 2019; Hwang et al., 2019). Moreover, a recent study reported that GGPP and GGT1 are important regulators of brown adipocyte function and contribute to systemic glucose metabolism (Balaz et al., 2019). Future in vivo studies will be needed to determine whether geranylgeranylation or Rap1a in different cell types contribute to the statin-T2D link.

Clinical trials of PCSK9 inhibitors did not show an effect of these drugs on glycemia or T2D diagnosis; however, an increase in T2D risk has been observed in individuals with PCSK9 loss-of-function variants (Colhoun et al., 2016; Fernandez et al., 2016; Lotta et al., 2016; Sabatine et al., 2017; Sattar et al., 2017; Schmidt et al., 2017). On the other hand, LDL-modulating allele in APOB showed no relationship with diabetes risk (Xu et al., 2017). Thus, the T2D link with statins and PCSK9 loss-of-function variants is complex and may not be simply attributed to their effects on liver LDLR expression or plasma LDL, which is in line with our data showing that intracellular cholesterol or LDLR do not impact statin-induced gluconeogenesis in hepatocytes. Finally, while our results have defined an important mechanism that may underlie statins’ hyperglycemic effects, it is important to note that the benefits of statin use for the reduction of cardiovascular disease risk outweigh the risk of developing T2D in the majority of patients, especially in individuals with higher cardiovascular disease risk (Mach et al., 2018; Sattar et al., 2014).

Figure 6. Rap1a inhibition or statin treatment increases gluconeogenesis via regulating FoxO1 activity (A and B) Igfbp1 mRNA levels were measured from the livers of DIO Rap1afl/fl mice that were injected with TBG-Cre or TBG-Gfp (A), and vehicle- or 5 μM rosuvastatin (rosuva)-treated primary hepatocytes (HCs) that were incubated with forskolin and dexamethasone (F + D) (B) (n = 7–8 mice/group and n = 3 biological replicates, respectively, mean ± SEM, *p < 0.05).

(C) Gck mRNA levels were measured from the livers of DIO Rap1afl/fl mice that were injected with TBG-Cre or TBG-Gfp (n = 7–8 mice/group, mean ± SEM, *p < 0.05).

(D) WT and Rap1a−/− primary hepatocytes were transfected with a luciferase fusion construct encoding nucleotides −1,227 to +57 of the G6pc promoter containing an intact FoxO binding site and FoxO1-Gfp or control plasmid. Relative luciferase activity and Gfp levels from WT and Rap1a−/− cells transfected with FoxO1-Gfp were measured (lower blots) (n = 3–4 biological replicates, mean ± SEM, *p < 0.05).

(E) Luciferase reporter assay of the Igfbp1 promoter in WT and Rap1a−/− cells transfected with control or FoxO1-Gfp. Gfp levels from WT and Rap1a−/− cells transfected with FoxO1-Gfp were assayed in the lower blots (n = 3–4 biological replicates, mean ± SEM, *p < 0.05).

(F) G6pc and Igfbp1 mRNA levels from F + D-treated WT and FoxO1,3,4−/− cells that were transfected with scrambled RNA (scr) or siRNA against Rap1a (si-Rap1a) (n = 4 biological replicates, mean ± SEM, *p < 0.05).

(G) Nuclear and whole-cell FoxO1 levels along with loading controls (lamin A/C and β-actin, respectively) in WT versus Rap1a−/− hepatocytes. Densitometric quantification of the nuclear FoxO1 immunoblot data is shown in the bar graph (n = 3 biological replicates).

(H) Nuclear and whole-cell FoxO1 levels along with loading controls (nucleophosmin, Np, and β-actin, respectively) in vehicle or 5 μM rosuvastatin (rosuva)-treated cells (n = 3 biological replicates).

(I) WT and Rap1a−/− hepatocytes were stimulated with vehicle control or insulin (100 nM) for 5 min, and p-Akt and total Akt levels were assayed (upper blots). Basal levels of phospho-Akt and total Akt in WT and Rap1a−/− cells are shown in the lower blots (n = 3 biological replicates).

(J) Same as in (I) except that p-FOXO1, total FOXO1, and β-actin levels were measured (n = 3 biological replicates).

(K–M) Vehicle or 5 μM rosuvastatin-treated cells were incubated with or without insulin (100 nM) for 5 min as indicated. p-Akt and total Akt (K), and p-FoxO1, total FoxO1 and β-actin levels (L–M) were assayed (n = 3 biological replicates). See also Figure S7.
Figure 7. Actin remodeling contributes to gluconeogenesis and glucose production

(A) Primary mouse hepatocytes (HCs) that were treated with scrambled control (scr), si-Rap1a, or cytochalasin D were stained with 488-phalloidin (green) to visualize F-actin. White arrows indicate fragmented F-actin filaments that form cytoskeletal clumps. Scale bar, 10 μm.

(B) Hepatocytes were treated with vehicle, 0.1 μM, or 1 μM cytochalasin D (cyto. D) for 8 h, and forskolin and dexamethasone (F + D)-stimulated G6pc and Pck1 mRNA levels were measured (n = 4 biological replicates, mean ± SEM, *p < 0.05).

(C) Same as in (B) except that glucose production was measured in cells treated with vehicle or 0.1 μM cytochalasin D (cyto. D) (n = 3 biological replicates, mean ± SEM, *p < 0.05).

(D) Hepatocytes were treated with 0.1 μM jasplakinolide (jasp.) for 6 h, and F + D-stimulated G6pc and Pck1 mRNA levels were measured (n = 3 biological replicates, mean ± SEM, *p < 0.05).

(legend continued on next page)
Limitations of the study
The data presented in this study demonstrate that Rap1α suppresses gluconeogenesis and inhibits the aberrant activation of HGP. As increased gluconeogenesis is a common feature of both fasting and obesity, we observed lower active-Rap1α levels in transition from fed to fasting state or in obese mice liver. However, we did not identify the upstream molecular mediators governing this regulation. Our data also suggest that hepatic Rap1α inhibition, through lowering intracellular GGPP isoprenoid levels, is a major mechanism by which statins increase gluconeogenesis and cause hyperglycemia. In this regard, one limitation of our study is that we did not evaluate the effects of statins and GGOH treatment on other insulin-sensitive tissues, an area that should be investigated using the hyperinsulinenic-euglycemic clamp studies. Finally, the mechanisms by which Rap1α and actin cytoskeleton organization regulate Akt-mediated FoxO1 activity remain to be determined.

STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111259.

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AUTHOR CONTRIBUTIONS
Y.W., S.S., J.A.Z., and A.S. designed and performed the experiments, analyzed the data, interpreted results, and edited the manuscript. L.O. conceived and supervised the project, designed experiments, analyzed data, interpreted results, and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.

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### STAR Methods

### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit anti-β-actin | Cell Signaling Technology | Cat # 4970, RRID: AB_2223172 |
| Rabbit anti-pan-Cadherin | Cell Signaling Technology | Cat # 4068, RRID: AB_2158565 |
| Rabbit anti-GFP     | Cell Signaling Technology | Cat # 2956, RRID: AB_1196615 |
| Rabbit anti-GAPDH   | Cell Signaling Technology | Cat # 5174, RRID: AB_10622025 |
| Rabbit anti-p-Akt   | Cell Signaling Technology | Cat # 4060, RRID: AB_2315049 |
| Rabbit anti-Akt     | Cell Signaling Technology | Cat # 4691, RRID: AB_915783 |
| Rabbit anti-p-FoxO1 | Cell Signaling Technology | Cat # 9464, RRID: AB_329842 |
| Rabbit anti-FoxO1   | Cell Signaling Technology | Cat # 2880, RRID: AB_2106495 |
| Mouse anti-Lamin A/C| Cell Signaling Technology | Cat # 4777, RRID: AB_10545756 |
| Rabbit anti-nucleophosmin (anti-NPM) | Cell Signaling Technology | Cat # 3542, RRID: AB_2155178 |
| Rabbit anti-p-CREB  | Cell Signaling Technology | Cat # 9198, RRID: AB_2561044 |
| Rabbit anti-CREB    | Cell Signaling Technology | Cat # 9197, RRID: AB_331277 |
| Rabbit anti-p-PKA substrate | Cell Signaling Technology | Cat # 9624, RRID: AB_331817 |
| Rabbit anti-HSP90   | Cell Signaling Technology | Cat # 4877, RRID: AB_2233307 |
| Goat anti-Rap1a     | R&D Systems | Cat # AF3767, RRID: AB_622167 |
| Rabbit anti-Na,K-ATPase | Abcam | Cat # ab76020, RRID: AB_1310695 |

| **Bacterial and virus strains** |        |            |
| AAV8-TBG-Gfp | Gene Therapy Resource Program (GTRP) of the NHLBI | N/A |
| AAV8-TBG-Cre | Gene Therapy Resource Program (GTRP) of the NHLBI | N/A |
| AAV8-TBG-Gfp | James M. Wilson, Addgene, Cat # 105535-AAV8 |
| AAV8-TBG-Cre | James M. Wilson, Addgene, Cat # 107787-AAV8 |
| AAV8-TBG-CA-Rap1a | Penn Vector Core | Custom |
| AAV8-H1-shPgggt1b sense: (5’-CACCACGCTAGAGCTAAGTCAAG-3’; 5’-TGTACGCTGATGCTT-3’) | Salk Institute Gene Transfer, Targeting, and Therapeutics Core | Custom |
| Adeno-LacZ | (Wittchen et al., 2011) | Custom |
| Adeno-CA-Rap1a | (Wittchen et al., 2011) | Custom |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Human liver samples | Liver Tissue Cell Distribution System at the University of Minnesota and Columbia University | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Forskolin | Sigma-Aldrich | Cat # F6886 |
| Dexamethasone | Sigma-Aldrich | Cat # D4902 |
| Geranylgeranyl transferase 1 inhibitor (GGT1i) | Sigma-Aldrich | Cat # G5294 |
| Geranylgeranyl transferase 1 inhibitor (GGT1i) | Sigma-Aldrich | Cat # G5169 |
| Farnesyl transferase 1 inhibitor (FT1i) | Sigma-Aldrich | Cat # F9803 |
| Glucose | Sigma-Aldrich | Cat # G7021 |
| Glucagon | Sigma-Aldrich | Cat # G2044 |
| Insulin | Sigma-Aldrich | Cat # I0516 |
| Simvastatin | Sigma-Aldrich | Cat # S6196 |
| Simvastatin | Tokyo Chemical Industry (TCI) | Cat # G50509 |
| Rosuvastatin | Cayman Chemicals | Cat # 18813 |
| Geranylgeranyl pyrophosphate (GGPP) | Cayman Chemicals | Cat # 63330 |
| Geranylgeraniol (GGOH) | Cayman Chemicals | Cat # 13272 |
| 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) | Avanti Polar Lipids | Cat # 850345 |
| Cholesterol | Sigma-Aldrich | Cat # C8667 |
| Heat-inactivated fetal bovine serum | GIBCO | Cat # 16140-071 |
| DMEM/F12 medium | ThermoFisher Scientific | Cat # 11320033 |
| Penicillin/streptomycin | Corning | Cat # 30-002-CI |
| Opti-MEM | ThermoFisher Scientific | Cat # 31985070 |
| Lipofectamine RNAiMAX | ThermoFisher Scientific | Cat # 13-778-150 |
| Lipofectamine 3000 | ThermoFisher Scientific | Cat # L3000015 |
| RIPA Buffer | ThermoFisher Scientific | Cat # P89901 |
| Supersignal West Pico enhanced chemiluminescent solution | ThermoFisher Scientific | Cat # P34580 |
| TRI Reagent | Sigma-Aldrich | Cat # T9424 |
| Cryopreserved hepatocyte recovery medium | ThermoFisher Scientific | Cat # CM7000 |
| Williams’ Medium E | ThermoFisher Scientific | Cat # A1217601 |
| Hepatocyte maintenance supplement pack | ThermoFisher Scientific | Cat # CM4000 |
| **Critical commercial assays** | | |
| Glucose (GO) Assay Kit | Sigma-Aldrich | Cat # GAGO20 |
| Active Rap1 Detection Kit | Cell Signaling Technology | Cat # 8818 |
| Cyclic AMP XP Assay Kit | Cell Signaling Technology | Cat # 4339 |
| Phalloidin-iFluor 488 reagent | Abcam | Cat # ab176753 |
| Ultrasensitive mouse insulin ELISA Kit | Crystal Chem | Cat # 90080 |
| Dual-Luciferase Reporter Assay System | Promega | Cat # E1960 |
| Nuclear Extract Kit | Active Motif | Cat # 40010 |
| **Experimental models: Cell lines** | | |
| Mouse: Primary hepatocytes | This paper | N/A |
| Human plateable hepatocytes | ThermoFisher Scientific | Cat # HMCPMS |
| AML12 cells | ATCC | Cat # CRL-2254 |
| **Experimental models: Organisms/strains** | | |
| Mouse: WT C57BL/6J | The Jackson Laboratory | JAX: 000664 |
| Mouse: db/db | The Jackson Laboratory | JAX: 000697 |
| Mouse: Diet-induced obese (DIO) | The Jackson Laboratory | JAX: 380050 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Lale Ozcan (<lo2192@cumc.columbia.edu>).

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**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: Controls for diet-induced obese | The Jackson Laboratory | JAX: 380056 |
| Mouse: Rap1a<sup>e3</sup>, C57BL/6J | The Jackson Laboratory | JAX: 021066 |
| Mouse: Foxo1<sup>3,4<sup>e3</sup></sup> | (Haeusler et al., 2010) | N/A |

**Oligonucleotides**

qPCR primers are listed in Table S1.

Mouse: IDT Rap1a DsiRNA sense: 5′-rCrArGrCrUrArGrUrArGrUrCrUrGrUrCrArGrGGA-3′

Mouse: IDT Rap1a DsiRNA antisense: 5′-rUrCrCrUrGrArArGrCrCrArArGrGrArUrCrUrUrCrUrAr-3′

Mouse: IDT Rapgef4 DsiRNA sense: 5′-rArArGrCrArArGrArUrCrGrGrUrUrUrGrUrArUrArArUGA-3′

Mouse: IDT Rapgef4 DsiRNA antisense: 5′-rUrCrArUrUrUArArArArCrCrGrArArUrCrUrArCrUrUrGrAr-3′

Mouse: IDT Pggt1b DsiRNA sense: 5′-rCrUrUrArArGrGrUrGrUrGrCrCrArArCrArUGT-3′

Mouse: IDT Pggt1b DsiRNA antisense: 5′-rArCrArUrGrUrUrArGrUrUrGrGrCrArCrArA-3′

Negative Control DsiRNA sense: 5′-rCrGrUrUrArArUrCrGrCrGrUrArUrArArUrArCrCrGrCrGrUAT

Negative Control DsiRNA antisense: 5′-rArUrArCrGrUrUrArArUrArArUrArCrGrCrGrUAT

Recombinant DNA

Igfbp1-promoter/pGL3 (Nakae et al., 2000) Addgene, Cat # 12146

GFP-FoxO1 (Frescas et al., 2005) Addgene, Cat # 17551

Renilla luciferase (Chen and Prywes, 1999) Addgene, Cat # 27163

Rap1-E63 (CA-Rap1a) (Lafuente et al., 2004) Addgene, Cat # 32698

Software and algorithms

ImageJ NIH https://imagej.nih.gov/ij/

PRISM GraphPad Software Version 9

SigmaPlot SigmaPlot Version 14.0

Other

High-fat high-calorie diet Research Diets Cat # D12492
**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animal models**
WT C57BL/6J (stock number: 000664), db/db and db/+ (stock number: 000697) and diet-induced obese (DIO, stock number: 380050) mice and their controls (stock number: 380056) were from Jackson Labs. Foxo1;3,4fl/fl mice were generously provided by Dr. Rebecca Haeusler from Columbia University (Haeusler et al., 2010). Rap1afl/fl mice were generated by crossbreeding Rap1ab double floxed mice (Jackson Labs, stock number: 021066) with WT C57BL/6J mice and confirmed by genotyping. For all experiments, twelve- to thirty-three-week-old male mice with similar weight were randomly assigned to experimental and control groups. The mice were housed in standard cages at 22°C under a 12-12-hour light-dark cycle in a barrier facility with ad libitum access to water and food. Animal studies were performed in accordance with the Columbia University Institutional Animal Care and Use Committee.

**Human liver samples and human primary hepatocytes**
Part of the human liver samples were obtained from the NIH-supported Liver Tissue Cell Distribution System at the University of Minnesota. The samples were collected postmortem on the date of liver transplantation and preserved as frozen samples. Part of the human liver samples were obtained from patients undergoing bariatric surgery or clinically indicated laparoscopic procedures at the New York Presbyterian Hospital, Columbia University Irving Medical Center. Samples were obtained from intra-operative needle biopsies of the liver at a standard anatomic location. The biopsy specimens were frozen immediately in liquid nitrogen and stored at −80°C until subsequent analyses. The mean age of the donors was 50 (ranging from 32 to 68 years old), and the donors included 4 males and 8 females, as noted in the figures. The Institutional Review Board at the Columbia University Medical Center approved the research protocol. All participants provided written informed consent.

Metabolism-qualified human plateable hepatocytes from a fifty-three-year-old male donor (ThermoFisher, cat # HMCPMS, lot # HU8272) was thawed using cryopreserved hepatocyte recovery medium (ThermoFisher, cat # CM7000) and cultured in Williams’ Medium E (ThermoFisher, cat # A1217601) containing hepatocyte maintenance supplement pack (ThermoFisher, cat # CM4000). The cells were grown at 37°C and 5% CO₂ and treated as noted in the figure legends.

**Primary mouse hepatocytes**
Primary mouse hepatocytes were isolated from 8- to 14-week-old male or female mice as described previously (Ozcan et al., 2012). Briefly, after the mice were euthanized and their abdomens were opened, a catheter was placed into the inferior vena cava. The liver was first perfused with Hanks’ balanced salt solution followed by perfusion with a collagenase solution (Sigma-Aldrich, cat # C2674). The liver was forceps-disaggregated, and the digested liver was centrifuged at 50 × g for 5 min. The supernatant fraction was removed, and the hepatocytes in the pellet were resuspended and cultured in DMEM/F12 (ThermoFisher, cat # 11320) containing 10% (vol/vol) heat-inactivated FBS (ThermoFisher, cat # 16140–071) and 1% penicillin-streptomycin solution (Corning, cat # 30-0002-CI). All cells were grown at 37°C and 5% CO₂.

**Cell lines**
AML-12 (alpha mouse liver 12) mouse hepatocyte cells were obtained from ATCC (cat # CRL-2254). Cells were cultured in DMEM/F12 medium (ThermoFisher, cat # 11320) with 10% (vol/vol) heat-inactivated FBS (ThermoFisher, cat # 16140–071) and 1% penicillin-streptomycin solution (Corning, cat # 30-0002-CI) and grown at 37°C and 5% CO₂. No authentication was done on AML-12 cells by the authors as the experiments were performed in cells cultured for less than 10 passages.

**METHOD DETAILS**

**Reagents and antibodies**
Forskolin (cat # F6886), dexamethasone (cat # D4902), GGT1i (cat # G5294 and G5169), FTi (cat # F9803), Fluvastatin (cat # SML0038), glucose (cat # G7021), glucagon (cat # G2044) and insulin (cat # I0516) were from Sigma-Aldrich. Simvastatin for cell treatment was from Sigma-Aldrich (cat # S6196) and simvastatin for mouse experiments was from Tokyo Chemical Industry (TCI) Company (cat # S0509). Rosuvastatin (cat # 18813), GGPP (cat # 63330) and GGOH (cat # 13272) were from Cayman Chemicals. Anti-β-actin (cat # 4970), anti-pan-Cadherin (cat # 4068), anti-GFP (cat # 2956), anti-GAPDH (cat # 5174), anti-p-Akt (cat # 4060), anti-Akt (cat # 4691), anti-FoxO1...
Mouse treatments and metabolic experiments

Four-week-old male Rap1afl/fl mice were fed with a high-fat diet with 60% kcal from fat (Research Diets, cat # D12492) for 12–20 weeks. The mice were injected with adenovirus-associated virus (AAV) expressing Cre recombinase, driven by the thyroxin-binding globulin (TBG) promoter (AAV8-TBG-Cre) to obtain hepatocyte-specific Rap1a deficient mice. Littermates injected with AAV8-TBG-Gfp were used as control mice. AAV8-H1-scrambled RNA (control), AAV8-H1-shPgg1t1b, AAV8-TBG-CA-Rap1a, AAV8-TBG-Gfp (control), adeno-CA-Rap1a and adeno-LacZ (control) viruses were delivered by tail vein injections of eighteen-week-old male db/db mice or six-week-old male DIO mice (Jackson Labs, stock number: 380050) were fed a high-fat diet containing 0.02% (w/w) simvastatin (Research Diets, cat #D12492) for 12 weeks. Geranylgeraniol (GGOH) was given by oral gavage at a dose of 100 mg/kg/day for 3 weeks.

Fasting blood glucose was measured in mice that were fasted for 5 h or overnight, with free access to water, using a glucose meter (OneTouch Ultra). Plasma insulin levels were measured in mice that were fasted for 5 h or overnight using ultrasensitive mouse insulin ELISA Kit (Crystal Chem, cat # 90080). Glucose tolerance tests (GTT) were performed in overnight-fasted mice by assaying blood glucose at various times after intraperitoneal injection of glucose (0.5 g/kg for db/db mice and 1.0–1.5 g/kg for DIO mice).

Gluconeogenesis and glucose production

For gluconeogenesis, the hepatocytes were serum-depleted overnight by incubation in medium containing 0.5% FBS and were then incubated in serum-free media containing 10 μM forskolin plus 100 nM dexamethasone (F + D) or 100 nM glucagon for three hours. For glucose production assays, primary hepatocytes were cultured in regular DMEM-F12 medium. Cells were then washed three times with PBS and incubated with glucose-free DMEM-F12 without phenol red media containing 2 mM sodium pyruvate, 20 mM lactate, 100 nM dexamethasone and 10 μM forskolin plus 100 nM dexamethasone for sixteen hours. Culture media was collected, and the cells were washed with PBS and lysed in RIPA buffer for protein concentration determination. Glucose concentration in the medium was measured using Glucose (GO) Assay Kit (Sigma-Aldrich, cat # GAGO20) and normalized to the protein amount of the cells. The data is presented as relative to control.

Immunoblotting

Total liver or cell protein was lysed using RIPA buffer (Thermo Scientific, cat # PI89901) containing 2 mM PMSF, 5 μg/mL leupeptin, 10 mM okadaic acid and 5 μg/mL aprotinin on ice. Protein concentration was measured. Protein extracts were electrophoresed on SDS-polyacrylamide gels and transferred to 0.45 μm or 0.2 μm PVDF membranes. Blots were blocked in Tris-buffered saline with 0.1% Tween 20 containing 5% BSA at room temperature for one hour. Membranes were then incubated overnight at 4°C with primary antibodies. The protein bands were detected with horseradish peroxidase-conjugated secondary antibodies and Supersignal West Pico enhanced chemiluminescent solution (ThermoFisher, cat# 34580). ImageJ was used for densitometric analysis of the immunoblots.

Membrane protein extraction

Membrane proteins were isolated as described previously (Petersen et al., 2016). Briefly, cells were washed with cold HBSS and lysed with buffer A containing 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 250 mM Sucrose, 10 mM NaF, and 2 mM
Na3VO4 on ice. Cell lysates were collected and centrifuged at 500 × g for 10 min at 4 °C. The supernatant was collected and centrifuged at 100,000 × rpm for 1 h at 4 °C. Supernatant was discarded, and the pellet was re-suspended with buffer B containing 250 mM Tris-Cl pH7.4, 1 mM EDTA, 0.25 mM EGTA, 10 mM NaF, 2 mM Na3VO4 and 2% Triton X- on ice for 30 min. Lysates were centrifuged at 15,000 × rpm for 20 min at 4 °C. Supernatant was collected, and protein concentration was measured.

**Nuclear protein extraction**
A modified Active Motif Nuclear Kit (cat # 40010) was used for nuclear protein extraction. Cells were scraped with cold PBS containing 2 mM PMSF, 5 μg/mL leupeptin, 10 nM okadaic acid and centrifuged at 200 × g for 5 min at 4 °C. Supernatant was discarded, and the pellet was re-suspended with 1X hypotonic buffer and incubated on ice for 15 min followed by detergent treatment. The lysates were then centrifuged at 14,000 × g for 30 s at 4 °C. Supernatant was discarded, and the pellet was re-suspended in RIPA buffer containing 2 mM PMSF, 5 μg/mL leupeptin, 10 nM okadaic acid and 5 μg/mL aprotinin for 30 min on ice. Lysates were centrifuged at 14,000 × g for 10 min at 4 °C, and the supernatant was collected, and protein concentration was measured.

**Rap1a activity assay**
Rap1a activity was assayed using Active Rap1 Detection Kit (Cell Signaling Technology, cat # 8818). Cells were washed with cold PBS and lysed with lysis/binding/washing buffer containing 2 mM PMSF, 5 μg/mL leupeptin, 10 nM okadaic acid and 5 μg/mL aprotinin. GST-RalGDS-RBD fusion protein was used to bind the activated form of GTP-bound Rap1, which was then immunoprecipitated with glutathione resin. Rap1a activation levels were determined by western blot using a Rap1a primary antibody.

**Measurement of cAMP**
Cyclic AMP XP® Assay Kit (Cell Signaling Technology, cat # 4339) was used for cAMP measurements. Cells were lysed with lysis buffer and centrifuged briefly to remove cell debris. Clear lysates were transferred to the cAMP rabbit monoclonal antibody-coated microwells, mixed with HRP-linked cAMP solution, and incubated at room temperature for 3 h. Following washing to remove excess sample cAMP and HRP-linked cAMP, HRP-coupled TMB is added to develop color and then absorbance was measured.

**Transcriptional reporter assays**
Hepatocytes were transfected with Igfbp1-promoter/pGL3 (Addgene, cat # 12146) and Gfp-FoxO1 plasmids (Addgene, cat # 17551), and renilla luciferase vector was co-transfected as an internal control (Addgene, cat # 27163) (Chen and Prywes, 1999; Frescas et al., 2005; Nakae et al., 2000). Cells were collected 72 h post-transfection with 1X Passive Lysis Buffer (Promega, cat # E1910). Firefly luciferase reporter was determined by addition of Luciferase Assay Substrate and quantification of luminescence on a FLUOstar Omega plate reader.

**Preparation of cholesterol rich liposomes**
DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, cat # 850345) and cholesterol (Sigma-Aldrich, cat # C8667) were dissolved in chloroform. For cholesterol rich liposomes, 40 mg of DMPC was mixed with 80 mg of cholesterol in a glass vessel and the solvent was dried under a stream of nitrogen gas. Multilamellar vesicles (large liposomes>1μM) were formed by addition of 10 ml of PBS to the lipid film and then shaking the mixture. The liposomes were reduced in size by probe sonication on ice for 10–15 min. The solution turned white and was centrifuged at 10,000 × g for 10 min. Supernatant was collected and extruded through 100 nm polycarbonate filter. Aliquots were stored in glass vials under argon at 4 °C and used within 2 weeks.

**Phalloidin staining**
Hepatocytes cultured in glass coverslips were washed with PBS and fixed with 4% formaldehyde for 20 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min, followed by 3 washes of PBS. F-actin fibers were stained for 1 h at room temperature with Phalloidin-iFluor 488 reagent (Abcam, cat # ab176753, 1:1000 diluted in 1%BSA). After washing three times with PBS, images were taken using a Leica epifluorescence microscope (DMi6000B).

**Quantitative PCR**
RNA was extracted from hepatocytes or liver tissue using TRI Reagent (Sigma-Aldrich, cat # T9424) and cDNA was synthesized from 1 μg of RNA using a cDNA synthesis kit (Invitrogen). Real-time PCR was performed using a 7500 Real-Time PCR system and SYBR Green reagents (Applied Biosystems). The primer sequences are listed in Table S1.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
All results are presented as mean ± SEM. Statistical significance was determined using SigmaPlot software. Data that passed the normality tests were analyzed using Student’s t-test and ANOVA. Data that were not normally distributed were analyzed using the nonparametric Mann-Whitney U test. Differences were considered statistically significant at p < 0.05.