The tight junction protein TJP1 regulates the feeding-modulated hepatic circadian clock

Yi Liu¹,², Yuanyuan Zhang¹,², Tong Li¹, Jinbo Han¹ & Yiguo Wang¹

Circadian clocks in the suprachiasmatic nucleus and peripheral tissues orchestrate behavioral and physiological activities of mammals in response to environmental cues. In the liver, the circadian clock is also modulated by feeding. However, the molecular mechanisms involved are unclear. Here, we show that TJP1 (tight junction protein 1) functions as a mediator of mTOR (mechanistic target of rapamycin) to modulate the hepatic circadian clock. TJP1 interacts with PER1 (period circadian regulator 1) and prevents its nuclear translocation. During feeding, mTOR phosphorylates TJP1 and attenuates its association with PER1, thereby enhancing nuclear shuttling of PER1 to dampen circadian oscillation. Therefore, our results provide a previously uncharacterized mechanistic insight into how feeding modulates the hepatic circadian clock.

¹MOE Key Laboratory of Bioinformatics, Tsinghua-Peking Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China. ²These authors contributed equally: Yi Liu, Yuanyuan Zhang. *email: yiguo@mail.tsinghua.edu.cn
The circadian clock is an endogenous timekeeper that coordinates behavior, physiology, and metabolism in a wide range of organisms. In mammals, the central pacemaker in the suprachiasmatic nucleus (SCN) synchronizes oscillations in peripheral organs in response to light–dark cycles. The molecular clock is driven by interconnected transcriptional feedback loops involving both transcriptional activators and repressors to produce self-sustained rhythmic transcription of target genes. In this process, a heterodimer of the transcription factors CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like 1) acts as a transcriptional activator that binds to E-box motifs in target genes and drives their transcription. These target genes include period (Per), cryptochrome (Cry), and Rev-erb, which encode repressors of CLOCK and BMAL1. PER and CRY rhythmically accumulate, shuttle to the nucleus and form a repressor complex that interacts with CLOCK:BMAL1 to inhibit transcription of Per and Cry. Another important diurnal loop involves the transcriptional repressor REV-ERB and a member of the ROR (retinoid–related orphan receptor) family of transcriptional activators, which together control the rhythmic expression of BMAL1. Unlike the SCN clock, the circadian clock in the liver is also modulated by feeding, such as refeeding after fasting, restricted feeding, and high fat diet feeding, to coordinate energy metabolism. However, the molecular mechanisms by which feeding affects the circadian clock remain unclear.

The liver, which is composed of polarized hepatocytes and other cells, is one of the most critical metabolic organs in mammals. The polarization of hepatocytes involves formation of distinct sinusoidal and bile canaliculus plasma membrane domains that are separated by tight junctions. These tight junctions form the blood–biliary-barrier, which keeps bile in bile canaliculi away from the blood circulation and also acts as a signaling platform for communicating between the inside and the outside of the cells. Although tight junction proteins are critical to maintain liver function, it is unknown whether they modulate the hepatic circadian clock.

In this study, we demonstrated that tight junction protein 1 (TJP1) functions as a mediator of mTOR to modulate the hepatic circadian clock. Mechanistically, feeding activates mTOR, which phosphorylates TJP1, thereby disrupting the TJP1:PER1 association and promoting PER1 nuclear translocation to inhibit the expression of CLOCK:BMAL1 target genes. This discovery provides a mechanistic insight into how feeding modulates the circadian clock in the liver and links the junction complex to circadian rhythm.

**Results**

**Tjp1 knockout suppresses hepatic circadian amplitude.** To maintain the characteristics of hepatocytes in vitro, we cultured mouse primary hepatocytes in a collagen sandwich configuration and evaluated the bile canaliculus-like structures by immunostaining of CLDN1 and CGN, which are tight junction markers (Supplementary Fig. 1a). These results showed that hepatocytes cultured in a collagen sandwich (SD+) have a clear bile canaliculus-like structure, similar to the previous reports. Interestingly, SD+ culture dramatically enhanced the expression of Rev-Erbα and circadian amplitude (Fig. 1a). Strikingly, knockout of Tjp1 (encoding tight junction protein 1) strongly repressed Rev-Erbα expression and circadian amplitude (Fig. 1a and Supplementary Fig. 1b–d), while having no effect on tight junction formation evaluated by CLDN1 and CGN staining (Supplementary Fig. 1a) and measurement of transepithelial electrical resistance (TER, Supplementary Fig. 1e), which reflects paracellular permeability regulated by the tight junction.

Previous reports also showed that the knockout of Tjp1 in epithelial cells did not affect tight junction formation.

We further tested the effect of Tjp1 on the circadian clock in Tjp1 liver-specific knockout (LKO) mice. Tjp1 LKO mice have similar tight junction structures and bile acid levels to wildtype ones (Supplementary Fig. 1f–h). However, Tjp1 deficiency not only repressed the expression of E-box-containing genes (Rev-Erbα, Dmp, and Per1) in the liver, but also in the SCN, and increased the nuclear translocation of PER1 and CRY1, but not PER2, in the liver (Fig. 1b–g and Supplementary Fig. 1i, j). Since liver-specific Tjp1 knockout has no effect on the circadian gene expression of SCN, these results show that Tjp1 autonomously modulates circadian amplitude in the liver.

**mTOR attenuates the association of TJP1 and PER1.** Next, we tested how TJP1 affects PER1CRY1 nuclear translocation. We identified TJP1-interacting proteins by immunoprecipitation (IP) of endogenous TJP1 in SD+ cultured primary hepatocytes and mass spectrometry (MS) analysis. We found that both PER1 and CRY1 were present in the TJP1 immunoprecipitates (Supplementary Fig. 2a). Co-IP assay showed that TJP1 is associated with both wildtype PER1 and the binding-defective mutant of PER1 (1–1147 aa on CRY1 (Supplementary Fig. 2b), indicating that PER1 but not CRY1 interacts with TJP1. In addition, TJP1 is not associated with other circadian core components, such as BMAL1 and CLOCK (Supplementary Fig. 2c). Further analysis by in vitro and in vivo co-IP assays showed that the C-terminus (511–1748 aa) of TJP1 is responsible for the TJP1:PER1 interaction (Supplementary Fig. 2d, e).

Recent evidence has shown that mTOR affects the circadian clock in cultured cells, SCN, and peripheral organs. However, the mechanisms by which mTOR modulates the circadian clock are largely unclear. We tested whether mTOR regulates the TJP1:PER1 association to modulate the circadian clock. Strikingly, addition of amino acids (AA) to activate mTOR, decreased the TJP1:PER1 association at the lysosome and promoted PER1 nuclear translocation, while addition of torin1, an mTOR inhibitor, abolished the effect of AAs (Fig. 2a–c and Supplementary Fig. 3a–c). mTOR does not affect the localization of TJP1 in lysosomal fractions or at junctions (Supplementary Fig. 3c, d). This indicates that mTOR promotes the dissociation of TJP1 and PER1 at lysosomal fractions. In addition, the TJP1:PER1 association was dynamically regulated in a manner related to mTOR activity in mice. There was lower mTOR activity and stronger binding of TJP1 to PER1 at ZT6, and vice versa at ZT18 (Fig. 2d and Supplementary Fig. 3e).

Although mTOR promotes PER1 nuclear translocation, neither PER1 nor CRY1 was phosphorylated by mTOR (Supplementary Fig. 3f–h). Activated mTOR had a stronger binding to TJP1 (Fig. 2e and Supplementary Fig. 3i, j). We therefore identified the preferred mTOR phosphorylation motifs in TJP1 and checked them by amino acid scanning. Alanine or aspartic acid mutations of six conserved AA affected the binding of TJP1 to PER1 and thus were judged to be critical for the TJP1:PER1 association (Supplementary Fig. 3k, l). We further confirmed that mTOR phosphorylates TJP1 by an in vitro kinase assay (Fig. 2f).

Phospho-specific antibodies indicated that phosphorylation of TJP1 is tightly modulated by mTOR both in cultured hepatocytes and in mice (Fig. 2a, d and Supplementary Fig. 3m). The phosphorylation-defective mutant of TJP1 (6A) constitutively interacted with PER1 and almost lost its response to AA stimulation, while the phosphorylation-mimic mutant of TJP1 (6D) did not bind to PER1 (Fig. 2g). Taken together, these results indicate that mTOR activation stimulated by AA attenuates the TJP1:PER1 association by phosphorylating TJP1.
**Fig. 1 Tjp1 deficiency suppresses circadian amplitude in the liver.** a Relative mRNA level of Rev-Erbα (left panel) and rhythmic amplitude (right panel) in wildtype (WT) or Tjp1 liver-specific knockout (LKO) mouse primary hepatocytes cultured in the presence (SD+) or absence (SD−) of a collagen sandwich configuration. Hepatocytes were exposed to dexamethasone (0.1 μM) and then harvested at different time points. n = 6. b qPCR results showing the expression of Rev-Erbα in liver extracts from WT and Tjp1 LKO mice. n = 3–7 mice. c Immunoblots showing the levels of core circadian components in different fractions (Nucl. for nuclear fraction, Cyto. for cytosolic fraction, and TCL for total cell lysate) of liver extracts from WT and Tjp1 LKO mice. d and e Statistical analysis of Rev-Erbα in TCL d, and PER1 and CRY1 in Nucl. e From immunoblots as shown in c. n = 6 mice. f and g Cellular localization of GFP-PER1, GFP-CRY1, or GFP-PER2 f and statistical analysis of the results g showing the percentage of nuclear-localized GFP-PER1, GFP-CRY1, or GFP-PER2 in the liver from WT and Tjp1 LKO mice at ZT6 and ZT18. Nuclei are stained by 4,6-diamidino-2-phenylindole (DAPI). n = 6 mice. Scale bars, 10 μm. ZT is the Zeitgeber time. Data are shown as mean ± s.e.m. Comparison of different groups was carried out using two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. NS, no statistical significance. Source data are provided as a Source Data file.

**Phosphorylation of TJP1 promotes nuclear shuttling of PER1.** Since the phosphorylation of TJP1 attenuates the TJP1:PER1 association, we checked whether the phosphorylation of TJP1 affects PER1 nuclear translocation. The nuclear translocation of PER1 stimulated by AA in primary hepatocytes was blocked by the phosphorylation-defective mutant of TJP1 (6A), while TJP1 6D constitutively promotes nuclear translocation of PER1 (Supplementary Fig. 4). We further confirmed these effects by adenoviral expression of mCherry-tagged TJP1 in Tjp1 LKO mice. Nuclear translocation of PER1 was blocked and expression of Rev-Erbα was enhanced in these animals (Fig. 3a–f). In addition, PER1 nuclear translocation in Tjp1 LKO mice was blocked by the mTOR-defective mutant of TJP1 (6A) but not the mTOR-mimic mutant (6D), which further supports the notion that phosphorylation of TJP1 by mTOR is critical for PER1 nuclear shuttling (Fig. 3a–f). Together, these results indicate that mTOR promotes nuclear translocation of PER1 by phosphorylating TJP1 and disrupting its association with PER1.

**mTOR dampens hepatic circadian amplitude.** Considering the effect of phosphorylation of TJP1 by mTOR on PER1 nuclear shuttling, we checked whether mTOR itself modulates the hepatic circadian clock. To address this, we interrupted mTOR activity by Torin1 treatment or by deleting the gene encoding RAPTOR, a subunit of mTOR complex 1 (mTORC1), and we enhanced mTOR activity by deleting the gene encoding TSC1, a GTPase-activating protein that inhibits mTORC1 activity30. In the absence of mTOR manipulations, Rev-Erbα expression, mTOR activity, and pTJP1 levels are dramatically enhanced in SD+ cultured hepatocytes compared to SD− cultured hepatocytes (Supplementary Fig. 5a–c). However, Torin1 treatment further...
enhanced both Rev-Erbα expression and circadian amplitude (Supplementary Fig. 5a, b). In addition, Raptor deficiency abolished mTORC1 activity and increased both Rev-Erbα expression and circadian amplitude, while Tsc1 deficiency enhanced mTORC1 activity and decreased Rev-Erbα expression and circadian amplitude (Fig. 4a and Supplementary Fig. 5d–f). These results were further confirmed by data showing that PER1 nuclear translocation was repressed and Rev-Erbα expression was enhanced in Raptor LKO mice (Fig. 4b–g and Supplementary Fig. 5g). Together, these results showed that mTOR modulates the circadian clock in the liver.

Feeding enhances PER1 nuclear shuttling via mTOR and TJP1. Feeding in mice mostly occurs at night and constitutes around 80% of daily food intake14. In addition, Raptor deficiency abolished mTORC1 activity and increased both Rev-Erbα expression and circadian amplitude, while Tsc1 deficiency enhanced mTORC1 activity and decreased Rev-Erbα expression and circadian amplitude (Fig. 4a and Supplementary Fig. 5d–f). These results were further confirmed by data showing that PER1 nuclear translocation was repressed and Rev-Erbα expression was enhanced in Raptor LKO mice (Fig. 4b–g and Supplementary Fig. 5g). Together, these results showed that mTOR and TJP1 modulates the circadian clock in the liver.

shown that both TJP1 and mTOR are critical for the feeding-modulated nuclear translocation of PER1 in the liver.

An mTOR–TJP1 axis regulates circadian clock during feeding. We next investigated whether the specific function of TJP1 as a downstream mediator of mTOR is important for the feeding-modulated hepatic circadian clock. Feeding-promoted nuclear translocation of PER1 was blocked by Raptor deficiency, while Raptor and Tip1 double knockout (DKO) in the liver restored nuclear translocation of PER1 and inhibited Rev-Erbα expression (Fig. 6a–f). Although mTOR is less active during fasting, Tsc1 deficiency constitutively activated mTOR, promoted PER1 nuclear translocation, and reduced Rev-Erbα expression (Fig. 6g–l). These effects were blocked by the mTOR-defective mutant of Tip1 (6A) (Fig. 6g–l). Together, these results indicate that mTOR modulates the hepatic circadian clock by phosphorylating TJP1 during feeding.

TJP1 deficiency improves insulin sensitivity. Altered circadian clock is tightly associated with insulin sensitivity, a hallmark of diabetes1,2,4,31. Considering the effect of TJP1 on the hepatic circadian clock, we tested whether Tip1 affects mouse insulin sensitivity. Tip1 knockout mice fed with a regular diet (RD) showed enhanced nuclear translocation of PER1, and reduced Rev-Erbα expression, blood glucose level, plasma insulin level, plasma triglyceride (TG) level, and hepatic TG accumulation (Fig. 7a–j). However, Tip1 knockout mice fed a RD had a similar body weight,
Fig. 3 Phosphorylation of TJP1 promotes PER1 nuclear translocation. 

a-d Effect of TJP1 or its mutants on cellular localization of PER1 in the liver evaluated by immunoblots a, and statistical analysis of PER1 b, CRY1 c in Nucl. and Rev-Erbα in TCL d of liver extracts harvested from mice at ZT6. e and f Effect of TJP1 or its mutants on cellular localization of GFP-PER1 e, and Rev-Erbα expression f in liver extracts harvested from mice at ZT6. 6A is the T589A/T709A/S927A/S1360A/S1460A/S1617A mutant of human TJP1, while 6D is the T589D/T709D/S927D/S1360D/S1460D/S1617D mutant of human TJP1. Data are shown as mean ± s.e.m. Comparison of different groups was carried out using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, n = 5 mice. Source data are provided as a Source Data file.

Fig. 4 mTOR dampens hepatic circadian amplitude. 

a Relative mRNA level of Rev-Erbα (left panel) and rhythmic amplitude (right panel) in wildtype (WT) or Raptor LKO mouse primary hepatocytes cultured in a collagen sandwich configuration. Hepatocytes were exposed to dexamethasone (0.1 μM) and then harvested at different time points. n = 6. 

b Effect of Raptor LKO on Rev-Erbα expression in the liver from mice at ZT6 and ZT18. n = 4–7 mice. 

c–e Effect of Raptor LKO on cellular localization of PER1 in the liver evaluated by immunoblots c, and statistical analysis of Rev-Erbα in TCL d, and PER1 and CRY1 in Nucl. e from immunoblots as shown in c, n = 4–7 mice. 

f and g Effect of Raptor LKO on cellular localization of PER1 in the liver visualized by mCherry-PER1 f and statistical analysis of the results g. n = 4–7 mice. Scale bar, 10 μm. Data are shown as mean ± s.e.m. Comparison of different groups was carried out using two-tailed unpaired Student’s t-test a or two-way ANOVA b, d, e and g. *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Data file.
fat mass, food intake, energy expenditure, circadian gene expression, and cholesterol levels to wildtype ones (Supplementary Fig. 6a–j). To evaluate the effect of TJP1 on insulin sensitivity, we performed hyperinsulinemic-euglycemic clamp studies. Compared to WT mice, the steady-state glucose infusion rate (GIR) in Tjp1 LKO mice was dramatically increased, reflecting enhanced whole-body insulin responsiveness, and was accompanied by an increase in the glucose disposal rate (GDR, Fig. 7k, l). Tjp1 LKO mice showed marked increases in the insulin-induced hepatic glucose production (HGP) suppression, which reflects liver insulin sensitivity; the insulin-stimulated GDR (IS-GDR), which primarily reflects skeletal muscle insulin sensitivity; and the insulin-induced suppression of plasma FFA levels, which indicates white adipose tissue insulin sensitivity (Fig. 7m–o).

Since mice fed with a high-fat diet (HFD) showed a dampened hepatic circadian amplitude, enhanced mTOR activity, and attenuated insulin sensitivity,14,31 we tested whether Tjp1 deficiency can improve insulin sensitivity in HFD-fed mice. Compared to WT mice fed with a HFD for 16 weeks, Tjp1 LKO mice showed enhanced PER1/CRY1 nuclear shuttling, an improved metabolic profile and restored insulin sensitivity (Fig. 7 and Supplementary Fig. 6). All these results demonstrate that TJP1 links hepatic circadian clock to insulin sensitivity.

**Discussion**

Although it is well known that the circadian clock in the liver is modulated by feeding to coordinate energy metabolism,2–4,14, the molecular mechanisms by which feeding modulates circadian clock remain unclear. Our results demonstrate that feeding activates mTOR and thereby phosphorylates TJP1, which disrupts the TJP1:PER1 association and thus promotes PER1/CRY1 nuclear translocation to inhibit the expression of CLOCK:BMAL1 target genes (Fig. 8). This discovery provides mechanistic insight into how feeding modulates the circadian clock in the liver and links the junction complex to circadian rhythm.

Since the circadian amplitude is affected in obese or diabetic models, modulating circadian amplitude can combat the negative effects of obesity.14,34–37 In support of this notion, Tjp1 deficiency restores the HFD-induced effect on circadian gene expression, metabolic profile, and insulin sensitivity (Fig. 7), although it is possible that TJP1 can also improve insulin sensitivity in a clock-independent manner. Therefore, our results demonstrate a potential molecular link between circadian amplitude and insulin resistance.

Activated mTOR phosphorylates TJP1 and disrupts the colocalization of TJP1 and PER1/CRY1 at lysosomes, thereby promoting the nuclear translocation of PER1 (Fig. 2b and
Supplementary Fig. 3a). It is unclear how phosphorylation of TJP1 affects the TJP1:PER1 interaction. Our results showed that the mTOR phosphorylation sites in TJP1 localize to the region of TJP1 that interacts with PER1, and some of these phosphorylation sites are also located in the protein domains of TJP1. Based on previous reports38,39, it is possible that the phosphorylation of TJP1 modulates TJP1:PER1 binding by directly disrupting the interaction or by changing the conformation of TJP1. More detailed structural information is needed to address this issue.

The lysosome is a central organelle in energy metabolism and nutrient sensing and recycling40–42. Recruitment of mTOR to lysosomes is required for mTOR activation, while amino acid stimulation enhances mTOR accumulation and activation at lysosomes40 (Supplementary Fig. 3c, i and 5c). It is reported that lysosome-dependent redistribution and degradation of TJP1 contributes to the hyperglycemia-induced increase in the permeability of the blood–brain barrier43. Although the function of lysosome-localized TJP1 and PER1/CRY1 is unclear, it is possible that lysosomes provide an interface to link nutrient status to the circadian clock. Consistent with this notion, autophagy promotes lysosome-dependent degradation of CRY1 and glucose metabolism during fasting44.

Previous studies showed that casein kinase phosphorylates PER1 and CRY1 to promote their nuclear shuttling, while AMPK, an energy stress sensor, phosphorylates CRY1 and enhances its ubiquitin-dependent degradation45–48. Our results showed that mTOR promotes the nuclear translocation of PER1/CRY1. All the results reflect the fact that different nutrient and energy statuses or metabolic conditions are tightly coupled to the circadian clock. Considering the ubiquitous expression of mTOR and TJP1, it will be interesting in the future to test whether the mTOR–TJP1 signaling axis also affects the circadian clock in other organs or non-polarized cells.

**Methods**

**Mouse strains.** Mice carrying FRT and a floxed allele of Tjp1 (Tjp1fl/+) were obtained from International Mouse Phenotyping Consortium (IMPC), and then crossed with mice expressing the FLP recombinase to obtain Tjp1fl/+.
mice were backcrossed to C57BL/6 mice for six generations, and then bred to homozygosity. For generation of liver-specific Tjp1 knockout (Tjp1/fl) mice, Tjp1/fl mice were crossed with mice expressing the Cre-recombinase transgene from the liver-specific albumin promoter (Alb-Cre mice) to delete exon 3. Study cohorts were generated by crossing Tjp1/fl (WT) mice with Alb-Cre Tjp1/fl (Tjp1 LKO) mice. PCR genotyping of Tjp1 LKO mice was performed with primers that detect the following: 3′ FlpO site of the targeted Tjp1 allele: forward, ACCCGTACCTGTACTTAGGTAAT; reverse, ACTCTCCTGTGAAAGGCCCT. PCR genotyping of Tsc1 LKO mice was performed with the following primers to detect the 3′ LoxP site of the targeted Tsc1 allele: forward, CAGGGTGTATAAGCAATCCC; reverse, GAATCAACCCCACAGAGCAT. To generate the liver-specific Raptor knockout (Raptor(fl)) mice, high-titer adenoviral-Cre (1 × 10^11 pfu) was delivered to Raptor(fl) (013188) mice by tail vein injection, and subsequent experiments were performed within 2 weeks. PCR genotyping for the floxed allele was performed with the following primers to detect the 3′ LoxP site of the targeted Raptor allele: forward, TTCAGTGTGGATGTGTTGAG; reverse, GGAGAAGC; reverse, GAATCAACCCCACAGAGCAT. To generate the liver-specific Mtor knockout (Mtor(fl)) mice, high-titer adenoviral-Cre (1 × 10^10 pfu) was delivered to Mtor(fl) mice by tail vein injection, and subsequent experiments were performed within 2 weeks. PCR genotyping for the floxed allele was performed with the following primers to detect the 3′ LoxP site of the targeted Mtor allele: forward, TTATGTTTGATA ATTGCAGTTTTGGCTAGCAGT; reverse, GAATCAACCCCACAGAGCAT.

**Fig. 7** TJP1 deficiency enhances insulin sensitivity. a–e Effect of Tjp1 LKO on nuclear PER1 levels evaluated by immunoblots a, statistical analysis of PER1 b, and CRY1 c in Nucl., statistical analysis of Rev-Erbα in TCL d, and Rev-Erbα expression e in liver extracts from mice fed on a RD or HFD for 16 weeks. n = 5 mice. f–i Effect of Tjp1 LKO on blood glucose f, plasma insulin levels g, plasma triglyceride levels h and hepatic triglyceride contents i from mice fed on a RD or HFD for 16 weeks. TGs triglycerides. n = 8 mice. j Hematoxylin and eosin staining of liver from WT and Tjp1 LKO mice fed on a RD or HFD for 16 weeks. Scale bar, 50 μm. k–o Glucose infusion rate (GIR, k), glucose disposal rate (GDR, l), percentage suppression of HGP m, insulin-stimulated GDR (15-GDR, n) and percentage of free fatty acid (FFA) suppression o from WT and Tjp1 LKO mice fed on a RD or HFD for 16 weeks. n = 6 mice. Data are shown as mean ± s.e.m. Comparison of different groups was carried out using two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Data file.

**Fig. 8** TJP1 couples mTOR signaling to the hepatic circadian clock. During fasting or in daytime, mTOR is less active and cannot disrupt the association of TJP1 and PER1CRY1, which sequesters PER1CRY1 in the cytoplasm. During feeding or at nighttime, active mTOR phosphorylates and interacts with TJP1, disrupts its interaction with PER1CRY1 and promotes nuclear translocation of PER1CRY1, which inhibits CLOCK: BMAL-dependent transcription.
Mouse experiments. Mice were housed in colony cages with a 12 h light/12 h dark cycle (lights on at 7:00 a.m. as ZT0, lights off at 7:00 p.m. as ZT12) in a temperature-controlled environment with free access to food and water. All of the experiments were conducted on 8–12-week-old male mice. To overspecify specific proteins in the liver, adenoviruses (5 × 10⁸ pfu per mouse) were injected through the tail vein. If needed, mice were fasted overnight from ZT12 to ZT4, then refed for 2 h and killed at ZT6. Animals were maintained with all relevant ethical regulations for animal testing and research. All animal experiments were approved by the Animal Care and Use Committee at Tsinghua University.

Metabolic studies and histology. Blood glucose values were determined using a LifeScan automatic glucometer. Triglyceride (TR0100, Sigma) and cholesterol (10007640, Cayman) levels in the liver and plasma, plasma insulin (10-1247-01, Merckodia), and plasma-free fatty acid levels (294-636, Wako) were measured according to the manufacturer’s instructions. Acidic cell extracts from tissue samples were homogenized in 200 μl tissue homogenization buffer. The homogenate was incubated at 50 °C for 2 h and centrifuged at 6000×g for 10 min. Total bile acid levels in plasma and liver tissues were determined using a Total Bile Acids Kit (Dyazyme, DZ902A) and normalized with the tissue weight of each sample. For tissue histology, mouse tissues were fixed in 4% paraformaldehyde (PFA) and paraffin embedded. Sections (5 μm) were used for hematoxylin and eosin staining.

Indirect calorimetry, physical activity, and food intake. Energy expenditure and food intake were simultaneously measured for individually housed mice with a PhenoMaster system (TSE Systems). Mice were allowed to acclimatize in the chamber at least 5 days before the clamp procedure. The clamp procedure was designed to induce hyperinsulinemia and euglycemia. Blood glucose, fat mass, and the rate of oxygen consumption were measured by the built-in automated instruments. Relative metabolic rates until the blood glucose concentration reached a constant level of about 100 ± 5 mg/dl. Blood glucose values were determined using a OneTouch® UltraMini analyzer.

Hyperinsulinemic-euglycemic clamps. The assays were performed as previously reported49,50. After mice were anesthetized, catheters (PE-10) were implanted in the right jugular vein and tunneled subcutaneously and exteriorized behind the neck. Mice were maintained in the clamp room for 4–5 days before the clamp procedure. Mice were fasted overnight, then weighed to calculate the insulin dose and placed in a restrainer. An equilibration syringe was connected to the catheter and an initial bolus of [6,6-2H]glucose (600 μg kg⁻¹) was administered, followed by continuous infusion of [6,6-2H]glucose (30 μg kg⁻¹ min⁻¹). Isotopic enrichment was achieved at -90 min before the onset of insulin infusion, and blood samples for isotopic measurements were collected at t = -10 and 0 min. Following this basal infusion period, insulin was constantly infused at the rate of 6 mU kg⁻¹ min⁻¹ until the termination of the study. [6,6-2H]glucose was infused together with glucose at various rates until the blood glucose concentration reached a constant level of about 100 ± 5 mg/dl. Blood samples were taken at time points 10 and 120 min. All plasma samples (15 μl) were deproteinized by gently mixing them with 60 μl cold methanol (pre-chilled at -80 °C) and incubating for 1–2 h at -80 °C. The samples were then centrifuged with 14,000 × g at 4 °C for 10 min. The supernatant was transferred to a new tube and lyophilized to produce a pellet. High performance liquid chromatography (UPLC) was used for the analysis. GDR was calculated as constant isotope infusion rate divided by enrichment of isotopes. HGP and GDR were calculated in the basal state and during the steady-state portion of the clamp. Tracer-determined rates were quantified using Steele’s equation. Under steady-state conditions, the rate of glucose disappearance, or total GDR, is equal to the sum of the rate of endogenous glucose production (HGP) and the exogenous GIR. The IS-GDR is equal to the total GDR minus the basal GDR.

Plasmids and adenoviruses. HA-tagged TIP1 was amplified from pEGFP-C1 (Addgene, 30313) and cloned into pcDNA3 vector containing a HA tag. Site-directed mutagenesis was performed using a PCR-based strategy with PluUltra II Fusion HS DNA polymerase (Agilent). FLAG-PER1 and FLAG-CRY1 were kindly provided by Dr. Erquan Eric Zhang (National Institute of Biological Sciences, Beijing). FLAG-PER1 and FLAG-CRY1 were cloned into pCMV6-XL1 (Agilent). HA-tagged TJP1 was amplified from pEGFP-C1 with or without plasmids from pAdTrack/pAdEasy or pShuttle/pAdEasy system and amplified in HEK293 cells. Adenoviruses (5 × 10⁸ pfu) were plated in 10 cm dishes and titrated for 1 h before adenovirus stimulation. All cell lines were routinely tested for mycoplasma using a PCR detection kit (Sigma, MP0035).

Collagen gel sandwich cultures were prepared by spreading 2 ml of collagen solution (BD Biosciences, at 1 mg ml⁻¹ in DMEM) over 10 cm dishes and allowing it to gel for 30 min. The gel was then cultured in an incubator at 37 °C. Hepatocytes (3 × 10⁶) were plated in collagen-coated 10 cm dishes. Hepatocytes were allowed to attach for 2 h. Mice were fasted overnight from ZT12 to ZT10 for 15 min before the clamp was started. After drying for 2 h, the medium was replaced with M199 supplemented with 2% FBS, 0.2% BSA and 100 μg ml⁻¹ penicillin–streptomycin. Cultures were aspirated and then the top layer of collagen gel was added. After drying for 2 h, the medium was replaced with M199 supplemented with 100 μg ml⁻¹ penicillin–streptomycin.

Subcellular fractionation. Hepatocytes or freshly harvested livers (100 mg) were placed in a glass homogenizer containing 1 ml cold lysis buffer (10 mM Tris–HCl pH 8.0, 10 mM NaCl, 0.2% NP-40) and then homogenized 30 times. The homogenates were incubated on ice for 15 min, and 200 μl was saved as the total cell lysate. The nuclear fraction was pelleted by centrifugation at 10,000 × g for 10 min at 4 °C and resuspended in 800 μl RIPA buffer (25 mM Tris–HCl pH 7.4, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl), then sonicated and centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was saved as the cytosolic fraction.

To evaluate the distribution of TIP1 in lysosome fractions, the lysosomes in cells were extracted using a Lysosome Isolation Kit (Sigma, LYSO01) according to the manufacturer’s instructions. After density gradient centrifugation of the cytosolic fraction, five bands were visible in the tube. These fractions were withdrawn using long tips pipetted from the top gradient, and number (with the top fraction as no. 1). Fractions numbered 1–4 were treated with 8 mM calcium chloride solution on ice for 15 min to precipitate the rough endoplasmic reticulum and mitochondria, and then centrifuged at 5000 × g for 15 min at 4 °C. This step, most of the lysosomes remained in the supernatant.

To test the distribution of TIP1 at junctions or in the cytoplasm, cells were incubated in ice-cold cell lysis buffer with 1% Triton X-100 for 15 min on ice. Cells were then harvested gently using a scraper and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was labeled as the soluble fraction. The cell pellets were resuspended in ice-cold RIPA buffer and pipetted gently to disperse the pellets. This suspension was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was labeled as the insoluble fraction.

Transintestinal electrical resistance. The TER assay was performed as previously described31,52. For co-IP experiments, TIP1-overexpressing cells were collected in cell lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 1% Triton X-100). HA-tagged TIP1 and mutants were immunoprecipitated with anti-HA antibody (Millipore, MAB1405). Immunoprecipitated samples were washed at least four times with lysis buffer. For all endogenous IP experiments, rabbit IgG was used as a negative control.

For immunoblotting, cells or mouse tissues were homogenized in cell lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 1% Triton X-100). HA-tagged TIP1 and mutants were immunoprecipitated with anti-HA antibody (Millipore, MAB1405). Immunoprecipitated samples were washed at least four times with lysis buffer. For all endogenous IP experiments, rabbit IgG was used as a negative control.

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IP, immunoblot, and immunostaining. Assays were performed as previously described31,52. For co-IP experiments, TIP1-overexpressing cells were collected in cell lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 1% Triton X-100). HA-tagged TIP1 and mutants were immunoprecipitated with anti-HA antibody (Millipore, MAB1405). Immunoprecipitated samples were washed at least four times with lysis buffer. For all endogenous IP experiments, rabbit IgG was used as a negative control.

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Transmission electron micrographs (TEM). Liver tissues were fixed with 2.5% glutaraldehyde in PBS by perfusion, then fixed in 2.5% glutaraldehyde and 2% PFA for 16 h. Tissues were post-fixed in 1% osmium tetroxide for 1 h and then dehydrated through an ethanol series. After changing the transitional solvent several times, tissues were embedded in Spurr’s resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, then analyzed using a transmission electron microscope (Tecnai Spirit 120 KV).

Protein expression and purification. Fusion proteins were purified as previously reported50. Briefly, His-HA-tagged TIP1, TIP1 mutant (924–1748aa) and His-PER1 fusion proteins were purified from Mtor knockout primary hepatocytes after 48-h adenoviral infection. His-HA-tagged TIP1 mutants (1-510aa, 511-923aa) and His-CRY1 were purified from E. coli. Cells or bacteria were collected, resuspended in buffer containing 25 mM Tris pH 8.0, 150 mM NaCl and protease inhibitors, sonicated and centrifuged at 16,000 × g for 10 min. The supernatant was then centrifuged at 300,000 × g for 60 min. After centrifugation at 300,000 × g for 30 min, the supernatant was applied to nickel affinity resin (Ni-NTA, Qiagen). The eluent was purified by size-exclusion chromatography (Superdex 200, GE Healthcare) in buffer (25 mM HEPES pH 7.4, 50 mM KCl, 5 mM MgCl₂, and 5 mM MnCl₂). The peak fractions were pooled and concentrated for subsequent analysis.

In vitro kinase assay. HA-tagged TIP1, TIP1/ΔA, and S6K fusion proteins were purified from Mtor knockout primary hepatocytes after 48-h infection. The kinase assay was performed as reported41. The reaction system (20 μL), containing 150 ng fusion protein, 20 ng truncated mTOR (Millipore, 14-770) in reaction buffer (25 mM HEPES pH 7.4, 50 mM KCl, 5 mM MgCl₂, and 5 mM MnCl₂), 0.5 μM ATP and 2 μCi [32P]ATP was incubated for 30 min at 30 °C. Reactions were stopped by adding 5 μL sample buffer, then boiled for 10 min and analyzed by SDS–PAGE followed by detection with phospho-specific antibodies.

RNA extraction and qPCR. Total RNA from cells or mouse tissues was extracted using a Total RNA Purification kit (GeneMark). cDNA was obtained using the RevertAid First Strand cDNA Synthesis kit (Thermo). RNA levels were measured with the LightCycler 480 II (Roche) as previously described11,22. The following primers were used for qPCR:

**Actin-forward:** TGGCATGGTGCTACTGTGTAAGG
**Actin-forward:** CACACACGCCATCACATCAA

**Cry1-forward:** TCTAGGGACACACCCAGTCC
**Cry1-forward:** CACTGGTTCCGAAAGGGACTC

**Per2-forward:** GTCCACCCCGGGGAAGGTGA
**Per2-forward:** GTAGATCCAAGGGGCGGACAGC

**Per1-forward:** CTTGAGCAGATCTTGCGAGGT
**Per1-forward:** GTGATCCAGAAGGGGCGGCACCT

**CLOCK-forward:** AGGCCCTGACACTGTCGCAA
**Dbf-forward:** TCTAGGGACACACCCAGTCC

**CLOCK-forward:** TGCACTGCTCTCAATCGAGC
**Dbf-forward:** ATGGCCCTGGAATCGTGACA

**Rev-erba-forward:** TGGCATGGTGGCTACTGTGTAAGG
**Per2-forward:** GAAACGTGCACCCATCGATA

**Rev-erba-forward:** ATATTCTGTTGTAGCTCGGGCG
**Per2-forward:** AAGAACGAGGCGGAGG

**Per1-forward:** CACACACGCCATCACATCAA
**Per1-forward:** TGAAGCAGGCGGAGG

**Per2-forward:** AAAAGGTGCACCCATCGATA
**Per2-forward:** AATTCGCGACTCTTTTTTTCAG

Mass spectrometry. To identify TIP1-interacting proteins, primary hepatocytes were infected with HA-TIP1 adenovirus. Immunoprecipitates of HA-TIP1 were prepared for MS studies as previously reported42,43, and analyzed by electrospray ionization tandem MS on a Thermo LTQ Orbitrap instrument. To identify the mTOR phosphate-site (s) in PER1 and CRY1, primary hepatocytes were infected with FLAG-PER1 or FLAG-CRY1 adenovirus and treated with or without 250 nM Torin1 for 2 h. Immunoprecipitates of FLAG-PER1 and FLAG-CRY1 were used to determine phosphate-site(s) by MS. The MS proteomics data have been deposited to the ProteomExChange with identifier PXD016917 and PXD016919. The source data under Figures 1–7 and Supplementary Figs. 1–6 are provided as a Source Data file.

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Author contributions
Y.L. Y.Z., and Y.W. designed the study and analyzed the data; Y.L., Y.Z. T. L. and J.H. performed experiments; Y.L. performed all the experiments during revision; Y.W. wrote the paper. All authors reviewed and commented on the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Y.W.

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