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Disruption of the AMPK–TBC1D1 signaling nexus increases lipogenesis and causes obesity in mice via promoting IGF1 secretion

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside; AMPK, AMP-activated protein kinase; GAP, GTPase activating protein; IGF1, insulin-like growth factor 1; mTOR, mammalian target of rapamycin; PKB, protein kinase B (also known as Akt); TBC1D1, tre-2/USP6, BUB2, cdc16 domain family member 1 (the TBC domain is the GAP domain); TSC2, tuberous sclerosis 2 (also known as tuberin).

Abstract

TBC1D1 is a Rab GTPase activating protein that is phosphorylated on Ser^{231} by the AMP-activated protein kinase (AMPK) in response to intracellular energy stress. However, the *in vivo* role and importance of this phosphorylation event remains unknown. To address this question, we generated a mouse model harboring a $TBC1D1^{Ser231Ala}$ knockin mutation and found that the knockin mice developed obesity on a normal chow diet. Mechanistically, TBC1D1 is located on IGF1 storage vesicles, and the knockin mutation increases endocrinal and paracrinal/autocrinal IGF1 secretion in a Rab8a-dependent manner. Hypersecretion of IGF1 causes increased lipogenesis via activating the protein kinase B (PKB, also known as Akt)–mammalian target of rapamycin (mTOR) pathway in adipose tissues, which contributes to the development of obesity, diabetes and hepatic steatosis as the knockin mice age. Collectively, these findings demonstrate that the AMPK–TBC1D1 signaling nexus interacts with the PKB–mTOR pathway via IGF1 secretion, which consequently controls lipogenesis.

INTRODUCTION

Due to changes in diet and lifestyle, metabolic syndrome including obesity, type 2 diabetes and non-alcoholic fatty liver disease are increasing in prevalence worldwide, putting a huge burden on modern society and motivating us to get better understanding of these syndromes and how to combat them.

High energy intake and lack of physical activity can result in imbalances of energy metabolism and changed energy status of the body, which is monitored by the energy sensing kinase AMPK (Hardie et al., 2012). The AMPK holoenzyme is a heterotrimer, consisting of catalytic α (α1 and α2), regulatory β (β1 and β2) and γ (γ1, γ2 and γ3) subunits (Hardie et al., 2012). AMPK is mainly activated through Thr^{172} phosphorylation in its catalytic T-loop by the upstream kinase LKB1 under energy stress conditions that increase the cellular AMP:ATP ratio (Hawley et al., 2003; Woods et al., 2003). The activation of AMPK also requires the v-ATPase–Ragulator protein complex, which recruits the AXIN–LKB1–AMPK complex onto late endosomes where LKB1 phosphorylates AMPK (Zhang et al., 2014). AMPK can regulate both glucose and lipid metabolism by its phosphorylation of multiple substrates (Hardie, 2013). For instance, acetyl-CoA carboxylase (ACC), an enzyme important for fatty acid synthesis and oxidation, is regulated through an inhibitory phosphorylation by AMPK, which is essential for the control of lipid metabolism (Fullerton et al., 2013). AMPK can also control glucose uptake into skeletal muscle by promoting translocation of the glucose transporter 4 (GLUT4) from its intracellular storage sites onto plasma membrane (Kurth-Kraczek et al., 1999). Genetic ablation of individual AMPK subunits (α2, β2 or γ3) or LKB1, or overexpression of a dominant inhibitory mutant of AMPK in mouse, inhibit muscle glucose uptake in response to a pharmacological AMPK activator, 5-aminoimidazole-4-carboxamide riboside (AICAR), and partially inhibit glucose uptake in response to muscle contraction (Barnes et al., 2004; Jorgensen et al., 2004; Mu et al., 2001; Sakamoto et al., 2005; Steinberg et al., 2010). However, the mechanism by which AMPK promotes translocation of GLUT4 onto plasma membrane is not well understood.

TBC1D1 is a Rab GTPase activating protein (RabGAP) and a R125W mutation on this protein has been linked to familial female obesity (Stone et al., 2006). A truncation or knockout of TBC1D1 confers leanness to mice fed on high fat diet with increased fatty acid oxidation in skeletal muscle (Chadt et al., 2008; Dokas et al., 2013). TBC1D1 has also been implicated in regulating trafficking of the glucose transporter 4 (GLUT4) (An et al., 2010; Peck et al., 2009)

while its deficiency causes a decreased expression level of GLUT4 in skeletal muscle (Chadt et al., 2008; Dokas et al., 2013; Stockli et al., 2015).

We previously identified TBC1D1 as an AMPK substrate that is phosphorylated on Ser^{231} by AMPK and interacts with 14-3-3 proteins upon Ser^{231} phosphorylation (Chen et al., 2008). However, the physiological role of this AMPK–TBC1D1 signal nexus remains elusive. In this study, we generated a TBC1D1^{Ser231Ala} knockin mouse model to address the functions of TBC1D1 Ser^{231} phosphorylation *in vivo*.

RESULTS

Generation and characterization of the TBC1D1^{Ser231Ala} knockin mice

The TBC1D1^{Ser231Ala} knockin mice were generated using the gene targeting strategy illustrated in Supplementary Fig. 1A. The expression levels of the TBC1D1^{Ser231Ala} mutant proteins were normal, and there was no detectable alteration in the expression of the related RabGAP AS160 (also known as TBC1D4) in various tissues from the TBC1D1^{Ser231Ala} knockin mice (Fig. 1A). As expected, the phosphorylation of Ser^{231} on TBC1D1 was not detectable in the tissues from the TBC1D1^{Ser231Ala} knockin mice (Fig. 1A). The pharmacological AMPK activator metformin induced phosphorylation of AMPK in primary hepatocytes from the knockin mice to a similar extent as in wild-type cells (Fig. 1B). In contrast and as anticipated, metformin-stimulated TBC1D1 Ser^{231} phosphorylation was only detected in the lysates of wild-type, but not knockin, hepatocytes (Fig. 1B). The metformin-stimulated 14-3-3–TBC1D1 interaction assessed by 14-3-3 overlay assays was diminished in the TBC1D1 immunoprecipitates from the knockin cell lysates as compared to the wild-type controls (Fig. 1B). Taken together, these data validate the suitability of the TBC1D1^{Ser231Ala} knockin mice and their derived cells for studying the specific *in vivo* and *in vitro* function of TBC1D1 Ser^{231} phosphorylation.

The TBC1D1^{Ser231Ala} knockin mice developed obesity and displayed characteristics of metabolic syndrome when they aged

We next monitored the growth of the TBC1D1^{Ser231Ala} knockin mice and found that they became significantly heavier than the wild-type littermates from 5 weeks after birth (Supplementary Fig. 1B). The TBC1D1^{Ser231Ala} knockin mice had longer bodies and tibias, and higher bone mineral density (BMD) than wild-types (Fig. 1C, Supplementary Fig. 1C-E). The growth plates of tibia from the TBC1D1^{Ser231Ala} knockin mice were significantly elongated, in both the zone of proliferation and of hypertrophy (Supplementary Fig. 1F-G). The increased body

mass of the young knockins (less than 4-month-old) was accounted for by their significantly increased lean mass (Fig. 1D, Supplementary Fig. 2A-B). In contrast, the fat mass in the young TBC1D1^{Ser231Ala} knockin mice was comparable to that of the wild-type littermate controls (Fig. 1D, Supplementary Fig. 2A-B). However, although young TBC1D1^{Ser231Ala} knockin mice contained a normal fat mass and enhanced lean mass, older TBC1D1^{Ser231Ala} knockin mice became fatter, and had higher lean and fat masses than wild-types (Fig. 1E-F, Supplementary Fig. 2C-D). Around five months of age was a transition period for increased fat mass in the TBC1D1^{Ser231Ala} knockin mice. Consistent with the increased fat mass, adipocytes were markedly enlarged in the older TBC1D1^{Ser231Ala} knockin mice (Fig. 1G-I).

Obesity is a high risk factor for the development of type II diabetes and hepatic steatosis. The young TBC1D1^{Ser231Ala} knockin mice had normal blood chemistry parameters including blood glucose, free fatty acid, triglyceride, total cholesterol and plasma insulin, and displayed normal glucose tolerance (Fig. 2A, and D-H). However, as these animals aged (~1-year-old), they developed hyperglycemia, hyperinsulinemia and hypercholesterolemia (Fig. 2D-F), and became glucose intolerant and insulin resistant (Fig. 2B-C). Furthermore, these mice developed hepatic steatosis, and their livers were significantly enlarged and contained larger lipid droplets (Fig. 2I-L).

Glucose uptake did not account for the obese phenotype of the TBC1D1^{Ser231Ala} knockin mice

TBC1D1 has been implicated in regulating GLUT4 trafficking (An et al., 2010; Peck et al., 2009), and its deficiency in mice decreases the GLUT4 expression and glucose uptake in skeletal muscle (Chadt et al., 2008; Dokas et al., 2013; Stockli et al., 2015). Therefore the TBC1D1^{Ser231Ala} knockin mutation could potentially cause obesity via regulating glucose uptake. However, we found that GLUT4 expression was normal in skeletal muscle from the TBC1D1^{Ser231Ala} knockin mice (Supplementary Fig. 3A). Furthermore, insulin- or contraction-stimulated glucose uptake rates in skeletal muscle from the TBC1D1^{Ser231Ala} knockin mice were indistinguishable from those in wild-type controls (Supplementary Fig. 3B-C). In primary adipocytes, the TBC1D1^{Ser231Ala} knockin mutation also did not affect insulin-stimulated GLUT4 translocation (Supplementary Fig. 3D-F).

Collectively, these data show that glucose uptake most likely did not account for the lower energy expenditure and obese phenotype of the TBC1D1^{Ser231Ala} knockin mice.

Lipogenesis was increased in the adipose of the TBC1D1^{Ser231Ala} knockin mice

We next investigated whether altered adipogenesis, lipogenesis or lipolysis in the adipose might account for the obese phenotype of the TBC1D1^{Ser231Ala} knockin mice. To this end, we analysed expression of key regulators for these processes in the adipose of the TBC1D1^{Ser231Ala} knockin mice. The expression of adipose triglyceride lipase (ATGL), an important enzyme for lipolysis, was not altered in the adipose of the TBC1D1^{Ser231Ala} knockin mice (Fig. 3A). Similarly, the expression of the adipogenic CEBPα and PPARγ genes remained normal in the adipose of the TBC1D1^{Ser231Ala} knockin mice (Fig. 3A-B). In contrast, the expression of fatty acid synthase (FASN), a key enzyme for lipogenesis, was significantly upregulated in the adipose of the TBC1D1^{Ser231Ala} knockin mice at both mRNA and protein levels, despite the normal expression of SREBP1 that is an upstream transcriptional regulator for FASN (Fig. 3A-B). It has recently been shown that lipin1, a downstream target of mTOR, can downregulate SREBP1 activity in the nucleus and consequently control the expression of FASN in adipose (Peterson et al., 2011). The entry of lipin1 into the nucleus is under control of its phosphorylation by mTOR, and this phosphorylation promotes lipogenesis by releasing the inhibitory effect of lipin1 on SREBP1 and consequently upregulating FASN expression (Peterson et al., 2011). Interestingly, phosphorylation of lipin1 was significantly upregulated in the adipose of the TBC1D1^{Ser231Ala} knockin mice (Fig. 3C), suggesting that upregulation of FASN in these mice was due to enhanced SREBP1 activity via lipin1 phosphorylation. Consistently, phosphorylation of PKB, tuberous sclerosis 2 (TSC2, also known as tuberin) and mTOR were all increased in the adipose of the TBC1D1^{Ser231Ala} knockin mice (Fig. 3C). This PKB activation in the adipose of the TBC1D1^{Ser231Ala} knockin mice selectively regulated the phosphorylation of TSC2–mTOR, but not AS160, which is a key regulator for insulin-stimulated glucose uptake (Fig. 3C). We further found that the phosphorylation of PKB, TSC2 and mTOR was also upregulated in skeletal muscle and liver of the TBC1D1^{Ser231Ala} knockin mice (Supplementary Fig. 4).

Together, these data show that the PKB–TSC2–mTOR–lipin1 pathway is activated in the TBC1D1^{Ser231Ala} knockin mice, which can increase lipogenesis through upregulation of FASN.

Plasma IGF1 levels were elevated in the TBC1D1^{Ser231Ala} knockin mice

We next sought to find out how the TBC1D1^{Ser231Ala} knockin mutation causes the activation of the PKB–TSC2–mTOR pathway. Considering the overgrowth phenotype of the TBC1D1^{Ser231Ala} knockins, we surmised that the levels of IGF1, a key growth promoter that

activates the PKB–TSC2–mTOR pathway in multiple tissues, might be elevated. Indeed, plasma IGF1 levels were significantly higher in both male and female TBC1D1^{Ser231Ala} knockin mice compared with their wild-type littermates (Fig. 4A, Supplementary Fig. 2G). In contrast, the plasma levels of the IGF1-binding protein IGFBP3 were normal in the knockin mice (Fig. 4B, Supplementary Fig. 2H). The expression levels of IGF1 and IGFBP3 were normal at both the mRNA and protein levels within the liver (Fig. 4D-G), which is the major endocrinal production site for these proteins. Expression of IGF1 and IGFBP3 in the liver is under control of growth hormone (GH). Consistent with unaltered expression of IGF1 and IGFBP3 in the liver, plasma GH levels were normal in the TBC1D1^{Ser231Ala} knockin mice (Fig. 4C, Supplementary Fig. 2I). We further found that the hepatic expression and phosphorylation of the GH receptor (GHR) and its downstream mediator, JAK2, were unaltered in TBC1D1^{Ser231Ala} knockin mice (Fig. 4D). These data show that plasma IGF1 levels were selectively elevated in the TBC1D1^{Ser231Ala} knockin mice, but with no alteration in the action of GH on its hepatic expression.

TBC1D1 colocalizes with IGF1 vesicles and regulates IGF1 secretion

IGF1 is synthesized and temporarily stored within vesicles in hepatocytes, and then secreted into the bloodstream. Interestingly, when TBC1D1 and IGF1 were co-expressed in human liver carcinoma HepG2 cells, the two proteins largely co-localized with each other (Supplementary Fig. 5A). We further found that endogenous TBC1D1 was localized on the IGF1 storage vesicles in primary hepatocytes (Fig. 5A) and in myotubes (Supplementary Fig. 5B). Together, these data suggest that TBC1D1 might be involved in the regulation of IGF1 secretion. Indeed, knockdown of TBC1D1 significantly decreased IGF1 secretion rates in primary hepatocytes with no alteration in IGF1 expression (Fig. 5B-C). Furthermore, in primary hepatocytes from our previously reported TBC1D1 knockout mice (Ducommun et al., 2012), IGF1 secretion rates were also significantly lower than those of the wild-type controls, despite normal hepatic IGF1 expression (Fig. 5D-E).

The TBC1D1^{Ser231Ala} knockin mutation increases both endocrinal and paracrinal/autocrinal IGF1 secretion

Consistent with the elevated levels of plasma IGF1 in the TBC1D1^{Ser231Ala} knockin mice, IGF1 secretion rates of primary hepatocytes from these mice were significantly higher than those of wild-type cells under unstimulated conditions (Fig. 5F). The widely-used AMPK activators, metformin and AICAR, significantly decreased IGF1 secretion rates of wild-type hepatocytes

(Fig. 5F). While the rates of IGF1 secretion by TBC1D1^{Ser231Ala} knockin hepatocytes decreased in response to metformin or AICAR, these secretion rates still remained significantly higher than those of wild-type cells treated with these drugs (Fig. 5F). We confirmed that the activation of AMPK by AICAR and metformin was comparable in primary hepatocytes from the knockin mice and in wild-type cells, with no effect on IGF1 protein expression (Fig. 5J). We further found that the TBC1D1^{Ser231Ala} knockin mutation also affected paracrinal/autocrinal IGF1 secretion. In primary chondrocytes, IGF1 secretion rates were significantly higher in the knockin cells than those in the wild-type cells under both unstimulated and AICAR-treated conditions (Fig. 5G and K). Similarly, IGF1 secretion rates were also significantly increased in the knockin adipocytes and isolated skeletal muscle *ex vivo*, compared with the wild-type cells or tissues (Fig. 5H-I).

Consistent with normal plasma levels of IGFBP3, secretion of IGFBP3 was unaltered in primary hepatocytes from the TBC1D1^{Ser231Ala} knockin mice (Supplementary Fig. 6). These data show that the TBC1D1^{Ser231Ala} knockin mutation selectively affects IGF1 secretion.

Inhibition of IGF1 secretion or action blunted the hyperactivation of the PKB pathway in primary cells from the TBC1D1^{Ser231Ala} knockin mice

We next sought to establish a causal relationship between the hypersecretion of IGF1 and the activation of the PKB–TSC2–mTOR pathway in the TBC1D1^{Ser231Ala} knockin mice. To this end, we first employed the non-IGF1 secretive cell line HEK293 (Supplementary Fig. 7A) to investigate the effect of the TBC1D1^{Ser231Ala} mutation on the activation of the PKB-TSC2-mTOR pathway. Over-expression of human TBC1D1 wild-type or TBC1D1^{Ser231Ala} mutant proteins in HEK293 cells did not affect the PKB–TSC2–mTOR pathway (Supplementary Fig. 7B), suggesting that the activation of the PKB–TSC2–mTOR pathway caused by the TBC1D1^{Ser231Ala} mutation requires IGF1 secretion. We next employed primary adipocytes and hepatocytes isolated from these knockin mice, in which the PKB–TSC2–mTOR pathway was activated under unstimulated conditions (Supplementary Fig. 7C-F). It is well known that activation of AMPK can inhibit mTOR through phosphorylation of TSC2 and raptor (Gwinn et al., 2008; Inoki et al., 2003). Inhibition of mTOR can decrease the serine phosphorylation of insulin receptor substrate-1 (IRS-1) and consequently increase its tyrosine phosphorylation that results in PKB activation (Tzatsos and Kandror, 2006). Consistent with these reports, AICAR could inhibit the phosphorylation of mTOR and consequently activate PKB in wild-type adipocytes (Fig. 6A-C). Both PKB and mTOR phosphorylation were again higher in TBC1D1^{Ser231Ala} knockin adipocytes

than wild-type cells under unstimulated conditions. As in wild-type cells, AICAR could inhibit mTOR phosphorylation in TBC1D1^{Ser231Ala} knockin adipocytes. However, in contrast to the increase of PKB phosphorylation in wild-type adipocytes, PKB phosphorylation was decreased in TBC1D1^{Ser231Ala} knockin adipocytes upon AICAR stimulation (Fig. 6A-C), which is most likely due to inhibition of IGF1 secretion by AICAR.

We further used an IGF1 receptor inhibitor, picropodophyllin (PPP) (Girnita et al., 2004), to treat primary hepatocytes from the wild-type and TBC1D1^{Ser231Ala} knockin mice. The phosphorylation of PKB was significantly increased in TBC1D1^{Ser231Ala} knockin hepatocytes under untreated conditions (Fig. 6D-E). Morover, blocking the IGF1 receptor with PPP strongly decreased PKB phosphorylation in TBC1D1^{Ser231Ala} knockin, but not wild-type, hepatocytes (Fig. 6D-E). These findings suggest that hyperactivation of PKB in TBC1D1^{Ser231Ala} knockin hepatocytes was most likely due to hypersecretion of IGF1. The reason for the unaltered PKB phosphorylation in wild-type cells upon PPP treatment is not clear, however we speculate that this basal PKB activation in wild-type hepatocytes is probably not elicited through the IGF1 receptor.

Together, these data strongly suggest a causal relationship between hypersecretion of IGF1 and activation of the PKB–TSC2–mTOR pathway in the TBC1D1^{Ser231Ala} knockin mice.

Rab8a plays a key role downstream of the TBC1D1 in regulating IGF1 secretion

We then sought to find out how TBC1D1 and its Ser^{231} phosphorylation regulate IGF1 secretion. IGF1 secretion rates were largely restored when a human recombinant TBC1D1 was expressed in the TBC1D1 knockout hepatocytes (Fig. 7A). Unlike the endogenous TBC1D1, the expressed human TBC1D1 was hypophosphorylated, and the expression of human TBC1D1^{Ser237Ala} mutant protein (Ser^{237} on human TBC1D1 corresponds to Ser^{231} on the mouse protein) did not increase IGF1 secretion rates over-and-above those achieved with the wild-type TBC1D1 (Fig. 7A). Interestingly, the expression of a GAP-deficient TBC1D1 mutant (Roach et al., 2007) could not restore IGF1 secretion rates (Fig. 7A), suggesting that the GAP activity of TBC1D1 is required for IGF1 secretion. We next investigated which Rab(s) downstream of TBC1D1 mediates IGF1 secretion by down-regulating four known *in vitro* TBC1D1 substrates, namely Rab2a, Rab8a, Rab10 and Rab14 (Roach et al., 2007). Knockdown of Rab8a caused a significant decrease in IGF1 secretion rates in wild-type primary hepatocytes (Fig. 7B-C) while down-regulation of Rab2a, Rab10 and Rab14 had no significant effect (Supplementary Fig. 8).

We also found that Rab8a largely colocalized with IGF1 in primary hepatocytes (Fig. 7D). Furthermore, knockdown of Rab8a normalized IGF1 secretion in primary hepatocytes from the TBC1D1^{Ser231Ala} knockin mice (Fig. 7E-F), suggesting that regulation of IGF1 secretion by the TBC1D1 Ser^{231} phosphorylation requires Rab8a. Overexpression of the wild-type Rab8a and a GDP-bound Rab8a^{T22N} mutant, but not a GTP-bound Rab8a^{Q67L} mutant, could restore IGF1 secretion in the TBC1D1 knockout hepatocytes (Fig. 7G-H). In an *in vitro* assay, immunoprecipitated recombinant hTBC1D1 displayed significant GAP activity towards Rab8a and enhanced the GTPase activity of Rab8a. The GAP activity of hTBC1D1 towards Rab8a was inhibited by its cellular phosphorylation in response to AMPK activator phenformin, and in contrast was enhanced by Ser237Ala mutation (Fig. 7I-J). These data suggest that the GDP-bound Rab8a promotes IGF1 secretion downstream of TBC1D1. Consistent with this proposal, we found that knockdown of Rabin8, a guanine nucleotide exchange factor (GEF) for Rab8a (Hattula et al., 2002), promoted IGF1 secretion in primary hepatocytes (Fig. 7K-L). Together, these data show that TBC1D1 and its Ser^{231} phosphorylation control IGF1 secretion by regulating Rab8a.

DISCUSSION

Our findings shed light on how energy status regulates lipogenesis and are consistent with a model in which the AMPK–TBC1D1 signaling nexus controls lipogenesis by regulating endocrinal and paracrinal/autocrinal IGF1 secretion. Inhibition of TBC1D1 Ser^{231} phosphorylation increases lipogenesis and consequently causes obesity in mice most likely due to hypersecretion of IGF1 and hyperactivation of the PKB–TSC2–mTOR pathway.

It has long been recognized that the energy and nutrient sensors, AMPK and mTOR, play opposing roles in governing cell growth in response to varying energy status (Inoki et al., 2003; Sarbassov et al., 2005). Energy deficiency activates AMPK, which in turn phosphorylates proteins including TSC2 and raptor. Phosphorylation of TSC2 leads to inhibition of the small GTPase Rheb, while phosphorylated raptor binds to the dimeric phosphoprotein-binding 14-3-3 proteins, and both of these mechanisms lead to inhibition of mTOR. The AMPK–TSC2–Rheb–mTOR and AMPK–raptor–mTOR pathways restrict cell growth in response to energy shortage (Gwinn et al., 2008; Inoki et al., 2003). Besides this opposing effect on growth, both AMPK and mTOR regulate lipogenesis. AMPK can phosphorylate ACC and thereby inhibit fatty acid

synthesis (Fullerton et al., 2013). In contrast, mTOR can phosphorylate lipin1 and the latter can promote lipogenesis by enhancing SREBP1 activity (Peterson et al., 2011).

Our findings reveal a previously unrecognized layer of regulation, involving IGF1 as a systemic signal that links these two signaling pathways to control lipogenesis in response to energy status. In our model, we propose that energy deficiency activates AMPK, which inhibits both endocrinal and paracrinal/autocrinal IGF1 secretion via phosphorylation of TBC1D1 and possibly other regulators of IGF1 secretion. Decreased endocrinal and paracrinal/autocrinal IGF1 secretion results in hypoactivation of the PKB–mTOR pathway, which may contribute to a decline in lipogenesis in adipose tissues. When energy is sufficient, AMPK activity and TBC1D1 phosphorylation decrease in the body, which promotes endocrinal and paracrinal/autocrinal IGF1 secretion. The elevated IGF1 activates PKB–mTOR signaling, which may promote lipogenesis in adipose tissues. Our findings not only elucidate the complexity of the regulatory mechanism linking energy status with lipogenesis but also provide novel potential targets for drug discovery to combat with obesity. These findings may also help to elucidate the therapeutic mechanisms for the anti-diabetic drug metformin. Though metformin has been used as the frontline anti-diabetic drug for many years (Wong et al., 2014), its therapeutic mechanisms are still not fully understood. Our observation that metformin inhibits IGF1 secretion opens a new possibility that this drug may regulate lipogenesis in the adipose through modulating IGF1 secretion.

Genetic evidence has suggested that TBC1D1 is linked to obesity although the molecular mechanisms are not fully understood. For instance, the TBC1D1^{R125W} mutation is a candidate for obesity susceptibility in human patients (Stone et al., 2006), while deficiency of TBC1D1 protects mice from diet-induced obesity (Chadt et al., 2008; Dokas et al., 2013). Serum IGF1 levels are associated with adiposity (Gayet et al., 2004): lower serum IGF1 levels in several mouse models correlate with reduced adipose tissue (Hesse et al., 2012; Wang et al., 2014), while higher IGF1 levels in the blood are associated with increased adiposity in a liver-specific PTPN2 knockout mouse (Gurzov et al., 2014). Our findings provide one mechanism linking TBC1D1 Ser^{231} phosphorylation to lipogenesis by controlling IGF1 secretion, which contributes to the development of obesity, type II diabetes and hepatic steatosis as the knockin mice age.

Clearly, TBC1D1 has other functions, and can regulate GLUT4 expression and fatty acid oxidation in skeletal muscle (Chadt et al., 2008; Dokas et al., 2013; Stockli et al., 2015). TBC1D1 can be phosphorylated on multiple sites besides Ser^{231} (Chen et al., 2008), and

electroporation of a TBC1D1-4P mutant (in which Ser^{231}, Thr^{499}, Thr^{590}, and Ser^{621} are mutated to Ala) into skeletal muscle decreases contraction-stimulated glucose uptake (An et al., 2010). Since our study does not support a role for TBC1D1 Ser^{231} phosphorylation in regulating GLUT4 expression or trafficking under the conditions tested here, it is possible that other phosphorylation sites on TBC1D1 might be important for contraction-stimulated muscle glucose uptake.

Our knowledge of the regulation of IGF1 secretion is surprisingly sparse, despite the many essential roles of this hormone and the intense study of how its downstream signaling pathways regulate cellular growth (Maggio et al., 2013). Besides the regulatory mechanism we describe here, the only previously known mechanism involves a calcium sensor synaptotagmin-10 that regulates activity-dependent IGF1 secretion in olfactory bulb neurons (Cao et al., 2011). Synaptotagmin-10 can bind to phospholipid and the SNARE-complex in a calcium-dependent manner, which triggers exocytosis. Given the important functions of IGF1 and potential for modulating its release for therapeutic purposes, we must elucidate the regulation of IGF1 secretion in more detail in future. There are several open questions stemming from our model, which deserve to be addressed. For instance, what are upstream regulators of AMPK that control IGF1 secretion under energy deficient conditions? Upon energy stress, the late endosomal/lysosomal protein complex v-ATPase–Ragulator recruits AXIN–LKB1–AMPK onto late endosome, which consequently activates AMPK (Zhang et al., 2014). We are therefore intrigued to know whether the v-ATPase–Ragulator–AXIN–LKB1 complex controls IGF1 secretion through activating AMPK in response to energy deficiency. Another question is how Rab8a regulates IGF1 secretion in a manner that is dependent on its GDP-bound form. Although Rabs usually regulate their effector proteins in their GTP-bound forms, there are precedents that GDP-bound forms of Rabs can have biological functions. For instance, Rab8a has recently been shown to promote fusion of lipid droplets in its GDP-bound form (Wu et al., 2014). Identification of downstream effector(s) for the GDP-bound form of Rab8a will help to elucidate the mechanism how Rab8a regulates IGF1 secretion and deepen our understanding of this process.

MATERIALS AND METHODS

Materials

Recombinant human insulin was purchased from Novo Nordisk (Denmark), IGF1 from Cell Signaling Technology (Danvers, MA, USA), AICAR from Toronto Research Chemicals (Toronto, Ontario, Canada), and metformin from Sigma-Aldrich (St. Louis, Missouri, USA).

Microcystin-LR was from Enzo Life Sciences (Farmingdale, NY, USA). Picropodophyllin (PPP) was from Selleck Chemicals (Shanghai, China). Protein G-Sepharose was from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and NHS-digoxygenin (NHS-DIG) from Roche Diagnostics (Basel, Switzerland). 2-deoxy-D-[1,2-^{3}H(N)]glucose and D-[1-^{14}C]-mannitol were from PerkinElmer. All other chemicals were from Sigma-Aldrich or Sangon Biotech (Shanghai, China).

Antibodies

The rabbit antibody that recognises phosphorylated Ser^{231} on TBC1D1 (#07-2268) and the mouse antibody against IGF1 (#05-172) were from Millipore. The anti-GH antibody (T-20, sc-10365), anti-Rab14 (sc-98610) and anti-GHR (S-19, sc-10355) were from Santa Cruz. The rabbit antibodies that recognise phosphorylated Thr^{172} on AMPK (#2531), phosphorylated Ser^{212} on ACC2 (#3661), phosphorylated Ser^{473} on PKB (#9271), phosphorylated Ser127 on YAP (#4911), anti-AMPK alpha subunit (#2532), anti-ACC (#3676), anti-PKB (#9272), anti-TBC1D1 (#4629), anti-Rab8a (#6975), anti-Rab10 (#4262), PhosphoPlus Jak2 antibody duet (#8224), PPARγ regulated fatty acid metabolism antibody sampler kit (#8660), fatty acid and lipid metabolism antibody sampler kit (#8335) and mTOR substrates antibody sampler kit (#9862) were from Cell Signaling Technology. The rabbit anti-IGF1, anti-Rab2 and anti-IGFBP3 antibodies were from Proteintech.

Generation of the TBC1D1^{Ser231Ala} knockin mouse

The TBC1D1^{Ser231Ala} knockin mouse was generated by the transgenic service facility at University of Dundee following the targeting strategy outlined in Fig. 1A. Briefly, the Ser^{231} (the surrounding sequence is MRKSF**s**QP, Ser^{231} shown in lower case bold) on TBC1D1 was mutated to alanine by point mutagenesis. The fourth exon (numbering according to Tbc1d1-002 ENSMUST00000121370) harbouring the point mutation was flanked by *lox*P sites to facilitate its excision by Cre recombinase. The TBC1D1^{Ser231Ala} knockin mouse was generated after *in vivo* Flp-mediated removal of selection marker, Neo gene, which was flanked by *frt* sites.

Mouse husbandry and genotyping

The Ethics Committees initially at University of Dundee, and latterly at Nanjing University approved all animal studies, breeding and husbandry. The TBC1D1^{Ser231Ala} knockin mice were back-crossed with C57BL6 for at least 8 generations before experiments. Mice were housed with a light/dark cycle of 12 h, and free access to food and water unless stated.

Genotyping of the TBC1D1^{Ser231Ala} knockin and wild-type alleles was performed by PCR using the following primers: 5'- TGGTTTACTGTGGCAGGAGGCAT-3' and 5'-CACTGGGCTTTGTCTCTGATACTG-3'. The TBC1D1 knockout mice were genotyped as previously described (Ducommun et al., 2012).

Body composition analysis

Mouse body composition was determined via dual-energy X-ray absorptiometry (DEXA) using a Lunar PIXImus II densitometer (GE Healthcare). Mice were anaesthetized with isoflurane, and images were taken with the PIXImus II densitometer and analyzed using the PIXImus II software following the manufacturer's instructions.

Glucose tolerance test and insulin tolerance test

After being denied access to food for 4 h (prior to insulin tolerance test) or overnight (16 h; for glucose tolerance test), mice were tail bled for measurement of basal blood glucose levels using a Breeze 2 glucometer (Bayer). Afterwards, mice were intraperitoneally injected either with a bolus of glucose (2 mg glucose per g of body weight) for intraperitoneal glucose tolerance test, or with a bolus of insulin (0.75 mU insulin per g of body weight) for insulin tolerance tests. In case of the oral glucose tolerance test, mice were orally administered via gavage with a bolus of glucose (1.5 mg glucose per g of body weight). Blood glucose levels were subsequently determined at the indicated times.

Blood chemistry and hormone analysis

Blood glucose levels were determined using a Breeze 2 glucometer (Bayer). For other blood parameters, blood was collected from tail veins of random-fed or overnight fasted mice. Plasma IGF1, IGFBP3, GH and insulin levels were determined using an IGF1 ELISA kit (Cat No. ELM-IGF1-1, RayBiotech Inc, USA), IGFBP3 ELISA kit (Cat No. ELM-IGFBP3, RayBiotech Inc, USA), GH ELISA kit (EZRMGH-45K, EMD Millipore, USA) and insulin ELISA kit (EZRMI-13K, EMD Millipore, USA), respectively. Plasma free fatty acid, triglyeride and total cholesterol levels were determined using a Wako LabAssay NEFA kit (294-63601), LabAssay Triglyceride (290-63701) and LabAssay Cholesterol kit (294-65801) (Wako Chemicals USA, Inc.).

Primary cell isolation and IGF1 secretion assay

Primary mouse hepatocytes were isolated using a collagenase-based method and cultured as previously described (Patel et al., 2014). Hepatocytes from preparations with a cell viability ≥

90% were seeded in 12-well plates at a density of 1.25 x 10^5 per well in a plating medium (M199 with GlutaMAX^{TM} supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1% bovine serum albumin (BSA), 10% (v/v) foetal bovine serum, 10 nM insulin, 200 nM triiodothyronine, 500 nM dexamethasone) for 4 h and then incubated overnight (16 h) in a recovery medium (M199 with GlutaMAX^{TM} supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% (v/v) foetal bovine serum). The next morning, cells were washed once with PBS and incubated in a serum-starvation medium (M199 with GlutaMAX^{TM} supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin) for 4 h. After serum-starvation, cells were washed once with PBS and further incubated in an assay medium (M199 with GlutaMAX^{TM} supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin) for another 4 h in the presence or absence of stimuli. The assay medium was collected for measurements of IGF1 using an ELISA kit (Cat No. ELM-IGF1-1, RayBiotech Inc, USA), and the cells were lysed and used for Western blot analysis.

Primary mouse chondrocytes were isolated from articular cartilage of hind legs of 2- to 7-day-old pups as previously described (Archer et al., 1990). Briefly, cartilage was digested in the digestion medium (DMEM/F12 supplemented with 2.5% FBS, 1% penicillin/streptomycin and 1 mg/ml type 2 collagenase), and chondrocytes were filtered and washed with DMEM/F12 medium. Chondrocytes were then cultured overnight in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Afterwards, chondrocytes were changed to fresh assay medium for another 4 h and the assay medium was collected for IGF1 measurement.

Primary preadipocytes were isolated from epididymal fat pad and differentiated into adipocytes as previously described (Wu et al., 2014). On day 6 after initiation of differentiation, adipocytes were changed to fresh assay medium for another 4 h and the assay medium was collected for IGF1 measurement.

For IGF1 secretion in *ex vivo* skeletal muscle, isolated EDL muscles were incubated in DMEM medium for 2 h, and the medium was collected for IGF1 measurement.

Cell culture and transfection

Human embryonic kidney HEK293 cells and human liver carcinoma HepG2 cells were obtained from the Cell Resource Center, Chinese Academy of Medical Sciences and Peking Union Medical College (China). Cells were cultured in DMEM medium containing 10% (v/v) foetal bovine serum, and subjected to tests for mycoplasma contamination on a regular basis.

PEI-mediated transfection of cells was carried out as previously described (Chen et al., 2014). siRNAs used for knockdown experiments are as previously described (Wu et al., 2014), and listed in Supplemental Table 1.

***In situ* muscle contraction**

In situ muscle contraction was performed as previously described (Ducommun et al., 2012). Briefly, electrodes were attached to surgically-exposed sciatic nerves of both hindlimbs of mice anaesthetised with sodium pentobarbital (90 mg/kg of body weight, administered intraperitoneally). Electrical stimulation of sciatic nerves to induce *in situ* contraction of hindlimb muscles was performed on one leg for 10 min while the other leg served as sham-operated control (basal). After contraction, mice were terminated by cervical dislocation, and EDL from both legs were isolated and used for glucose uptake.

Muscle incubation and glucose uptake *ex vivo*

Isolated soleus or EDL muscles were incubated with or without stimuli in KRB buffer that was continuously gassed with 95% O_2/5% CO_2 for 50 min. Muscles were then either snap-frozen in liquid nitrogen for subsequent biochemical studies or used for glucose uptake as previously described (Chen et al., 2011). Briefly, glucose uptake was carried out in KRB buffer containing 2-deoxy-D-[1,2-^{3}H(N)]glucose for another 10 min with or without stimuli. After termination of the assay, muscles were blotted dry, snap-frozen in liquid nitrogen, weighed and lysed. Radioisotopes in muscle lysates were measured using a Tri-Carb 2800TR scintillation counter (PerkinElmer).

Liver triglyceride measurement

Liver triglyceride content was measured as previously described (Norris et al., 2003). Briefly, frozen liver chunks were saponified in ethanolic KOH, and the extracts were subsequently neutralized. The resultant free glycerol was determined with the Free Glycerol Reagent (F6428, Sigma-Aldrich) using glycerol (G7793, Sigma-Aldrich) as standard for calculation.

Tissue lysis and immunoprecipitation

Mouse tissues were homogenized as previously described (Ducommun et al., 2012), and protein concentrations of tissue lysates were determined using Bradford reagent (Thermo Scientific).

The anti-TBC1D1 antibody (1 µg antibody/mg cell lysate protein) was used to immunoprecipitate TBC1D1 proteins from cell lysates. Briefly, lysates were mixed with the antibody-coupled protein G-Sepharose. After overnight incubation at 4^{o}C, the suspension was

centrifuged to sediment and non-specific binding proteins were then washed off from the resins. The immunoprecipitates were extracted in SDS sample buffer for subsequent analysis via Western blot.

Rab8a GTPase activity measurement

The GTPase activity of recombinant GST-Rab8a was determined through measuring phosphate released during reaction in the presence or absence of immunoprecipitated HA-hTBC1D1 as previously described (Li et al., 2015).

Western blot and 14-3-3 overlay

Proteins were electrophoretically separated on SDS-PAGE gels, and subsequently immunoblotted onto nitrocellulose membranes that were blocked in milk and incubated at 4^{o}C for 16 h using the indicated antibodies or digoxygenin-labelled 14-3-3 proteins in case of 14-3-3 overlay. Detection was carried out using horseradish-peroxidase-conjugated secondary antibodies (Promega) and ECL® (enhanced chemiluminescence reagent; GE Healthcare).

RNA isolation and QPCR

Total RNA was isolated using the TRIzol® Reagent (Life Technologies) and reverse-transcribed into cDNA using a PrimeScript® RT reagent kit (DRR047A, TaKaRa). The expression levels of target genes were determined via QPCR using an Applied Biosystems® StepOnePlus^{TM} Real-Time PCR system (Life Technologies) and the primers listed in Supplementary Table 2.

Immunofluorescence staining and imaging

Immunofluorescence staining was carried out as previously described (Chen et al., 2014). Briefly, primary hepatocytes fixed in paraformaldehyde were sequentially incubated with primary antibodies and Alexa Fluor®-conjugated or Cy3/Cy5-conjugated secondary antibodies. After decoration with the antibodies, slides were mounted, photographed with a Leica confocal microscope, and representative images are shown.

Statistical analysis

Data were checked for normal distribution and similar variance. Unless stated, data were analyzed via two-sided student's t-test, and differences were considered statistically significant at $P<0.05$. For animal experiments, number of animals was kept as small as possible, and yet still resulted in statistically meaningful data.

Reproducibility

Results for animal experiments are representative of at least two similar experiments.

Author contributions

L.C., B.X.X., Q.L.C., C.Q., Y.S. and S.L.Z. performed experiments, analyzed data, reviewed and edited the manuscript. K.S. and C.M. were involved in the earlier stages of this study, and reviewed and edited the manuscript. H.Y.W. and S.C. designed and performed experiments, analyzed data, and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1 Generation and characterization of the TBC1D1^{Ser231Ala} knockin mice

A. TBC1D1 expression and Ser^{231} phosphorylation and AS160 expression in various tissues of the TBC1D1^{Ser231Ala} knockin mice.

B. Ser^{231} phosphorylation and 14-3-3 binding of TBC1D1 isolated from primary hepatocytes that had been treated with metformin.

C. Representative picture of the male TBC1D1^{Ser231Ala} knockin mice at 52 weeks of age. The bar indicates 1 cm in length.

D-F. Body composition of the male TBC1D1^{Ser231Ala} knockin mice measured at the age of 14 weeks (D, WT: n=18; KI: n=19), 20-22 weeks (E, WT: n=15; KI: n=15) and 52 weeks (F, WT: n=8; KI: n=9).

G-I. Histology (G) and cell size (H and I) of the adipose from the wild-type and TBC1D1^{Ser231Ala} knockin male mice. Bars indicate 50 μm in length. The sizes of wild-type cells were set to one, and the sizes of knockin cells were then normalized with the respective wild-type cells. The numbers shown in the bar graphs refer to the number of cells measured in the experiments.

The data are given as the mean ± SEM, and the asterisk indicates $p < 0.05$.

Figure 2 Insulin sensitivity, blood chemistry and hepatic lipid contents in the TBC1D1^{Ser231Ala} knockin mice

A-B. Oral glucose tolerance test in the male TBC1D1^{Ser231Ala} knockin mice at age of 14 weeks (A, WT: n=5; KI: n=7) and 40 weeks (C, WT: n=6; KI: n=7). The values show the glucose area under the curve during glucose tolerance test.

C. Insulin tolerance test in the male TBC1D1^{Ser231Ala} knockin mice at age of 32 weeks. The data are relative to the basal blood glucose levels before insulin injection. n=5.

D-H. Blood glucose (D), plasma insulin (E), total cholesterol (F), NEFA (G) and triglyceride (H) in the overnight-fasted male TBC1D1^{Ser231Ala} knockin mice at 4.5 (young) and 12 (old) months of age. WT: n=7; KI: n=9.

I-L. Liver morphology and histology (I), fresh weight (J), triglyceride (K), lipid droplet (LD) size distribution (L) in the old male TBC1D1^{Ser231Ala} knockin mice and wild-type littermates (13-months-old). Bars for liver morphology indicate 0.5 cm in length while those for liver sections indicate 20 μm in length. n=8-9.

The data are given as the mean ± SEM, and the asterisk indicates $p < 0.05$.

Figure 3 Lipogenesis in the TBC1D1^{Ser231Ala} knockin mice

A. Protein expression of FASN, ATGL and PPARγ, and phosphorylated and total AMPK were measured in the adipose from the wild-type and TBC1D1^{Ser231Ala} knockin male mice (16-week-old) using GAPDH as a loading control.

B. Phosphorylated and total lipin1, PKB, TSC2, mTOR, AS160 and TBC1D1 were measured in the adipose from the TBC1D1^{Ser231Ala} knockin mice and wild-type littermates (16-week-old) using GAPDH as a loading control.

C. mRNA expression of Fasn, Srebp1, PPARγ and Cebpα in the adipose. n=5.

The data are given as the mean ± SEM, and the asterisk indicates $p < 0.05$.

Figure 4 Plasma IGF1, IGFBP3 and GH, and hepatic GH signaling and IGF1 and IGFBP3 expression in the TBC1D1^{Ser231Ala} knockin mice

A-C. Plasma levels of IGF1 (A), IGFBP3 (B) and GH (C) in the male wild-type and TBC1D1^{Ser231Ala} knockin mice (8-week-old). n=7.

D. Protein expression and phosphorylation in the liver of the wild-type and TBC1D1^{Ser231Ala} knockin mice.

E. QPCR analysis of *igf1* and *igfbp3* in the liver of the wild-type and TBC1D1^{Ser231Ala} knockin mice. n=6.

F-G. Quantitation of protein expression of IGF1 (F) and IGFBP3 (G) in the liver. n=6.

The data are given as the mean ± SEM, and the asterisk indicates $p < 0.05$.

Figure 5 IGF1 secretion in primary cells/tissues from the TBC1D1^{Ser231Ala} knockin mice

A. Co-localisation of TBC1D1 and IGF1 in primary hepatocytes. Bars indicate 10 μm in length.

B-C. Down-regulation of TBC1D1 in primary hepatocytes via siRNA (B), and IGF1 secretion in TBC1D1-depleted primary hepatocytes (C). NC, negative control. n=6.

D-E. IGF1 secretion, and expression levels of indicated proteins in primary hepatocytes from the TBC1D1 knockout mice. n=6.

F. IGF1 secretion in primary hepatocytes from the TBC1D1^{Ser231Ala} knockin mice. a indicates $p < 0.05$ (WT metformin or AICAR vs WT untreated) and b indicates $p < 0.05$ (KI metformin or AICAR vs KI untreated). n=8.

G. IGF1 secretion in primary chondrocytes from the TBC1D1^{Ser231Ala} knockin mice. a indicates $p < 0.05$ (WT AICAR vs WT untreated) and b indicates $p < 0.05$ (KI AICAR vs KI untreated). n=6.

H. IGF1 secretion in primary adipocytes from the TBC1D1^{Ser231Ala} knockin mice. WT: n=5; KI: n=6.

I. IGF1 secretion in *ex vivo* EDL muscles from the TBC1D1^{Ser231Ala} knockin mice. WT: n=11; KI: n=12.

J. Phosphorylated and total TBC1D1 and AMPK, and IGF protein levels were measured in primary hepatocytes from the TBC1D1^{Ser231Ala} knockin mice using GAPDH as a loading control. Met, metformin.

K. Phosphorylated and total TBC1D1 and AMPK were measured in primary chondrocytes from the TBC1D1^{Ser231Ala} knockin mice using GAPDH as a loading control.

The data are given as the mean ± SEM, and the asterisk indicates $p < 0.05$ (WT vs KI or KO). Statistical analyses for F and G were carried out using two-way ANOVA.

Figure 6 IGF1 action and activation of the PKB-TSC2-mTOR pathway in primary cells from the TBC1D1^{Ser231Ala} knockin mice

A-C. Phosphorylated and total PKB and mTOR, and phosphorylated TBC1D1 and AMPK were measured in AICAR stimulated or unstimulated primary adipocytes from the TBC1D1^{Ser231Ala} knockin and wild-type mice using GAPDH as a loading control. A, representative blots; B and C, quantified data, n=4.

D-E. Phosphorylated and total PKB and TBC1D1 were measured in PPP treated or untreated primary hepatocytes from the TBC1D1^{Ser231Ala} knockin and wild-type mice using GAPDH as a loading control. D, representative blots; E, quantified data, n=6. n.s. not significant.

The data are given as the mean ± SEM, and the asterisk indicates $p < 0.05$. Statistical analyses for B, C and E were carried out using two-way ANOVA.

Figure 7 Rab8a and IGF1 secretion in primary hepatocytes

A. IGF1 secretion, and expression and phosphorylation levels of indicated proteins in TBC1D1 knockout primary hepatocytes expressing human TBC1D1 (hTBC1D1) wild-type and mutant proteins. Ser^{237} on human TBC1D1 is a paralogous site to Ser^{231} on the mouse protein. The substitution of Arg^{854} with lysine inactivates the RabGAP of TBC1D1. n=6.

B-C. Down-regulation of Rab8a in primary hepatocytes via siRNA (B), and IGF1 secretion in Rab8a-depleted primary hepatocytes (C). NC, negative control. n=6.

D. Co-localisation of Rab8a and IGF1 in primary hepatocytes. Bars indicate 10 μm in length.

E-F. Down-regulation of Rab8a in primary hepatocytes from the wild-type and TBC1D1^{Ser231Ala} knockin mice via siRNA (E), and IGF1 secretion in Rab8a-depleted primary hepatocytes (F). n=6.

G-H. Expression of indicated proteins (G) and IGF1 secretion (H) in TBC1D1 knockout primary hepatocytes expressing Rab8a wild-type and mutant proteins. n=6.

I-J. The GTPase activity of recombinant GST-Rab8a was measured in the presence of immunoprecipitated wild-type or R854K mutant hTBC1D1 upon phenformin treatment (I) or in the presence of hTBC1D1^{S237A} mutant protein (J).

K-L. Down-regulation of Rabin8 in primary hepatocytes via siRNA (K), and IGF1 secretion in Rabin8-depleted primary hepatocytes (L). NC, negative control. n=6.

The data are given as the mean ± SEM, and the asterisk indicates $p < 0.05$.