Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21

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The liver is a key metabolic organ that controls whole-body physiology in response to nutrient availability. Mammalian target of rapamycin (mTOR) is a nutrient-activated kinase and central controller of growth and metabolism that is negatively regulated by the tumor suppressor tuberous sclerosis complex 1 (TSC1). To investigate the role of hepatic mTOR complex 1 (mTORC1) in whole-body physiology, we generated liver-specific Tsc1 (L-Tsc1 KO) knockout mice. L-Tsc1 KO mice displayed reduced locomotor activity, body temperature, and hepatic triglyceride content in a rapamycin-sensitive manner. Ectopic activation of mTORC1 also caused depletion of hepatic and plasma glutamine, leading to peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α)-dependent fibroblast growth factor 21 (FGF21) expression in the liver. Injection of glutamine or knockdown of PGC-1α or FGF21 in the liver suppressed the behavioral and metabolic defects due to mTORC1 activation. Thus, mTORC1 in the liver controls whole-body physiology through PGC-1α and FGF21. Finally, mTORC1 signaling correlated with FGF21 expression in human liver tumors, suggesting that treatment of glutamine-addicted cancers with mTOR inhibitors might have beneficial effects at both the tumor and whole-body level.

TSC | hepatocellular carcinoma | metabolic stress | behavior

The atypical Ser/Thr kinase target of rapamycin (TOR) is a central controller of cell growth and metabolism, conserved from yeast to human. TOR exists in two structurally and functionally distinct complexes, TORC1 and TORC2 (1–4). Mammalian TOR complex 1 (mTORC1) consists of mTOR, raptor, and mLST8. mTORC1 is activated by nutrients, growth factors, and cellular energy and is acutely inhibited by rapamycin. Growth factors activate mTORC1 via the PI3K-PDK1-Akt signaling pathway. Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC) heterodimer TSC1-TSC2. The TSC complex is a GTPase activating protein (GAP) toward the small GTPase ras-homolog enriched in brain (Rheb) that directly binds and activates mTORC1. Thus, deletion of either Tsc1 or Tsc2 causes ectopic activation of mTORC1. mTORC1 promotes anabolic processes such as protein, lipid, and nucleotide synthesis and ribosome biogenesis and inhibits catabolic processes such as autophagy (4–8). The best-characterized substrates of mTORC1 are 4E-BP and S6 kinase (S6K). Deregulation of the mTOR signaling network is associated with aging and several diseases, including diabetes, obesity, and cancer (9–11). In the tumor syndrome tuberous sclerosis complex and lymphangioleiomyomatosis (LAM), mTORC1 is deregulated due to mutations in the tumor suppressor gene Tsc1 or -2. Rapamycin and rapamycin analogs (rapalogs) are currently used as immunosuppressive agents and as anticancer drugs (12, 13).

In mammals, behavior and physiology display 24-h oscillations controlled by environmental cues such as light and feeding. Light activates the suprachiasmatic nucleus (SCN) in the hypothalamus. The SCN synchronizes cells in other brain regions and peripheral organs, such as the liver, either by humoral and neuronal signals (14) or by regulating body temperature and the fasting/feeding cycle (15–18). The fasting/feeding cycle is a particularly important synchronizer (Zeitgeber) of the liver (19, 20). Expression of several hepatic enzymes and hormones involved in carbohydrate, lipid, cholesterol, and xenobiotic metabolism are regulated over a 24-h cycle in response to nutrient availability (21–28). However, the molecular mechanism(s) by which nutrients control behavior and metabolism is poorly understood but may involve TORC1, a nutrient sensor and key regulator of metabolism. In Drosophila, neuronal TORC1 signaling affects circadian behavior (29). In the SCN, mTORC1 signaling is activated by light and controls behavior in a circadian manner (30–32). In the liver, fasting/feeding cycles regulate the energy sensor AMPK and mTORC1 (24, 33–36). Hepatic mTORC1 and the NAD+–dependent deacetylase SIRT1 are active during the night whereas AMPK is active during the day (34, 35, 37, 38). Moreover, in the liver and other tissues, AMPK (35) and SIRT1 (37, 38) control the core clock machinery. The core clock machinery, composed of the transcription factors CLOCK, BMAL1, PER, CRY, ROR, and REV-ERB, determines circadian oscillations. Disruption of core clock components in the liver strongly alters the regulation of hepatic as well as whole-body glucose and lipid metabolism (39–41). Importantly, time of feeding profoundly affects hepatic gene expression as well as mTORC1 and AMPK signaling (24, 33). Thus, time-restricted feeding improves metabolic rhythms and protects against obesity and liver diseases (33). However, the

Significance

The mammalian target of rapamycin complex 1 (mTORC1) controls cell growth and metabolism in response to nutrients, growth factors, and cellular energy. Aberrant mTORC1 signaling is implicated in human diseases such as diabetes, obesity, and cancer. Our results reveal that ectopic mTORC1 activation in the liver controls the stress hormone fibroblast growth factor 21 (FGF21) in a peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α)-dependent manner via glutamine depletion, which in turn affects whole-body behavior and metabolism. mTORC1 signaling correlates with FGF21 expression in human liver tumors, suggesting that our findings in mice may have physiological relevance in glutamine-addicted human cancers. Thus, treatment with the anticancer drug rapamycin may have beneficial effects by blocking tumor growth and by preventing deregulation of whole-body physiology due to FGF21 expression.


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role of hepatic mTORC1 in the control of whole-body behavior and metabolism has not been investigated.

FGF21 is a hormone produced mainly in the liver (42) and whose expression oscillates over a 24-h cycle (43–45). Upon fasting, peroxisome proliferator-activated receptor α (PPARα) (46–48) and possibly PPARγ coactivator (PGC)-1α (49) activate FGF21 expression in the liver. FGF21 in turn controls behavior and whole-body metabolism by acting on the nervous system and peripheral organs (46, 47, 50–52). Via the central nervous system, FGF21 decreases locomotor activity and body temperature. In the liver, FGF21 stimulates fatty-acid oxidation and gluconeogenesis. Thus, the liver modulates glucose and lipid metabolism, locomotor activity, and body temperature, at least in part, through FGF21.

Glutamine, the most abundant amino acid in the body, plays an important role in growth and metabolism. A TSC1 or TSC2 deficiency causes metabolic/energetic stress by increasing anabolic processes and thereby increasing energy consumption (53, 54). Glucose-limited TSC-deficient cells are addicted to glutamine as an alternative carbon source (54). In addition to being a carbon source used for energy generation, glutamine is also a precursor for nucleotides and other amino acids. Finally, glutaminolysis and/or biogenesis. Consistent with the observed decrease in triglyceride content, expression of the PGC-1α gene decreased in fasted L- Tsc1 KO mice (Fig. S1 A–E). Thus, constitutive hepatic mTORC1 activation, due to Tsc1 and Tsc2 deficiency, causes glutamine addiction.

Results
Hepatic mTORC1 Controls Locomotor Activity, Body Temperature, and Lipid Metabolism. To investigate the role of hepatic mTORC1 in whole-body physiology, we generated mice lacking Tsc1 exclusively in hepatocytes (L-Tsc1 KO mice). The L-Tsc1 KO mice displayed reduced levels of Tsc1 and Tsc2 specifically in the liver (Fig. 1 A and Fig. S1 A). The decrease in Tsc2 is consistent with previous reports indicating that TSC1 stabilizes TSC2 (60, 61). Knockout mice fed ad libitum exhibited unchanged body weight and composition, compared with controls (Fig. S1 B and C). Mice were also subjected to fasting and refeeding to evaluate the effect of Tsc1 knockout on mTORC1 signaling. The L-Tsc1 KO mice displayed constitutively active mTORC1 signaling in the liver, as indicated by high levels of S6 phosphorylation upon both fasting and feeding. Akt phosphorylation was significantly reduced in refed L-Tsc1 KO mice, as expected due to both the S6K-mediated negative feedback loop (62–64) and ER stress (65, 66) (Fig. 1 A). We next examined the effect of constitutive hepatic mTORC1 signaling on glucose and lipid homeostasis. Blood-glucose levels and hepatic expression of the gluconeogenic gene G6Pase were increased in fasted L-Tsc1 KO mice (Fig. S1 D and E). Triglyceride content was reduced in the liver of L-Tsc1 KO mice upon both fasting and refeeding (Fig. 1 B). Thus, constitutive hepatic mTORC1 signaling disrupts glucose and lipid homeostasis. As shown by Yecies et al., the decreased hepatic triglyceride content observed in refed L-Tsc1 KO mice is due to attenuation of Akt signaling by the negative feedback loop (67) (Fig. 1 A). To better understand the decrease in hepatic triglyceride content observed in fasted L-Tsc1 KO mice, we measured expression of Pgc-1α involved in mitochondrial oxidation and/or biogenesis. Consistent with the observed decrease in triglyceride content, expression of Pgc-1α and the PGC-1α target gene CD36 was increased twofold specifically in fasted knockout mice (Fig. S1 E). This suggests that the decrease in triglyceride levels in fasted mice was due to an increase in fatty acid oxidation. The above results are in agreement with previous studies (68, 69), thereby confirming our L-Tsc1 knockout.

The above results taken together indicate that loss of TSC confers a phenotype mainly under fasted conditions. This is expected because the TSC complex inhibits mTORC1 under fasting, but not fed, conditions. Because loss of TSC confers a phenotype mainly under starvation conditions, all subsequent

Fig. 1. Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism. (A) Immunoblots of liver extracts from L-Tsc1 KO and control mice fasted overnight or refed for 4 h. Each lane consists of a mixture of liver extracts obtained from three animals. (B) Representative images of hematoxylin/eosin (H&E) and Oil red O staining of liver sections from L-Tsc1 KO and control mice. (C) Locomotor activity was measured by the Comprehensive Laboratory Animal Monitoring System (CLAMS) (C), and body temperature was measured by rectal thermometer every 4 h over a 24-h cycle (D) (n = 8 per group). (E) Hepatic triglyceride content from L-Tsc1 KO and control mice was measured (n = 6 per time point and per genotype). (F) Locomotor activity was measured during the dark phase in L-Tsc1 KO and control mice. Animals were treated with rapamycin (2 mg/kg) or vehicle at ZT11.5. Data are presented as total counts from ZT12 to ZT24 (n = 6 per group). (G) Body temperature was measured by rectal thermometer at ZT24 in L-Tsc1 KO and control mice. Animals were treated with rapamycin (2 mg/kg) or vehicle at ZT18 (n = 6 per group). (H) Hepatic triglyceride content from L-Tsc1 KO and control mice at ZT20. Animals were treated with rapamycin (2 mg/kg) or control vehicle at ZT18 (n = 6 per group). White bars or squares represent control mice and black bars or squares represent L-Tsc1 KO mice. Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (**P < 0.01, ***P < 0.001).
experiments designed to investigate the effect of mTORC1 activation on whole-body physiology were performed in fasted mice. Furthermore, examining only fasted mice circumvents complications due to the negative feedback loop that inhibits Akt during feeding.

To examine the role of hepatic mTORC1 in behavior and lipid metabolism, L-Tsc1 KO and control mice were monitored for locomotor activity, body temperature, and hepatic triglyceride content over a 24-h cycle. The mice were fasted for the entire 24 h starting at ZT0. ZT is Zeitgeber time within a 24-h light/dark cycle, with ZT0 and ZT12 corresponding to the appearance and disappearance of light, respectively. We note that, in our experimental conditions, ZT24 corresponds to 24 h of fasting and is thus different from ZT0. L-Tsc1 KO mice displayed reduced locomotor activity and body temperature during the dark phase, compared with controls (Fig. 1 C and D). Body temperature decreased by ZT4 in both L-Tsc1 KO and control mice but dropped further during the dark phase in knockout mice (Fig. 1D). Plasma levels of thyroid (T4) hormone and adipose-secreted leptin, key regulators of locomotor activity and thermogenesis, were similar in L-Tsc1 KO and control mice (Fig. S1F). Thus, mTORC1 appears to control locomotor activity and body temperature independently of leptin and thyroid hormone. Similar to locomotor activity and body temperature, triglyceride levels were reduced during the dark phase in the knockout mice (Fig. 1E), suggesting that mTORC1 also controls the daily levels of hepatic lipid metabolism.

To determine whether the observed decrease in locomotor activity, body temperature, and triglyceride was indeed due to mTORC1, animals were treated with rapamycin. The i.p. injection of rapamycin abolished hepatic mTORC1 signaling in L-Tsc1 KO mice (Fig. S1G) and restored normal levels of locomotor activity, body temperature, and hepatic triglycerides (Fig. 1F–J). Collectively, these data demonstrate that hepatic mTORC1 controls the daily levels of locomotor activity, body temperature, and lipid metabolism.

To investigate whether mTORC1 signaling itself oscillates, we examined S6 phosphorylation in the liver of wild-type mice killed every 4 h over a 24-h cycle. S6 phosphorylation was low during the light phase and high during the dark phase, indicating that mTORC1 controls the daily levels of locomotor activity, body temperature, and lipid metabolism.

Figure 2. Hepatic mTORC1 controls FGF21 expression. (A and B) Mice were fasted at ZT0 and killed every 4 h over a 24-h cycle. (A) Immunoblot of total liver extracts from L-Tsc1 KO and control mice. Each lane consists of a mixture of total liver extracts obtained from six animals. (B) Expression of Fgf21 mRNA in the liver of L-Tsc1 KO and control mice was measured by quantitative reverse transcription PCR (qRT-PCR). Total liver RNA was extracted. White squares represent control mice, and black squares represent L-Tsc1 KO mice (n = 6 per time point and per genotype). (C) Plasma Fgf21 levels in L-Tsc1 KO and control mice. Food was removed at ZT0, and plasma Fgf21 levels were measured at ZT8 and ZT20. White bars represent control mice, and black bars represent L-Tsc1 KO mice (n = 6 per time point and per genotype). (D) Expression of Fgf21 mRNA at ZT20 in the liver of L-Tsc1 KO and control mice was measured by qRT-PCR. Food was removed at ZT0. Animals were treated with rapamycin (2 mg/kg) or vehicle at ZT14. Total liver RNA was prepared from L-Tsc1 KO and control animals. White bars represent control mice, and black bars represent L-Tsc1 KO mice (n = 6 per condition and per genotype). Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (*P < 0.05, **P < 0.01, ***P < 0.001).

To determine the role of the TSC complex in the daily regulation of mTORC1 signaling, we next examined hepatic S6 phosphorylation in L-Tsc1 KO mice. S6 phosphorylation displayed the similar diurnal rhythm, but at higher absolute levels at all time points, compared with the wild-type control (Fig. 2A and Fig. S1H). Thus, mTORC1 signaling oscillates daily, and this regulation is independent of the TSC complex.

**Hepatic mTORC1 Controls FGF21 Expression.** How does hepatic mTORC1 control locomotor activity, body temperature, and lipid metabolism? Several observations suggest that mTORC1 may control locomotor activity and lipid metabolism via FGF21. The hormone FGF21, expressed mainly in the liver, decreases locomotor activity, body temperature, and hepatic lipid accumulation (46, 47, 51). Furthermore, mTORC1 activates PGC-1α (Fig. S1E), and PGC-1α has been shown to promote FGF21 expression (49). To determine whether mTORC1 controls FGF21 expression, we examined Fgf21 mRNA levels in the liver and FGF21 protein levels in plasma in L-Tsc1 KO and control mice killed every 4 h over a 24-h cycle. Similar to mTORC1 activity (Fig. 2F), FGF21 expression was low during the light phase and high during the dark phase in both L-Tsc1 KO and control mice, but the increase during the dark phase was significantly higher in the knockout mice (Fig. 2B and C). The i.p. injection of rapamycin restored normal Fgf21 mRNA levels in L-Tsc1 KO mice (Fig. 2D). The above results suggest that hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism via FGF21 expression.

Core clock components, in addition to FGF21, are also key regulators of locomotor activity, body temperature, and hepatic glucose and lipid metabolism (40, 41). In agreement with previous studies, Crys1, Crys2, Per1, Per2, Rora, and Rorγ mRNA levels and Cry2 and Pher2 protein levels in the liver were low during the light phase and high during the dark phase in control animals (Fig. S2A and B) (70–72). Also in agreement with previous studies, we observed that Rev-erbα and Rev-erbβ expression was the inverse of the above (Fig. S2A and B) (70, 71, 73). L-Tsc1 KO mice displayed little-to-no change in (i) expression of the core clock components, as measured at the mRNA and protein levels (Fig. S2A and B), (ii) interaction of circadian clock components (Fig. S2C), and (iii) binding of CLOCK to the fgf21 promoter (Fig. S3). Thus, mTORC1 appears not to control the core clock components, again suggesting that mTORC1 may control whole-body physiology through FGF21.

**Hepatic mTORC1 Controls Locomotor Activity, Body Temperature, and Lipid Metabolism Through FGF21.** To investigate whether FGF21 is responsible for the decreased locomotor activity, body temperature, and triglycerides in L-Tsc1 KO mice, we examined the effect of FGF21 knockdown. L-Tsc1 KO and control mice were infected with adenovirus expressing shRNA against FGF21. The knockdown was confirmed by loss of FGF21 mRNA in the liver and reduced FGF21 protein in plasma (Fig. 3A and B). Knockdown of
FGF21 restored normal locomotor activity, body temperature, and hepatic triglycerides in L-Tsc1 KO mice (Fig. 3 C–E). Thus, hepatic mTORC1 inhibits locomotor activity, body temperature, and lipid metabolism through FGF21.

FGF21 controls carbohydrate metabolism in the liver (50) by inducing expression of gluconeogenic genes (74–76). L-Tsc1 KO mice display an increase in expression of gluconeogenic genes in the liver and increased blood glucose levels (Fig. S1 D and E). We investigated whether mTORC1 controls glucose metabolism through FGF21. Knockdown of FGF21 in the liver had no effect on blood glucose levels or expression of Pgc-1α, G6Pase, and PEPCCK (Fig. S4 A and B). Thus, mTORC1 controls carbohydrate metabolism independently of FGF21.

**Hepatic mTORC1 Controls FGF21 and Behavior and Lipid Metabolism Through PGC-1α.** How does hepatic mTORC1 control FGF21 expression? Purushotham et al. (49) reported that the transcriptional coactivator PGC-1α promotes FGF21 expression, and our results (Fig. S1E) indicate that Pgc-1α is up-regulated in L-Tsc1 KO mice. Furthermore, PGC-1α controls locomotor activity and body temperature (77) and promotes expression of genes involved in mitochondrial fatty acid oxidation, thereby preventing hepatic steatosis (78–80). Thus, we investigated whether hepatic mTORC1 controls FGF21 and whole-body physiology via PGC-1α.

First, we examined PGC-1α mRNA and protein levels in the liver of L-Tsc1 KO and control mice killed every 4 h over a 24-h cycle. PGC-1α expression was low during the light phase and high during the dark phase, reaching a peak at ZT20 in both L-Tsc1 KO and control mice, but was significantly increased overall in the knockout compared with the control (Fig. 4 A and B). Expression of PGC-1α target genes, such as Fgf21 and the gluconeogenic genes G6Pase and PEPCCK, was also low during the light phase and high during the dark phase (Figs. 2 B and 4 C). Second, we determined whether i.p. injection of rapamycin reduced expression of Pgc-1α and G6Pase in the liver of L-Tsc1 KO mice (Fig. 4 D). As observed...
previously for Fgf21 expression (Fig. 2D), rapamycin restored normal expression of Pgc-1α and G6Pase. Third, we examined Fgf21 expression upon knockout of Pgc-1α in the liver. Mice containing a floxed Pgc-1α allele were infected with Cre-expressing adenovirus. Hepatic deletion of Pgc-1α (L-Pgc-1α KO) was confirmed by loss of Pgc-1α mRNA in the liver (Fig. 4E). L-Pgc-1α KO mice displayed a strong decrease in FGF21 protein in the plasma (Fig. 4F), a twofold increase in triglyceride content in the liver (Fig. 4G), and a >1 °C increase in body temperature (Fig. 4H). Fourth, we examined the effect of PGC-1α knockdown in L-Tsc1 KO mice. Infection of L-Tsc1 KO mice with adenovirus expressing shRNA against PGC-1α strongly decreased hepatic Pgc-1α expression and reduced expression of G6Pase, PEPC, and CD36 (Fig. 5A and Fig. S5). Importantly, knock down of Pgc-1α also decreased FGF21 expression, as measured at both the mRNA and plasma protein level (Fig. 5A and B), and restored normal locomotor activity, body temperature, and hepatic triglyceride content in L-Tsc1 KO mice (Fig. 5C–E). Collectively, the above results suggest that mTORC1 controls FGF21 and ultimately behavior and lipid metabolism via PGC-1α.

The transcriptional repressor E4BP4 inhibits FGF21 expression by binding a d-box in the Fgf21 promoter (45). Although E4BP4 expression increased in the dark phase, its expression was similar in L-Pgc-1α KO and control mice (Fig. S6A). Importantly, E4BP4 expression increased in the dark phase, its expression was similar in L-Pgc-1α KO and control mice (Fig. S6A). Importantly, E4BP4 expression increased in the dark phase, its expression was similar in L-Pgc-1α KO and control mice (Fig. S6A). Importantly, E4BP4 expression increased in the dark phase, its expression was similar in L-Pgc-1α KO and control mice (Fig. S6A).

Fig. 5. Hepatic mTORC1 controls FGF21 and behavior and lipid metabolism through Pgc-1α. (A–E) L-Tsc1 KO and control mice were infected either with an adenovirus expressing shRNA against PGC-1α or LacZ. Four days after infection, food was removed at ZT0. (A and B) Pgc-1α and Fgf21 mRNA (A) and plasma FGF21 levels (B) were measured at ZT20 (n = 10 per condition and per genotype). (C) Locomotor activity was measured during the dark phase. Data are presented as total counts between ZT12 and ZT24 (n = 8 per condition and per genotype). (D) Body temperature was measured by rectal thermometer at ZT24 (n = 8 per condition and per genotype). (E) Representative images of hematoxylin/eosin (H&E) and Oil red O staining of liver sections. Animals were killed at ZT24. [Original magnification: 40x (Left.)] Hepatic triglyceride content was measured (Right) (n = 8 per condition and per genotype). White bars represent control mice, and black bars represent L-Tsc1 KO mice. Values are expressed as mean ± SEM; the * indicates a statistically significant difference between the indicated groups (*P < 0.05, **P < 0.01, ***P < 0.001).

ER stress response as assessed by eIF2α phosphorylation (Fig. S6F) (84). However, ATF4 expression was not affected in L-Tsc1 KO mice, as measured at both the mRNA and protein level (Fig. S6C). Thus, mTORC1 does not appear to regulate FGF21 via E4BP4 or ATF4, further suggesting that mTORC1 activates FGF21 through PGC-1α. Of note, L-Tsc1 KO mice displayed similar PPARα mRNA levels compared with controls, suggesting that PPARα is not the limiting factor controlling FGF21 expression (Fig. S6D).

Hepatic mTORC1 Activates PGC-1α and FGF21 via Glutamine Depletion. The hormone FGF21 is produced in the liver (42) in response to metabolic stress such as carbon depletion (46, 47, 83, 85). Glutamine, via glutaminolysis, can serve as an alternative carbon source to prevent metabolic/energetic stress in glucose-deprived cells (53, 54, 59). mTORC1 promotes glutaminolysis, and consequently mTORC1 hyperactivation in Tsc2-deficient cells leads to glutamine depletion (56). Thus, we investigated whether L-Tsc1 KO mice display glutamine depletion that may turn induce FGF21. First, we examined glutamine levels in the liver and in plasma of L-Tsc1 KO mice. Glutamine levels were decreased during the dark phase in both L-Tsc1 KO and control mice, but the decrease in the dark phase was significantly more pronounced in the knockout mice (Fig. 6A). Asparagine is synthesized by amidotransfer from glutamine to aspartate. Consistent with the reduced hepatic glutamine levels, pancreatic levels were also reduced in the liver whereas aspartate levels were unaffected (Fig. S7A). The levels of branched-chain amino acids were also unchanged (Fig. S7A). Thus, L-Tsc1 KO mice are depleted specifically for glutamine. Second, to determine whether glutamine depletion was due to mTORC1 hyperactivity, animals were treated with rapamycin. The i.p. injection of rapamycin restored normal glutamine levels in L-Tsc1 KO mice (Fig. 6B and C). Third, we examined whether glutamine depletion is responsible for PGC-1α and FGF21 expression. The i.p. injection of glutamine decreased Pgc-1α and Fgf21 mRNA levels in the liver, reduced FGF21 protein levels in plasma, and increased body temperature (Fig. 6D–F). Interestingly, glutamine also increased hepatic mTORC1 signaling in both L-Tsc1 KO and control mice (Fig. 6G), consistent with previous in vitro studies (55, 56). Collectively, these data suggest that hepatic mTORC1 controls whole-body glutamine levels and thereby whole-body physiology through PGC-1α–dependent FGF21 expression.

mTORC1 Signaling Correlates with FGF21 Expression in Human Liver Tumors. Several observations suggest that mTORC1, often deregulated in cancer, may lead to FGF21 expression in tumors. First, as described in Fig. 6 A–C and in the literature (56), mTORC1 hyperactivation causes glutamine depletion. Second, tumors are often glutamine-addicted, causing cancer patients to display glutamine depletion (57–59). Third, FGF21 is expressed in response to metabolic stress such as carbon limitation (46, 47, 83, 85). To determine whether mTORC1 signaling correlates with FGF21 expression in tumors, we examined S6 phosphorylation and FGF21 protein levels in human hepatocellular carcinoma (HCC) biopsies. Histological examination of HCC from 10 separate patients revealed a strong correlation between mTORC1 signaling and FGF21 expression. Four HCCs expressed both phospho-S6 and FGF21 whereas six expressed neither (Fig. 6H and Fig. S7B). Thus, our findings in L-Tsc1 KO mice may be physiologically relevant in tumors and, furthermore, suggest that treatment of glutamine-addicted cancers with mTOR inhibitors might have beneficial effects at both the tumor and whole-body level.

Discussion
To obtain insight on how nutrient availability controls whole-body behavior and metabolism, we investigated the role of the nutrient sensor mTORC1 on locomotor activity, body temperature, and glucose and lipid homeostasis. In particular, we examined the
Fig. 6. (A) Hepatic and plasma glutamine levels in L-Tsc1 KO and control mice were quantified by LC-MS/MS. 8–9-wk-old mice were fasted at ZT0 and killed at ZT8 and ZT20 (n = 6 per time point and per genotype). (B) Hepatic glutamine levels in L-Tsc1 KO and control mice were quantified by LC-MS/MS. 12-wk-old animals were fasted at ZT0, treated with rapamycin (2 mg/kg) or control vehicle and killed at ZT20 (n = 6 per condition and per genotype). (C) Plasma glutamine levels in L-Tsc1 KO and control mice were quantified by LC-MS/MS. Twelve-week-old animals were fasted at ZT0, treated with rapamycin (2 mg/kg) or control vehicle and killed at ZT20 (n = 6 per condition and per genotype). (D–G) Food was removed at ZT0 and L-Tsc1 KO mice and controls were treated with L-Glutamine (1 g/kg) or saline solution, 2 h before sacrifice at ZT20. (D) Expression of Fgf21 and Pgc-1α mRNA in the liver of L-Tsc1 KO and control mice was measured by qRT-PCR. Total liver RNA was extracted (n = 6 per condition and per genotype). (E) Plasma Fgf21 levels in L-Tsc1 KO and control mice were measured (n = 6 per condition and per genotype). (F) Body temperature was measured by rectal thermometer at ZT20 (n = 6 per condition and per genotype). (G) Immunoblots of liver extracts from L-Tsc1 KO and control mice injected with glutamine or saline solution. (H) FGF21 and phospho-S6 staining in human HCC. Representative images of an HCC showing little-to-no staining of either (a) FGF21 or (b) phospho-S6. Images of HCC presenting diffuse staining of both (c) FGF21 and (d) phospho-S6. (e–h) Examples of HCCs exhibiting medium to high staining of both (e and g) FGF21 and (f and h) phospho-S6. White bars represent control mice, and black bars represent L-Tsc1 KO mice. Values are expressed as mean ± SEM; the * indicates statistical significant difference between the indicated groups (*P < 0.05, **P < 0.01, ***P < 0.001).

Fig. 7. Model of hepatic mTORC1 controlling whole-body physiology. Hepatic mTORC1 causes glutamine depletion and thereby PGC-1α-dependent FGF21 expression, which in turn affects whole-body physiology.
We found that RORγ expression is slightly increased in the liver of L-Tsc1 KO mice during the dark phase whereas RORα and PPARα were reduced and unchanged, respectively (Figs. S2A and S6D). This suggests that PGC-1α may activate Fgf21 via RORγ but does not rule out PPARα.

Our finding that PGC-1α promotes Fgf21 expression is consistent with the previous observation that PGC-1α promotes FGF21 through PPARα (49). However, Estill et al. showed that PGC-1α indirectly represses FGF21 through the transcriptional repressor Rev-erβ (90). These seemingly conflicting findings on the role of PGC-1α in the regulation of Fgf21 may be due to different experimental conditions. We (Fig. S2 A and B) and others (70, 71, 73) observed that Rev-erβ is expressed only during the light phase whereas glutamine depletion increases PGC-1α and FGF21 expression only during the dark phase. PGC-1α may inhibit FGF21 expression via Rev-erβ during the light phase.

We and others observed that hepatic mTORC1 activity is circadian (24, 33, 34, 36). Two previous studies showed that hepatic mTORC1 rhythmicity is controlled by the circadian clock (34, 36). Furthermore, Jouffe et al. (34) speculated that the circadian clock controls mTORC1 signaling via autophagy. Interestingly, hepatic mTORC1 signaling remains rhythmic in L-Tsc1 KO mice. It is well established that loss of the TSC complex prevents autophagy, as a consequence of mTORC1 activation, and autophagy is indeed inhibited in L-Tsc1 KO mice (92). Thus, at least in the absence of the TSC complex, mTORC1 rhythmicity appears not to be regulated by autophagy. Recently, Khapre et al. showed that Bmal1 negatively regulates mTORC1 rhythmicity appears not to be regulated by autophagy. Furthermore, Jouffe et al. (34) speculated that the circadian clock controls mTORC1 signaling via autophagy. In contrast, we and others observed that hepatic mTORC1 activity is rhythmic in L-Tsc1 KO mice during the dark phase whereas glutamine depletion increases PGC-1α and FGF21 expression only during the dark phase. PGC-1α may inhibit FGF21 expression via Rev-erβ during the light phase.

We and others observed that the expression of Fgf21 in the liver of L-Tsc1 KO mice during the dark phase whereas RORα and PPARα were reduced and unchanged, respectively (Figs. S2A and S6D). This suggests that PGC-1α may activate Fgf21 via RORγ but does not rule out PPARα.

Our finding that PGC-1α promotes Fgf21 expression is consistent with the previous observation that PGC-1α promotes FGF21 through PPARα (49). However, Estill et al. showed that PGC-1α indirectly represses FGF21 through the transcriptional repressor Rev-erβ (90). These seemingly conflicting findings on the role of PGC-1α in the regulation of Fgf21 may be due to different experimental conditions. We (Fig. S2 A and B) and others (70, 71, 73) observed that Rev-erβ is expressed only during the light phase whereas glutamine depletion increases PGC-1α and FGF21 expression only during the dark phase. PGC-1α may inhibit FGF21 expression via Rev-erβ during the light phase.

We and others observed that hepatic mTORC1 activity is circadian (24, 33, 34, 36). Two previous studies showed that hepatic mTORC1 rhythmicity is controlled by the circadian clock (34, 36). Furthermore, Jouffe et al. (34) speculated that the circadian clock controls mTORC1 signaling via autophagy. Interestingly, hepatic mTORC1 signaling remains rhythmic in L-Tsc1 KO mice. It is well established that loss of the TSC complex prevents autophagy, as a consequence of mTORC1 activation, and autophagy is indeed inhibited in L-Tsc1 KO mice (92). Thus, at least in the absence of the TSC complex, mTORC1 rhythmicity appears not to be regulated by autophagy. Recently, Khapre et al. showed that Bmal1 negatively regulates mTORC1 rhythmicity appears not to be regulated by autophagy. Furthermore, Jouffe et al. (34) speculated that the circadian clock controls mTORC1 signaling via autophagy. In contrast, we and others observed that hepatic mTORC1 activity is rhythmic in L-Tsc1 KO mice during the dark phase whereas glutamine depletion increases PGC-1α and FGF21 expression only during the dark phase. PGC-1α may inhibit FGF21 expression via Rev-erβ during the light phase.

We and others observed that hepatic mTORC1 activity is circadian (24, 33, 34, 36). Two previous studies showed that hepatic mTORC1 rhythmicity is controlled by the circadian clock (34, 36). Furthermore, Jouffe et al. (34) speculated that the circadian clock controls mTORC1 signaling via autophagy. Interestingly, hepatic mTORC1 signaling remains rhythmic in L-Tsc1 KO mice. It is well established that loss of the TSC complex prevents autophagy, as a consequence of mTORC1 activation, and autophagy is indeed inhibited in L-Tsc1 KO mice (92). Thus, at least in the absence of the TSC complex, mTORC1 rhythmicity appears not to be regulated by autophagy. Recently, Khapre et al. showed that Bmal1 negatively regulates mTORC1 signaling over a 24-h cycle by affecting the expression of mtor and depetr in the liver (36). This provides a mechanism through which the transcription factors of the circadian clock impinge on mTORC1 signaling. However, further investigation is required to determine the role of other circadian clock components in the regulation of mTORC1 signaling. The rhythmicity of mTORC1 signaling accounts for the rhythmicity of glutamine depletion and PGC-1α-dependent FGF21 expression. Thus, disruption of the circadian clock frequency observed in cancer or upon jet lag might alter mTORC1 signaling and thereby whole-body physiology. Finally, we note that, although the circadian clock affects mTORC1 signaling (34, 36), mTORC1 itself appears not to control the core clock components (Fig. S2 A and B).

In conclusion, our findings underscore the importance of hepatic mTORC1 in the regulation of whole-body physiology. Hyperactivation of mTORC1 in the liver causes glutamine depletion, which leads to FGF21 expression and, in turn, changes in whole-body physiology. Moreover, mTORC1 signaling correlates with FGF21 expression in human liver tumors. This latter observation is relevant because human cancers often exhibit hyperactive mTORC1 signaling and glutamine addiction (9–11, 59). Thus, treatment of glutamine-addicted cancers with mTOR inhibitors might have beneficial effects at both the tumor and whole-body level.

## Materials and Methods

### Mice

Generation of liver-specific Tsc1 knockout (L-Tsc1 KO) mice has been described previously (6). Where indicated, mice were intraperitoneally injected with rapamycin (LC Laboratories) at 2 mg/kg or vehicle. Rapamycin solvent was composed of 5% (v/vol) FEG-400, 4% (v/vol) ethanol, and 91% PBS (w/vol) during 80. Additionally, where indicated, animals were in traperitoneally injected with -glutamine (Sigma) at 1 g/kg or with saline solution. For details see SI Materials and Methods.

### Other Methods

For other methods, see SI Materials and Methods. The primer sequences used in this study are listed in Table S1.

### Statistical Analyses

Statistical significance was measured using a Student’s unpaired t test to determine differences among two groups. The differences were considered to be significant if P < 0.05. Data are presented as mean ± SEM. Immunoblot quantification was assessed using Image J software.

## ACKNOWLEDGMENTS

We thank Christoph Handschin for reagents and Annette Roullet for the illustration. This work was supported by the Swiss National Science Foundation, the Canton of Basel, the Louis Jeantet Foundation (M.N.H.), the Société Francophone du Diabète-Association de Langue Française pour l’Étude du Diabète et des Maladies Métaboliques (M.C.), and the Werner Siemens Foundation (A.M.R. and V.A.)


Liver-specific tuberous sclerosis complex 1 knockout (L-Tsc1 KO) mice were obtained by crossing Tsc1<sup>fl/fl</sup> mice (1) purchased from The Jackson Laboratory (005680) with transgenic mice expressing Cre recombinase under the control of the hepatocyte-specific albumin promoter (Alb-Cre<sup>E1b</sup>) (2). Littermate Tsc1<sup>fl/fl</sup> mice without the Cre gene were used as controls. The mice produced were on a mixed genetic background (C57BL/6J, 129SvJae, BALB/cJ). Liver-specific Tsc1 knockout (Tsc1<sup>fl/fl</sup> Alb-Cre<sup>E1b</sup>) mice (L-Tsc1 KO mice) were born viable at the expected Mendelian ratio and displayed normal fertility. PCR genotyping for Tsc1 and Cre was performed as described (1). Generation of PGC-1α<sup>lox/lox</sup> mice has been described previously (3). The generated animals were on a C57BL/6. Mice were maintained under temperature- and humidity-controlled conditions with 12-h light/12-h dark (LD) cycles (lights on at 6:00 AM, lights off at 6:00 PM) with free access to food and water. All of the experiments were conducted on male mice between 8 wk and 16 wk of age. All experiments were performed in accordance with federal guidelines and were approved by the Kantonale Veterinaeramt of Kanton Basel-Stadt.

Quantitative PCR. Total RNA was isolated from ~50 mg of mouse livers. Tissues were homogenized by 30 s bead beating in lysing matrix D tubes (Q-Biogene) containing 1 mL of TRIzol reagent (Sigma). After the chloroform extraction and centrifugation, samples were mixed with 600 μL of 70% ethanol, and the extraction was continued with the RNeasy kit (Qiagen). cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was done using the power SYBR green mix (Applied Biosystems) and quantitated using Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems). Duplicate runs of each sample were normalized to TBP or cyclophilin to determine relative expression levels. Values plotted represent averages from at least six different animals. The sequences for the primer pairs used in this study are listed in Table S1.

Western Blot. Mouse livers were quickly isolated, flash-frozen in liquid nitrogen, and stored at ~80 °C. For whole protein extraction, tissues were homogenized by polytron in cold lysis buffer [100 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 0.5 M mannitol] supplemented with 1% Triton X-100, protease and phosphatase inhibitor mixture tablets (Roche). Lysates were centrifuged at 10,000 × g for 5 min at 4 °C, cell debris were removed, and total protein concentration was assessed (BCA Protein Assay; Thermo Scientific). For protein nuclear extraction, mouse livers were homogenized, and nuclear extracts were prepared according to the NUN procedure (4). Then, 30 μg of proteins were loaded on SDS/PAGE and transferred to a nitrocellulose membrane. The following antibodies were used for immunoblotting: TSC1 (A300-316A), Bmal1 (A302-616A) and E4BP4 (A302-606A) from Bethyl; TSC2 (1613-1) from Epitomics; Akt (9272), Phospho-Akt (Thr473) (2920), Phospho-Akt (Ser473) (9271), S6 Ribosomal Protein (2317), Phospho-S6 Ribosomal Protein (Ser235/236) (2655), CRY2 (kindly made from Cell Signaling; PER2 (AB5428P), Rev-Erb (AB10130), and α-actin (MAB1501) from Millipore; PGC-1α (H-300); sc-13067 from Santa Cruz Biotechnology, and CRY2 (kindly made available by U. Schibler, University of Geneva, Geneva). For detection, Supersignal west femto maximum sensitivity substrate (Pierce) was used.

Histology. For hematoxylin/eosin (H&E) stainings, liver samples were fixed in 4% paraformaldehyde immediately after euthanization. Tissues were cut into 5-μm-thick paraffin sections, and the slides were stained with hematoxylin/eosin according to the standard protocol. For Oil Red O staining, liver pieces were snap-frozen in 2-methyl butane precooled in liquid nitrogen. Tissues were cut into 5-μm-thick cryo-sections and stained with Oil Red O (Sigma). Morphology and staining were assessed by a pathologist.

Patient’s Samples and Immunohistochemistry. All patients’ specimens and clinico-pathological data including complete follow-up were obtained from the Institute of Pathology, University Hospital of Basel, Switzerland. Hepatocellular carcinoma (HCC) diagnosis was verified by an experienced pathologist (L.T.) according to the World Health Organization (WHO) classification and Edmondson and Steiner grading system. The clinico-pathologic features of the samples used in this study are summarized in Fig. S7B. The study was approved by the review board of the Institute of Pathology and by the University of Basel Ethics Committee. For immunohistochemical analysis (IHC), 5-μm sections of formalin-fixed paraffin (FFPE)-embedded tissue were immunostained for Phospho-S6 Ribosomal Protein (Ser235/236) (4857) from Cell signaling and FGF21 (ab66564) from Abcam as previously described (5). Their levels were assessed in n = 10 human HCCs, obtained from patients that underwent surgical resection. Immunoreactivity was scored semiquantitatively (0 for no staining, 1 for low staining, II for medium, and III for high signal) by evaluating the staining intensity as described by Allred et al. (6).

Hepatic Triglyceride Measurement. Approximately 50 mg of mouse liver tissue were homogenized with 1 mL chloroform:methanol (2:1). The homogenizer was washed in 1 mL of this solution and transferred to a new glass tube. Afterward, 1 mL of distilled water was added into the tubes and vortexed vigorously for 2 min. Tubes were centrifuged at 800 × g for 10 min at 4 °C, and then the upper aqueous phase was discarded. The lower solvent phase was dried under nitrogen at 50 °C and then resuspended with 1 mL of chloroform. Triglycerides were subsequently separated by SPE column (Interchim) and dissolved in chloroform/Triton X-100 (1%). Triglyceride levels were measured using a triglyceride determination kit (TG PAP 150; BioMérieux).

Locomotor Activity, Body Temperature, and Body Composition. Locomotor activity was measured using a comprehensive laboratory animal monitoring system (CLAMS) (Linton Instrumentation and Columbus Instruments). Locomotor activity was defined as successive linear infrared beam breaks recorded every 30 min on 16 mice simultaneously and measured for 1 light (6:00 AM to 6:00 PM) and 1 dark (6:00 PM to 6:00 AM) period after an acclimatization of 48 h. Mice were fed ad libitum during the 48 h of adaptation, and the food was removed at ZT0 on the same day of locomotor activity measurement. Body temperature was measured by rectal thermometer. Body composition was determined using EchoMRI-100 nQMR (EchoMRI Medical Systems) according to the manufacturer’s instructions.

Blood Analysis. The levels of blood glucose were measured with an Aviva glucometer (Accu-check; Roche). Blood was drawn from...
the posterior vena cava immediately after euthanization and collected in heparinized tubes. After centrifugation, plasma was collected, and parameters were determined with the following kits: Mouse and Rat FGF-21 ELISA kit (BioVendor), Quantikine Mouse Leptin ELISA kit (R&D Systems), and Thyroxine (T4) ELISA kit (USCN Life Science, Inc.).

Adenovirus Generation. Murine FGF-21 shRNA or LacZ shRNA-expressing adenovirus were generated with the previously described sequences (7), and murine PGC-1α shRNA-expressing adenovirus was kindly made available by C. Handschin (University of Basel, Basel). Null or Cre-expressing adenovirus were purchased from Vector BioLabs. Adenoviruses were amplified by infecting 293T cells and purified on CsCl gradients, and the titer was determined. Mice were infected with adenovirus by tail-vein injection. Four days after injection, mice were fasted at ZT0, and experiments were subsequently performed as described.

Chromatin Immunoprecipitation. Mouse livers were homogenized with a dounce tissue grinder in cold PBS solution supplemented with protease inhibitor mixture tablets (Roche). Cross-linking was performed with 1% formaldehyde incubating the homogenate for 10 min at 37 °C. The cross-linking was stopped by adding 0.125 M glycine, and the homogenates were centrifuged at 239 × g for 10 min. The cell pellets were resuspended in cell lysis buffer (5 mM Pipes, 85 mM KCl, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture tablets (Roche) and incubated for 30 min on a rotating platform. Cell lysates were then centrifuged at 2,655 × g for 5 min. Nuclei were resuspended for 10 min in nuclei lysis buffer [50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS]. Chromatin was sonicated two times for 15 s to obtain ~200-bp fragments. Then, 50 μg of DNA were incubated with 1 μg of the indicated antibodies at 4 °C for 12 h and further incubated with a mix of protein A/G beads for an additional 2 h. The beads were collected by centrifugation at 106 × g for 2 min and then washed. The immunoprecipitation complexes were eluted with elution buffer (0.1 M NaHCO3 and 1% SDS), and the cross-linking was reversed. DNA purification was performed using a phenol-chloroform–isoamyl alcohol (25:24:1) extraction, and DNA was precipitated with ethanol 70%. The recovered DNA was quantified by quantitative PCR, and data were expressed as a percentage of the input.

Metabolic Measurements. Animals were fasted at ZT0 and killed at the indicated time. Where indicated, mice were intraperitoneally injected with rapamycin or vehicle at ZT0 and ZT12. Liver pieces were flash-frozen in liquid nitrogen to quench hepatic metabolism. Afterward, samples were homogenized and extracted twice in lysis matrix D tubes (Q-Biogene) by adding 0.5 mL of 60% ethanol: 40% 10 mM ammonium acetate (pH 7.2) at 80 °C for 2 min. 13C internal standard was added to diluted cell-free lysates in a 1:1 ratio after centrifugation at 18,363 × g for 5 min, and the mix was dried in a speed vac and stored at −80 °C. Plasma samples were mixed 1:1 with 13C internal standard and subsequently incubated with 5x volume of cold MeOH at −30 °C for 1 h to precipitate protein. Protein-free supernatant was obtained after centrifugation at 18,363 × g for 10 min, dried in a Speed-Vac, and stored at −80 °C.

Dried extracts were resuspended in ddH2O and analyzed using a targeted liquid chromatography mass spectrometry (LC-MS/MS) method as described previously (8). Intracellular hepatic concentrations of metabolites were calculated using calibration curves with standard compounds and normalized to a 13C internal standard and liver sample volume assuming a liver density of 1.05 g/mL (9). Plasma concentrations were calculated using calibration curves with standard compounds and normalized to a 13C internal standard.

For gas chromatography mass spectrometry (GC-MS/MS)-based amino acids quantification, dried extracts were resuspended in water-free N,N-Dimethylformamide mixed with N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchlorosilane in a 30:70% ratio and derivatized for 1 h at 80 °C. Then, 3 μL of derivatized sample were then injected into a GC-MS instrument (6890N GC and 5875 Inert XL MS system, both Agilent), and mass spectra were recorded as described previously (10). Absolute concentrations were calculated by calibration curves with a mix of pure amino acids after normalization to an internal standard (U-13C6 glutamate signal) and liver sample volume.


Fig. S1. Phenotypic analysis of L-Tsc1 KO mice fed a chow (related to Fig. 1). (A) Immunoblots of liver, brown adipose tissue (BAT), epididymal white adipose tissue (WAT), brain, and muscle extracts from L-Tsc1 KO and control mice. (B) Body weight of 8-wk-old L-Tsc1 KO and control mice. White bars represent control mice (n = 17), and black bars represent L-Tsc1 KO mice (n = 14). (C) Body composition of 8-wk-old L-Tsc1 KO and control mice was determined by Echo-MRI analysis. White bars represent control mice (n = 17), and black bars represent L-Tsc1 KO mice (n = 14). (D) Blood glucose levels of L-Tsc1 KO and control mice. White bars represent control mice, and black bars represent L-Tsc1 KO mice fasted overnight (n = 8 for controls and n = 7 for L-Tsc1 KO) or refed for 4 h (n = 9 for controls and n = 7 for L-Tsc1 KO). (E) Expression of Pgc-1α, G6Pase, and CD36 mRNA in the liver of L-Tsc1 KO and control mice was measured by qRT-PCR. White (n = 8) and light gray (n = 9) bars represent control mice, and black (n = 7) and dark gray (n = 7) bars represent L-Tsc1 KO mice fasted overnight or refed for 4 h, respectively. (F) Plasma leptin and T4 levels in L-Tsc1 KO and control mice were measured at ZT8 and ZT20 in mice fasted at ZT0. White bars represent control mice, and black bars represent L-Tsc1 KO mice (n = 6 per time point and per genotype). (G) Immunoblots of total liver extracts from L-Tsc1 KO and control mice. Food was removed at ZT0. Animals were treated either with rapamycin (2 mg/kg) or vehicle at ZT14 and killed at ZT20. (H) Quantitation of S6 phosphorylation relative to S6 expression in the liver of L-Tsc1 KO and control mice observed in Fig. 2A. Values are expressed as mean ± SEM; the * indicates statistical significance difference between the indicated groups (*P < 0.05, **P < 0.01, ***P < 0.001).
Fig. S2. Hepatic mammalian target of rapamycin complex 1 (mTORC1) does not control core clock components (related to Fig. 2). (A–C) L-Tsc1 KO and control mice were fasted at ZT0. Animals were killed every 4 h over a 24-h cycle. (A) Temporal analysis of core clock mRNA levels in the liver. Total liver RNA was extracted from L-Tsc1 KO and control mice (n = 6 per time point and per genotype). White squares represent control mice, and black squares represent L-Tsc1 KO mice. Values are expressed as mean ± SEM; the * indicates a statistical significant difference from control mice (*P < 0.05, **P < 0.01, ***P < 0.001). (B) Immunoblots of liver nuclear extracts. Each lane consists of a mixture of liver nuclear extracts obtained from six animals per genotype. (C) CLOCK was immunoprecipitated from mouse liver nuclear extracts, and the immunoprecipitated proteins were analyzed by immunoblotting. Rabbit IgG antibody was used for the mock control.
**Fig. S3.** Hepatic mTORC1 does not affect Clock binding to the E box promoter (related to Fig. 2). Clock binding profiles to the E box promoter in the liver. L-Tsc1 KO and control mice were fasted at ZT0. Animals were killed every 4 h over a 24-h cycle. Chromatin immunoprecipitation assays were performed with chromatin extracts from mouse livers using anti-CLOCK antibody. qRT-PCR analysis were performed with primers surrounding the canonical E box of Cry2, Fgf21, Per1, and Cry1 (n = 6 per time point and per genotype). White squares represent control mice, and black squares represent L-Tsc1 KO mice. Values are expressed as mean ± SEM; the * indicates a statistical significant difference from control mice (*P < 0.05, **P < 0.01).

**Fig. S4.** Hepatic mTORC1 controls carbohydrate metabolism independently of FGF21 (related to Fig. 3). (A and B) L-Tsc1 KO and control mice were infected with an adenovirus expressing shRNA against FGF21 or LacZ. Four days after infection, food was removed at ZT0, and the experiments were performed at the indicated time. (A) Blood glucose levels were measured at ZT20 (n = 6 per condition and per genotype). (B) mRNA expression levels of Pgc-1α, G6Pase, and PEPCK were measured at ZT20 (n = 6 per condition and per genotype). White bars represent control mice, and black bars represent L-Tsc1 KO mice. Values are expressed as mean ± SEM; the * indicates a statistical significant difference between the indicated groups (*P < 0.05, **P < 0.01, ***P < 0.001).
Fig. S5. Additional analysis of Pgc-1α knockdown in L-Tsc1 KO mice (related to Fig. 5). mRNA levels of the PGC-1α target genes G6Pase, PEPCK, and CD36 were measured in L-Tsc1 KO and control mice, which were infected with an adenovirus expressing shRNA against PGC-1α or LacZ. Four days after infection, food was removed at ZT0, and animals were killed at ZT20. White bars represent control mice, and black bars represent L-Tsc1 KO mice (n = 10 per condition and per genotype). Values are expressed as mean ± SEM; the * indicates a statistically significant difference between the indicated groups (*P < 0.05, **P < 0.01, ***P < 0.001).

Fig. S6. Additional analysis of the regulation of Fgf21 in L-Tsc1 KO mice (related to Fig. 5). (A–C) L-Tsc1 KO and control mice were fasted at ZT0, and animals were killed every 4 h over a 24-h cycle. (A) Temporal analysis of E4BP4 mRNA levels in the liver. Total liver RNA was extracted from L-Tsc1 KO and control mice (n = 6 per time point and per genotype). (B) Immunoblots of total liver extracts from L-Tsc1 KO and control. (C) Temporal analysis of ATF4 mRNA (Left) and protein (Right) levels in the liver. Total liver RNA was prepared from L-Tsc1 KO and control mice. Each lane consists of a mixture of total liver extracts (Right) (n = 6 per time point and per genotype). (D) Temporal analysis of PPARα mRNA levels in the liver. Total liver RNA was extracted from L-Tsc1 KO and control mice (n = 6 per time point and per genotype). White squares represent control mice, and black squares represent L-Tsc1 KO mice. Values are expressed as mean ± SEM.
Fig. S7. Additional analysis of metabolites in L-Tsc1 KO mice and information about the human samples (related to Fig. 6). (A) Hepatic metabolites in L-Tsc1 KO and control mice were quantified by LC- and GC-MS/MS. Mice were fasted at ZT0 and killed at ZT8 and ZT20. White bars represent control mice, and black bars represent L-Tsc1 KO mice \((n = 6\) per time point and per genotype). Values are expressed as mean \(\pm\) SEM; the * indicates statistical significant difference between the indicated groups \((*P < 0.05, **P < 0.01, ***P < 0.001)\). (B) Clinico-pathologic features and staining intensity for FGF21 and phospho-S6 of the human samples used in this study.

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Table S1. Primer sequences used for quantitative PCR

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