Clock is important for food and circadian regulation of macronutrient absorption in mice

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Abstract **Clock genes respond to external stimuli and exhibit circadian rhythms. This study investigated the expression of clock genes in the small intestine and their contribution in the regulation of nutrient absorption by enterocytes. We examined expression of clock genes and macronutrient transport proteins in the small intestines of wild-type and Clock mutant ($Clk^{mt/mt}$) mice with free or limited access to food. In addition, we studied absorption of macronutrients in these mice. Intestinal clock genes show circadian expression and respond to food entrainment in wild-type mice. Dominant negative Clock in $Clk^{mt/mt}$ mice disrupts circadian expression and food entrainment of clock genes. The absorption of lipids and monosaccharides was high in $Clk^{mt/mt}$ mice whereas peptide absorption was reduced. Molecular studies revealed that Clock regulates several transport proteins involved in nutrient absorption. Clock plays an important role in light and food entrainment of intestinal functions by regulating nutrient transport proteins. Disruptions in intestinal circadian activity may contribute to hyperlipidemia and hyperglycemia.**—Pan, X., and M. M. Hussain. **Clock is important for food and circadian regulation of macronutrient absorption in mice.** ***J. Lipid Res.*** **2009.** 50: **1800–1813.**

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Various physiologic and behavioral activities show diurnal variations. These activities are centrally controlled by suprachiasmatic nuclei (SCN) (1–5). Although neurons in the SCN exhibit endogenous rhythms, they require entrainment by light. Daily changes in light are sensed by neurons in the retina and this information is transmitted to the SCN. In the SCN, this information is processed into molecular events leading to changes in the expression of a set of "clock genes" that include Clock, Bmal1, Per1, Per2, Per3, Cry1, and Cry2 transcription factors. These factors form specific partnerships and constitute auto-regulatory feedback loops. Clock and Bmal1 heterodimers activate *Per* and *Cry* genes initiating the positive feed forward loop. Per and Cry heterodimers oppose the action of Clock/Bmal1, forming a negative feedback loop. In mammals, these circadian clocks exist not only in the SCN but also in most peripheral tissues such as liver, heart, adipose tissue, and intestine (6–14). However, their role in intestinal functions is unknown.

Besides light, food is a potent synchronizer of peripheral clocks and entrains various behavioral and physiologic activities (5, 8, 15–19). There is evidence to suggest that the food-entrainable oscillator (FEO) is distinct from the light-entrainable oscillator (LEO). It is unknown whether FEO acts alone or there is significant cooperation between FEO and LEO during food entrainment. However, it is known that ablation of *Per1* and *Bmal1*, but not *Clock*, abolishes food entrainment (20, 21). Knowledge about the role of clock genes in food entrainment may be useful in explaining etiologies of some gastrointestinal, metabolic, and behavioral disorders.

The role of Clock in circadian control has largely been derived from mice that express a dominant negative Clock protein in C57Bl/6J background and are arrhythmic (22). They exhibit longer periodicity (26–29 h instead of 23–24 h) in their locomotor activity (22). The Clock mutant protein (encodes a protein with 51 amino acid deletion in its putative transcriptional regulatory domain and is defective in transcriptional activity) interacts with Bmal1, binds to E-box sequences, and acts in a dominant negative fashion (23) and decreases the transcription of per and other circadian clock genes and disables the negative feedback

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Abbreviations: αMG, α-methyl-glucopyranoside; apo, apolipoprotein; BBMV, brush border membrane vesicle; $Clk^{mt/mt}$, Clock mutant; FEO, food-entrained oscillator; Dgat, diacylglycerol acyltransferase; GLUT, glucose transporter; gly-sar, glycyl-sarcosine; LD, light/dark; LEO, light-entrained oscillator; MGAT, monoacylglycerol acyltransferase; MTP, microsomal triglyceride transfer protein; PEPT1, proton-coupled, oligopeptide transporter 1; SCN, suprachiasmatic nuclei; SGLT1, Na^+/glucose cotransporter 1; SR-B, scavenger receptor-B; WT, wild type.

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loop of circadian rhythm. These Clock mutant ($Clk^{mt/mt}$) mice are entrainable during a normal light/dark (LD) cycle but lose this ability when placed in the dark (22). In addition, they show physiologic abnormalities such as reduced fertility, obesity, and metabolic syndrome (24). Nevertheless, they can be entrained by food as seen by changes in locomotor activity (24–26). $Clk^{mt/mt}$ are obese and show hyperleptinemia, hyperlipidemia, hepatic steatosis, and hyperglycemia (27). The etiologies of these metabolic diseases are unknown.

For unknown reasons, the major complaints in shift workers are related to gastrointestinal disturbances (28). Various functions of the intestine, such as gastric emptying, colonic motility, DNA synthesis, and epithelial cell renewal, exhibit circadian activities (5, 29, 30). The major function of the small intestine is to digest and absorb food. Macronutrients, carbohydrates, lipids, and proteins are hydrolyzed in the lumen of the intestine and products are retrieved by enterocytes involving various transporters. Several studies indicate that transporters involved in the absorption of fat, carbohydrates, and proteins show diurnal rhythms. For example, apical Na^+/glucose cotransporter 1 (SGLT1) and fructose transporter 5 (GLUT5) as well as the basolateral hexose transporter GLUT2 show rhythmic expression (31–36). In addition, the expression of proton-coupled, oligopeptide transporter 1 (PEPT1), which is also a nutrient and drug transporter, shows diurnal expression (32, 37). We reported that intestinal and hepatic gene expression of microsomal triglyceride transfer protein (MTP), a protein involved in intracellular neutral lipid transfer (38, 39), shows rhythmic expression (40). Furthermore, MTP expression is significantly changed when mice are subjected to food entrainment. Mechanisms pertaining to the rhythmic expression of these nutrient transport proteins have not been elucidated. Moreover, it is unknown whether clock genes important in light entrainment play a role in food-entrained regulation of intestinal proteins.

Our aim was to study light- and food-entrained regulation of clock and intestinal genes as well as absorptive functions of the gut. In addition, we wanted to find out the importance of clock genes in intestinal activities. We studied the expression of clock genes and different nutrient transporters in mice expressing normal and dominant negative Clock protein. Our data show that intestinal epithelial cells express canonical clock genes in a circadian manner and are susceptible to attunement by food. Normal Clock expression is important for the circadian and food-entrained expression of nutrient transport proteins as well as in the absorption of macronutrients. Thus, this study has uncovered a role for circadian rhythm and Clock protein in macronutrient absorption by the intestine.

EXPERIMENTAL PROCEDURES

Materials

[4-^{14}C]cholesterol (58.0 mCi/mmol), [9,10-^{3}H(N)]triolein (22.0 Ci/mmol), [9,10-^{3}H(N)]oleic acid (7.0 Ci/mmol), and Solvable were from PerkinElmer Life Sciences. [^{3}H]glycyl-sarcosine (gly-sar, 1.78 GBq/mmol) and [^{14}C]methyl-α-D-[U-^{14}C]glucopyranoside (αMG, 9.66 GBq/mmol) were obtained from Moravek Biochemicals, Inc.. Gly-sar was from Sigma. Rabbit polyclonal antibodies were purchased from different companies and used at indicated dilutions for Western blotting: GAPDH (1:1000, Abcam), PER1 (1:100, Abcam), PER2 (1:200, Chemicon), Bmal1 (1:400, Novus) and Alexa-conjugated secondary antibodies (1:200, Molecular Probes). MTP (1:1000), Cry1 (1:100), and Clock (1:50) antibodies were from Santa Cruz Biotechnology, Inc..

Animals

All the animal studies were approved by an institutional animal care and use committee. Heterozygous breeding pairs of C57BL/6J-*Clock*^{m1Jt} ($Clk^{mt/mt}$ mouse) (22) were from the Jackson Laboratories. Male, wild-type (WT), and homozygous mutant siblings (4–6 months) were used for experiments and heterozygous mice were used for breeding. Mice were maintained in an accredited animal facility on a 12-h LD schedule (0700 hours–1900 hours) and fed normal chow ad libitum. For food entrainment, animals were divided into three groups of 36 animals; all groups had access to food for two h (0930 hours–1130 hours) for 10 days. The second and third groups of animals were continued on the same feeding regimen for an additional 5 days but were placed in constant dark or light during these days. Animals (n = 6, at each time) were euthanized at 4 h intervals (0400, 0800, 1200, 1600, 2000, and 2400 hours), and the intestines from stomach to cecum were collected and washed. Duodenum (3–5 cm) was severed from the stomach. Next, three consecutive 5 cm segments were collected and designated as proximal jejunum, distal jejunum, and ileum. The first 3 cm segments were used for protein analyses and the last 2 cm segments were used for RNA isolation. For this purpose, the intestinal mucosa was scraped and rapidly frozen in liquid nitrogen. For circadian and food entrainment experiments, small intestines were divided into eight equal segments. Segments 4 and 5 were used for protein measurements whereas segments 3 and 6 were used for mRNA quantifications. Brush border membrane vesicles (BBMV) and enterocytes were prepared from 10 cm long segments of jejunum obtained from the ligament of Treitz. Intestinal segments 2–3 cm in length, taken from the mid-point of these regions, were used for uptake experiments and for immunohistochemistry.

In situ loop technique

Two small incisions were made in the gut of anesthetized mice and flushed with PBS. A proximal jejunal loop (5 cm) was made by tying with strings (40). For lipid absorption studies, PBS (0.5 ml) containing [^{3}H]triolein or [^{14}C]cholesterol (2.5 μCi/ml) and cholesterol (0.2 mg/ml) was introduced into the loop with a microsyringe at 1200 hours or at 2400 hours. After 1 h, entire loops were collected. Total counts in plasma were measured as before (40). For carbohydrate and peptide absorption, in situ loops were prepared at 1200 hours or 2400 hours and a cannula (0.5 mm inner diameter and 0.8 mm outer diameter polyethylene tube) was inserted in the portal vein. Experiments were initiated by the injection of 0.5 ml of PBS containing [^{14}C] αMG with 10 mM αMG or [^{3}H]gly-sar (40 nmol/mg body wt) with gly-sar (10 mM) into the loops with a microsyringe. Blood was withdrawn from the portal artery at designated times, centrifuged (10 min, 12,000 *g*), and 50 μl of plasma was solubilized in 0.5 ml of OptiSolv. Radioactivity was counted after mixing with 5 ml of scintillation cocktail.

Determination of MTP activity

After extensive washes with ice-cold PBS, 1 cm segments of proximal jejunum were homogenized in 1 ml of ice-cold 1 mM Tris-HCl (pH 7.6), 1 mM EGTA, and 1 mM $MgCl_2$ in a glass

homogenizer. The homogenates were centrifuged (SW55 Ti rotor, 50,000 rpm, 10°C, 1 h), and supernatants were used for an MTP assay (40, 41) using a kit (Chylos, Inc.).

Uptake by enterocytes

Enterocytes were isolated at 1200 hours from WT and $Clk^{mt/mt}$ mice as before (42, 43). Mice were anesthetized, intestinal contents removed, washed with 117 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, 5.5 mM glucose, filled with 67.5 mM NaCl, 1.5 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, 27 mM sodium citrate, 5.5 mM glucose, and bathed in oxygenated saline at 37°C for 10 min. The buffer was discarded, intestines were refilled with the same buffer containing 1.5 mM EDTA and 0.5 mM dithiothreitol, and bathed in oxygenated saline at 37°C for 10 min. Lumenal contents were collected and centrifuged (1,500 rpm, 5 min) to collect enterocytes. All buffers were adjusted to pH 7.4, gassed with 95% O_2, 5% CO_2 for 20 min, and maintained at 37°C.

BBMV preparation

BBMV were prepared at 4°C according to (32, 44). Jejunal segments (proximal 10 cm) were opened and the mucosal layers were scraped off and added to homogenization buffer (50 mM mannitol, 2 mM Hepes, 0.25 mM phenylmethylsulphonyl fluoride, pH 7.1; 28 ml g^{-1} mucosa). The mixture was homogenized, $MgCl_2$ was added to obtain 10 mM concentration, stirred on ice for 20 min, and centrifuged (3000 *g*, 15 min). The supernatant was centrifuged (27,000 *g*, 30 min). The pellet was resuspended in buffer (100 mM mannitol, 0.1 mM $MgSO_4$, and 0.4 mM Hepes-Tris, pH 7.2) and centrifuged (6000 *g*, 15 min). The supernatant was then centrifuged again (27,000 *g*, 30 min) and the pellet was suspended in 300 mM mannitol, 10 mM Hepes-Tris, 0.1 mM $MgSO_4$, and 0.25 mM PMSF, buffer pH 7.4 by passing six times through a 25-gauge needle to obtain a protein concentration of 2 mg·ml^{-1}. The experimental buffer for D-glucose uptake consisted of 300 mM mannitol and 10 mM HEPES (pH 7.5). For gly-sar uptake, 10 mM HEPES buffer pH 7.5 containing 100 mM mannitol and 100 mM potassium chloride was used. The pH of both buffers was adjusted with potassium hydroxide. The uptake of [^{14}C] αMG or [^{3}H]gly-sar by BBMV was measured by a rapid filtration technique (44). The radioactivity of [^{14}C] αMG or [^{3}H]gly-sar trapped in the membrane vesicles was solubilized in 0.5 ml of OptiSolv and radioactivity was determined in 2.5 ml of scintillation cocktail.

Western blot analysis

For subcellular fractionation, tissues were homogenized, filtered, and centrifuged (500 *g*, 10 min, 4°C; Beckman GS-15R centrifuge). Supernatant was the cytosolic fraction. Pellet was suspended and used as the nuclear fraction. Cytosolic and nuclear fractions were separated under nonreducing conditions, transferred to nitrocellulose membranes, blocked for 2 h in 20 mM Tris, 137 mM NaCl, pH 7.5, containing 0.1% Tween 20 and 5% nonfat dry milk at room temperature. The blots were washed three times and incubated overnight at 4°C in the same buffer containing 0.5% dry milk and a primary antibody, washed, and then incubated with mouse horseradish peroxidase-conjugated secondary antibody (1:1000-1:4000) in 1% skim milk for 1 h. Immune reactivity was detected by chemiluminescence (40, 45).

Immunohistochemistry

Immunohistochemistry was performed as described (32, 46). Briefly, fresh specimens were cut transversely, and the lumens were washed with PBS. Samples were embedded in optimal cutting temperature compound, rapidly frozen in liquid nitrogen, and stored at −80°C. Frozen sections were cut with a cryostat (8 μm thick), mounted on glass slides, fixed in ethanol for 15 min at −15°C, and washed with PBS at room temperature. The sections were then covered with 5% normal goat serum for 60 min and incubated with anti-Bmal1, Per1, Per2 or Cry1 antibodies at a dilution of 1:50-1:250 for 1 h, washed with PBS for 10 min three times; the sections were incubated with Alexa-conjugated secondary antibodies 1:200 for 1 h and then washed with PBS. These sections were treated with Vectashield to prevent fluorescent bleaching and visualized using a Biorad Radiance 2000 confocal microscope.

Real-time PCR analysis

Total RNA was isolated using TRIzol™ (Invitrogen) and used to reverse transcribe and the resultant reaction mixtures were used for real-time PCR (40, 47). Primers used for different genes are in **Table 1**. 18S rRNA was used for reference.

Statistical analyses

Each time point or each group represents mean from 5–6 animals. Data are presented as means ± SD. Statistical significance was evaluated using Student's *t*-test. $P < 0.05$ was considered significant. GraphPad Prism was used for graphing and statistical evaluations.

RESULTS

Expression of Clock genes in the intestinal epithelium

Western blotting of proteins (**Fig. 1A**) and quantitative RT-PCR of RNA (Fig. 1B) revealed that clock genes are expressed in all regions of the gut. To identify cells expressing these proteins, we used immunohistochemistry (Fig. 1C). Bmal1 was detectable mainly in the epithelial cells of the proximal and distal jejunum (Fig. 1C). This was further confirmed by separating enterocytes from the intestinal mucosa (42, 43). Bmal1 was mainly in enterocytes (Fig. 1D, EDTA) with small amounts in nonepithelial cells (Fig. 1D, mucosa). Clock genes are transcription factors. Therefore, we looked for the presence of Bmal1 in the nuclear fractions of the intestine. Bmal1 was detectable in both cytosolic and nuclear fractions (Fig. 1E). These studies indicate that clock genes are expressed throughout the small intestine.

Circadian expression of clock genes in the gut

Clock genes showed diurnal expression in the small intestine of normal mice subjected to a 12 h LD cycle (**Fig. 2**). Bmal1 and Cry1 proteins (Fig. 2A) and Bmal1, Cry1, and Cry2 mRNA (Fig. 2B) levels were high at 2400 hours to 400 hours. The Per proteins and mRNA levels peaked at 1600 hours to 2000 hours, consistent with (11, 12, 48). Intestinal Clock protein showed weak rhythm but GAPDH did not. These studies indicate that proteins and mRNA levels of clock genes in the small intestine show diurnal variations. Their diurnal expression is similar to that seen in the colon (12) and liver and is phase delayed with respect to the expression of these genes in the SCN (18).

Food entrainment of clock genes

Feeding schedule can shift the expression of peripheral clock genes (18). Thus, we wondered whether clock genes

TABLE 1. Primers used for the quantification of different mouse mRNA

Gene ID	Forward (5′-Primer)	Reverse (3′-Primer)
Per1	CTCAGGTATTTGGAGAGCTGCAA	TTGCTGACGACGGATCTTTCT
Per2	CTGGCTTCACCATGCCTGTT	AAGGCCTGAGGCAGGTTTG
Per3	GTGTACACAGTGTGCAAGCAAACA	ACGGCCGCGAAGGTATCT
Clock	CACCGACAAAGATCCCTACTGAT	TGAGACATCGCTGGCTGTGT
Bmal1	CAACCTTCCCGCAGCTAACA	TCCGCGATCATTCGACCTAT
Cry1	CCTCTGTCTGATGACCATGATGA	CCCAGGCCTTTCTTTCCCAA
Cry2	AGGGCTGCCAAGTGCATCAT	AGGAAGGGACAGATGCCAATAG
MTP	ACGGCCATTCCCATTGTG	GCCAGAGCTCCGAGAGAGAA
18S rRNA	AGTCCCTTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
GAPDH	GCAGTGGCAAAGTGGAGATTG	GTGAGTGGAGTCATACTGGAACATG
ApoB	GATCAGGCTTTGCCGCAATA	CATCAGGAGAGGCCAATCC
DGAT1	GTTCAGGTCAGACAGTGGTTTCA	TCAGCATCACCACACACACCAA
DGAT2	AGCTGCAGGTCATCTCAGTACTACA	CTGCAGGCCACTCCTAGCA
SR-B1	ACGGCCAGAAGCCAGAAGCCAGTAGTC	GACCTTTTGTCTGAACTCCCTGTAG
ApoA1	GATGCGGTGGAACACTTTCT	ACTGTCCTCGACTTCCGAGA
MGAT2	TGGGATTTGAACCTGTAGCC	TCCCTGTTTGTCCTTTGGTC
ACAT2	CCACCACCTTGGACAGTTCT	AGCCCTTGATGACTGATTGG
ApoA-IV	GCACAACAAGCTGGTGCCC	CAGGTGCTCCTGCAACTTCTG
FABP-I	CATATTCCGTTCCCTGCACT	TTGGGGTCTGTGTTCATTCA
FAS	GGAAGGCTGGGCTCTATGG	GGCGTCGAACTTGGAGAGATC
SCD-1	CTACAAGCCTGGCCTCCTGC	GGACCCCAGGGAAACCAGGA
ACAT2	GGTGGAACTATGTGGCCAAGA	CCAGGATGAAGCAGGCATAGA
SGLT1	ATGCACCCATCCCGTCCTA	ACCACGGAGTAGATGACGATGACT
GLUT5	TCAGTCCGGTAGCAGTTGAACA	CAGCAATCTGCCCACAAGCT
GLUT2	GGTCACTGCCATCCACACAGT	TCAGTCCGGTAGCAGTTGAACA
PEPT1	CGTGCACGTAGCACTGTCCAT	GGCTTGATTCCTCCTGTACCA
ARPp0	GTCCAACTACTTCCTCAAGATCATCCA	ACATGCGGATCTGCTGCAT

in the gut respond to food. Mice were fed for 10 days from 930 hours to 1130 hours. Food entrainment impacted significantly on the expression of clock genes in the gut. All mRNA (**Fig. 3A**) and protein (Fig. 3D) levels were high at or after mealtime. To study the importance of the LD cycle, food-entrained animals were exposed to constant dark or light for an additional 5 days. Also during this time, food was available for 2 h. The circadian expression of clock genes was significantly dampened when exposed to dark (Fig. 3B, E) and was abolished when exposed to constant light (Fig. 3C, F). These studies showed that food entrainment significantly alters expression of clock genes and that this entrainment requires a 12 h LD cycle.

Clock is important for the circadian and food entrainment of clock genes

It is known that Clock is critical for the diurnal regulation of clock genes in the SCN. Therefore, we hypothesized that it might play a role in the diurnal expression of clock genes in the gut. To test this, we studied the expression of clock genes in the jejunum of $Clk^{mt/mt}$ mice that express a dominant negative form of Clock (22) and their WT siblings. In WT mice, clock genes showed circadian expression (**Fig. 4A**) as in Fig. 2B. However, in $Clk^{mt/mt}$ mice, these changes were significantly dampened or not evident (Fig. 4A). These data indicate that normal Clock function is important for the circadian expression of clock genes in the gut.

To determine whether Clock also plays a role in food entrainment, WT and $Clk^{mt/mt}$ mice were subjected to food entrainment for 10 days. Food entrainment significantly altered the expression of clock genes in the small intestine of WT mice (Fig. 4B): *Per1, Per2,* and *Per3* mRNA increased before or at the time of food availability, whereas *Cry1*, *Cry2,* and *Bmal1* levels increased 4 h later. In $Clk^{mt/mt}$ mice, however, these changes were not seen. *GAPDH* expression was resistant to changes induced by food in both WT and $Clk^{mt/mt}$ mice. These data indicate that Clock is important for the regulation of small intestinal clock genes by food.

Role of Clock in glucose and peptide absorption

Next, we studied the role of Clock in carbohydrate and peptide absorption. To document further the importance of Clock in carbohydrate and peptide absorption, we studied the uptake of αMG, a substrate for SGLT1, and gly-sar, a substrate for PEPT1, using in situ loops, enterocytes, and BBMV. [^{14}C] αMG and [^{3}H]gly-sar were injected into in situ loops prepared in WT and $Clk^{mt/mt}$ mice at 1200 h (**Fig. 5A**). Absorption of αMG was high in $Clk^{mt/mt}$ mice compared with WT mice. In contrast, absorption of gly-sar peptide was low in mutant mice. We next studied their absorption from in situ loops at 1200 hours and 2400 hours to study diurnal variations (Fig. 5B). WT mice absorbed higher amounts of αMG and gly-sar at 2400 hours than at 1200 hours. The lower plasma counts at 1200 hours in these mice were due to increased retention in the intestinal lumen and tissue (data not shown). In contrast, $Clk^{mt/mt}$ mice absorbed these compounds to similar extent at both times. We next examined the uptake of aMG and gly-sar at 1200 hours by enterocytes (Fig. 5C), and BBMV (Fig. 5D). The uptake of [^{14}C]αMG by enterocytes (Fig. 5C) and BBMV (Fig. 5D) was high in $Clk^{mt/mt}$ mice, but the uptake of gly-sar was low. These results indicate higher monosaccharide and lower peptide absorption in $Clk^{mt/mt}$ mice. Therefore, Clock exerts differential effects on carbohydrate and peptide transporters.

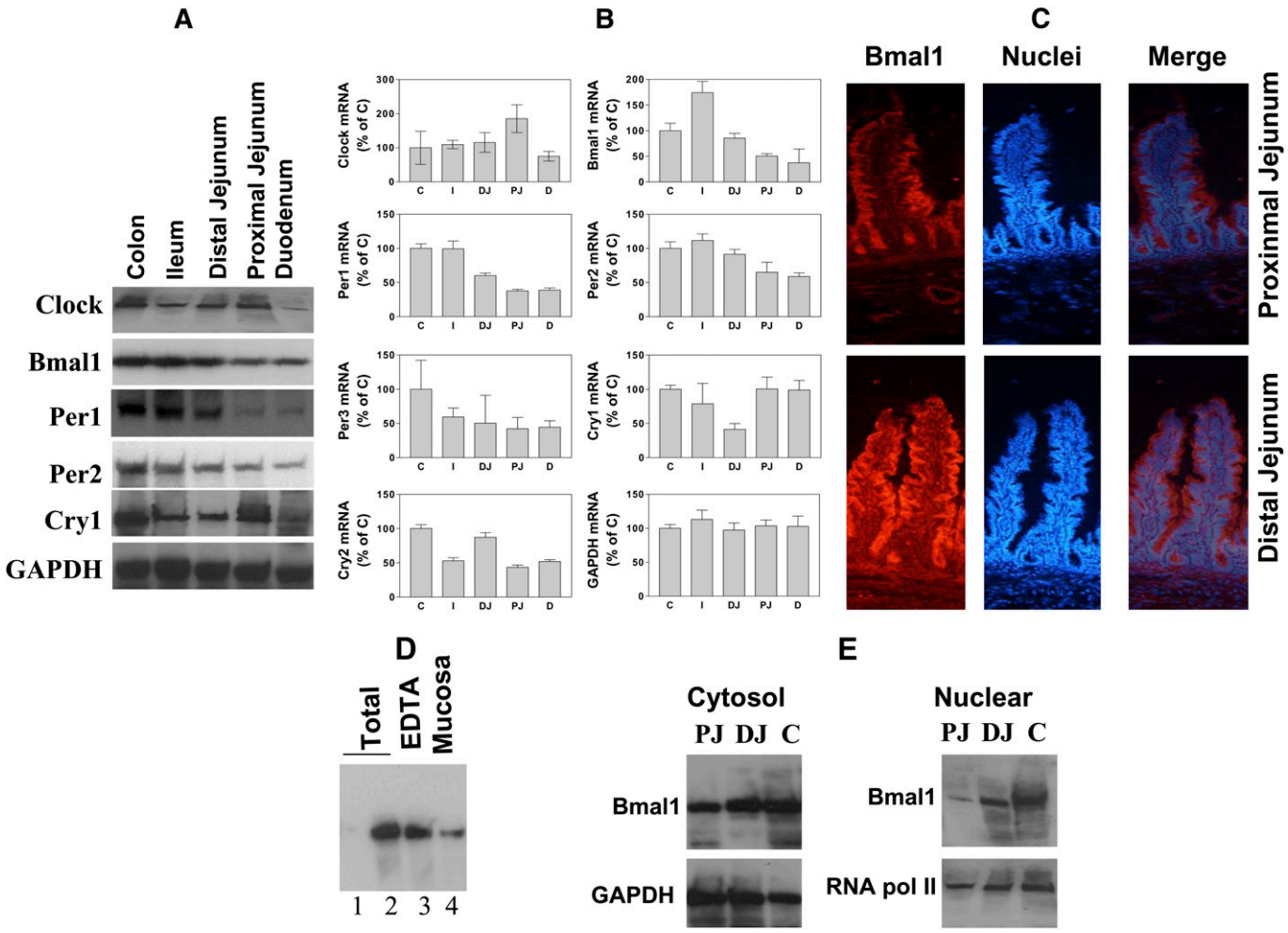

Fig. 1. Expression of clock genes in the intestine. A, B: Intestine from stomach to cecum was collected and washed. Segments of 3–5, 5, 5, and 5 cm were designated as duodenum, proximal jejunum, distal jejunum, and ileum, respectively. In addition, 5 cm of colon was obtained for analyses. The first 3 cm were used for immunoprecipitation followed by Western blotting (A) and the next 2 cm segments were used for RNA isolation (B). Clock gene mRNA was normalized to 18S rRNA (mean ± SD, n = 6). C, colon, I, ileum, DJ, distal jejunum, PJ, primal jejunum, D, duodenum. C: Immunohistochemistry of frozen sections of mouse gut tissues revealed presence of Bmal1 in the villi. D: Presence of Bmal1 in the intestinal epithelial cells. Lanes 1 and 2 are lysates from total tissue. Lane 3 has EDTA eluted cells, and lane 4 shows Bmal1 associated with the remaining tissue. Lane 1 was immunoprecipitated with preimmune serum, whereas lanes 2–4 were immunoprecipitated with anti-Bmal1 anti-serum. Proteins were immunoblotted with anti-Bmal1 antibodies. E: Distribution of Bmal1 in the cytosol and nucleus. Mucosa from proximal jejunum, distal jejunum, and colons were subjected to subcellular fractionation. Bmal1 was immunoprecipitated and subjected to immunoblotting. GAPDH and RNA polymerase (pol) II were used as markers.

To identify molecular mechanisms for differences in carbohydrate and peptide absorption in $Clk^{mt/mt}$ mice, we studied the expression of carbohydrate and peptide transporters. SGLT1, GLUT5, and GLUT2 are important for carbohydrate absorption (49), whereas PEPT1 is important for protein absorption (50, 51). In WT siblings, small intestinal SGLT1, GLUT2, and GLUT5 mRNA (Fig. 5E) were high at night and low during the day, consistent with Northern data (32). In contrast, PEPT1 mRNA was high during the day. In $Clk^{mt/mt}$ mice, however, intestinal SGLT1, GLUT5, GLUT2, and PEPT1 did not show any significant variations within 24 h (Fig. 5E). $Clk^{mt/mt}$ mice had high levels of intestinal SGLT1, GLUT2, and GLUT5 mRNA. In contrast, PEPT1 mRNA was low in these mice compared with WT mice. Food entrainment shifted the peak of their expression to before mealtime in WT mice (Fig. 5F). $Clk^{mt/mt}$ mice, however, failed to respond to food entrainment and the expression of these transporters remained unchanged during the day (Fig. 5F). These data indicate that circadian and food-entrained regulation of intestinal macronutrient transporters requires normal Clock activity and Clock controls circadian and food-entrained regulation of intestinal carbohydrate and peptide absorption by regulating key transporters.

Clock is important for lipid absorption

Next, we addressed whether Clock also plays a role in lipid absorption. We studied lipid absorption in WT and $Clk^{mt/mt}$ mice. It is known that gastric emptying and pH changes show rhythmic behavior (52). Thus, in vivo studies may not differentiate between the role of Clock expressed in the stomach and the gut. To specifically address the role of Clock in lipid absorption by the small intestine, we studied the absorption of lipids from mouse jejunal

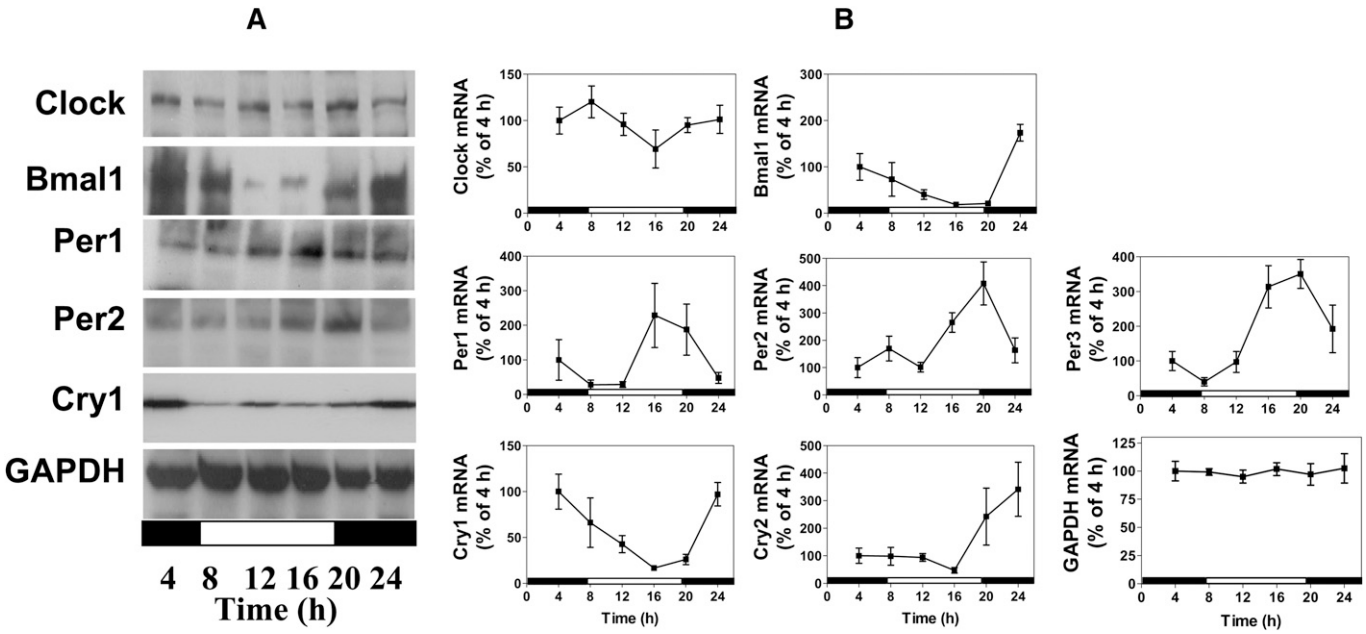

Fig. 2. Circadian rhythms of clock genes Proximal jejunum was collected as in Fig 1. A: Proteins were immunoprecipitated and immunoblotted as in Fig 1. B: Clock gene mRNA levels were normalized using 18S rRNA (mean ± SD, n = 6). After normalizing with 18S rRNA, values at 400 hours were set to 100% and other data were presented as % of this value. Solid circles, wild type (WT); open circles, Clock mutant ($Clk^{mt/mt}$) mice. Mean ± SD, n = 5–6.

loops severed from gastric apposition at 1200 hours. [^{3}H] triolein-derived counts in the plasma were higher in a time-dependent manner in $Clk^{mt/mt}$ mice compared with their WT siblings and a similar trend was observed for cholesterol (**Fig. 6A**). Next, we studied lipid absorption at 12 h and 24 h from in situ loops of WT and mutant mice (Fig. 6B). WT mice absorbed significantly more triglyceride and cholesterol at night than during the day. The lower counts in the plasma during the day in WT mice were due to significant retention of counts in the intestinal lumen and tissue (data not shown). In contrast, $Clk^{mt/mt}$ mice absorbed similar amounts of lipids during the day and night. The amounts of lipids absorbed by mutant mice during the day and night were similar to those absorbed by WT mice during the night.

It was previously shown that absorption from in situ loops can still be affected by hormonal and other factors that affect intestinal function (53). Therefore, we used isolated primary enterocytes to study lipid uptake and secretion (Fig. 6C, D). Uptake of [^{3}H]oleic acid and [^{14}C] cholesterol (Fig. 6C) was high in enterocytes isolated from $Clk^{mt/mt}$ mice. Similarly, $Clk^{mt/mt}$ enterocytes secreted more lipids compared with their WT enterocytes at 1200 hours (Fig. 6D). These studies indicate that intestinal lipid uptake and secretion is high in enterocytes obtained from mutant mice. Hence, $Clk^{mt/mt}$ mice absorb more lipids than their WT siblings.

Regulation of lipid absorption genes during food entrainment

To understand mechanisms for enhanced lipid absorption in Clock mutant mice, we hypothesized that Clock might control genes involved in lipid synthesis and transport. We have shown that intestinal lipid absorption plays a role in the circadian regulation of plasma lipids and lipoproteins (40). Furthermore, circadian regulation of plasma lipids and lipoproteins was correlated with diurnal changes in the hepatic and intestinal MTP expression. In mice, MTP activity, protein, and mRNA (**Fig. 7A**) levels were high at night and low during the day as before (40). Food restriction changed MTP expression. Higher MTP activity, protein, and mRNA levels were now present at mealtime (Fig. 7B, LD). Constant exposure to dark attenuated (Fig. 7B, DD) these increases, whereas constant light abolished (Fig. 7B, LL) this induction. These data indicate that LD cycle is required for the regulation of intestinal MTP by food.

Next, we studied changes in the expression of apoB (**Fig. 8A**), another protein essential for apoB-lipoprotein assembly. Mice fed ad libitum kept in 12 h LD showed significant variations within a day; apoB mRNA levels were high during the night and low during the day. Similar expression pattern was observed for apoAIV. ApoAIV modulates the amounts of lipids secreted with these particles. Food restriction altered the expression of apoB and apoAIV; they were now predominantly expressed at mealtime (Fig. 8B). Exposure to constant dark attenuated (Fig. 8C), whereas exposure to light abolished, their response to food (Fig. 8D). These data indicate that the LD cycle is required for the regulation of intestinal apoB and apoAIV by food.

Lipoprotein assembly not only depends on apoB and MTP but also requires triglycerides; therefore, we studied expression of genes involved in triglyceride synthesis. Under a normal LD cycle and free access to food, diacylglycerol acyltransferase (DGAT)2 (Fig. 8A) and I-fatty acid binding

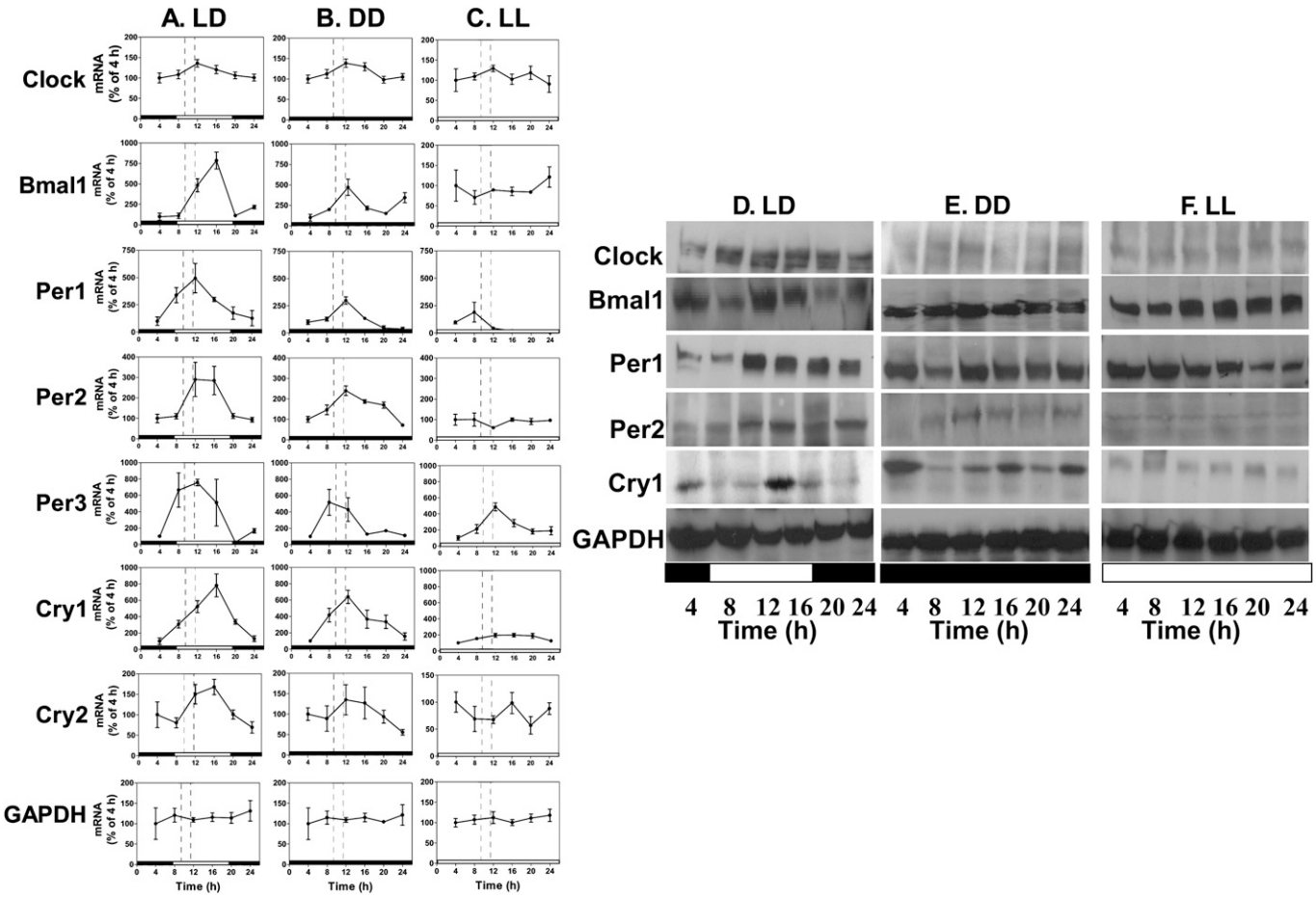

Fig. 3. Regulation of clock genes by food. A, D: Mice in 12 h light/dark (LD) cycle were fed from 0930 hours to 1130 hours for 10 days. B, E: Mice in 12 h LD cycle were fed from 0930 hours to 1130 hours for 15 days. During the last 5 days, mice were in dark (DD). C, F: Mice in 12 h LD cycle were fed from 930 hours to 1130 hours for 15 days. During the last 5 days, mice were in constant light (LL). Intestines were collected and used to measure mRNA (A–C) or protein (D–F) levels of different clock genes as in Fig 2. Mean ± SD, n = 5–6.

protein (FABP) (**Fig. 9A**) mRNA showed diurnal variations, but DGAT1, monoacylglycerol acyltransferase (MGAT)2, ApoA1 (Fig. 8A), ACAT2, and scavenger receptor-B (SR-B)1 (Fig. 9A) did not. Despite differences in circadian variations, the highest expressions of DGAT1, DGAT2, MGAT2, and FABP-I were seen at mealtime (Figs. 8B, 9B). These changes were suppressed in mice kept in constant dark (Figs. 8C, 9C) and were absent in mice exposed to constant light (Figs. 8D, 9D). In contrast, apoAI (a protein not involved in triglyceride transport but important for HDL biogenesis) mRNA levels did not show diurnal variations (Fig. 8A). It responded modestly to food entrainment (Fig. 8B) and exposure to constant light or dark attenuated this response (Fig. 8C, D). Therefore, intestinal apoAI is resilient to circadian as well as food-induced regulations.

We also looked at the fatty acid synthesizing enzymes FAS and stearoyl CoA desaturase (SCD)-1. They showed areciprocal diurnal expression pattern. SCD-1 expression was high during the day, whereas FAS expression was high at night (Fig. 8A). Nevertheless, both of these enzymes were significantly induced at mealtime (Fig. 8B). These changes were significantly dampened when the LD cycle was altered (Fig. 8C, D). These data indicate that some genes involved in triglyceride biosynthesis and secretion show diurnal variations. However, all the genes respond robustly to food entrainment. Therefore, genes involved in triglyceride synthesis and secretion can be divided into those that respond to food or food and light. Food entrainment might be a more dominant regulator of intestinal genes compared with circadian regulation.

Clock is important for food entrainment of the genes involved in lipid absorption

To determine the role of Clock in lipid absorption, we measured changes in the intestinal expression of MTP in WT and $Clk^{mt/mt}$ mice. In WT intestines, MTP activity, protein, and mRNA (Fig. 7C) showed diurnal variations. MTP expression was high during the night compared with day as described before (40). In mutant mice, however, intestinal MTP did not show variations during 24 h (Fig. 7C). In these mice, MTP activity was high during the day, a time of low activity in WT mice. GAPDH mRNA showed no rhythm in WT and mutant mice (data not shown). Expression analyses of genes involved in triglyceride synthesis and secretion indicated that several genes showed circadian rhythms in WT mice and these rhythms were absent in $Clk^{mt/mt}$ mice (Figs. 8E, 9E). These data indicate that Clock

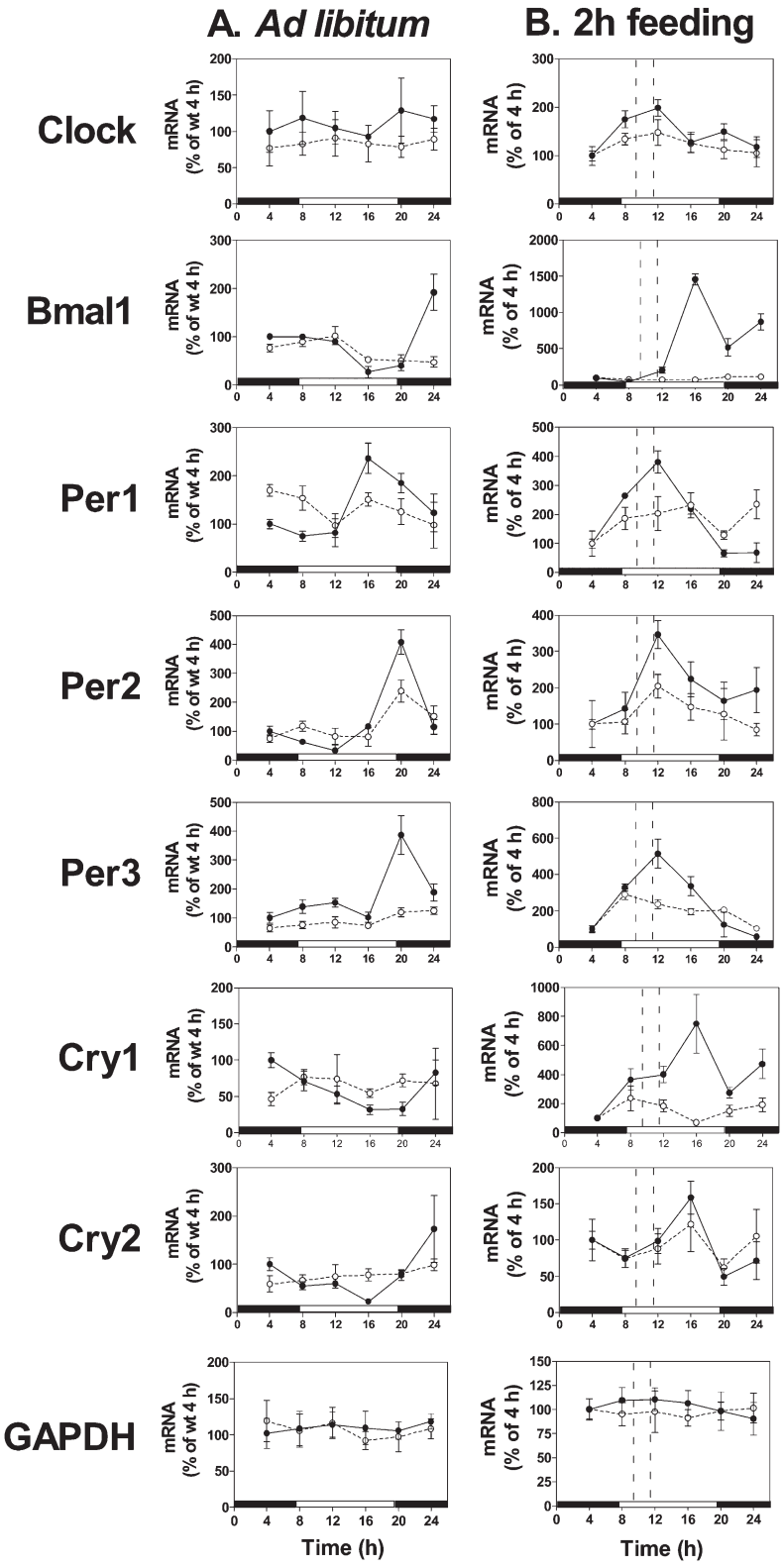

Fig. 4. Importance of Clock in circadian and food-entrained regulation of clock genes. A: WT (solid circles) and $Clk^{mt/mt}$ (open circles) siblings in 12 h LD cycle were fed ad libitum. B: WT and $Clk^{mt/mt}$ siblings in 12 h LD cycle were fed from 0930 hours to 1130 hours for 10 days. Intestines were collected and used to measure mRNA levels of different clock genes as in Fig 2. Mean ± SD, n = 5–6.

plays an important role in the daily regulation of genes involved in lipid transport.

Next, we studied the regulation of intestinal MTP in WT and $Clk^{mt/mt}$ mice subjected to food entrainment for 10 days. Food entrainment significantly enhanced intestinal MTP activity, protein, and mRNA (Fig. 8D) at mealtime. These changes were attenuated or not seen in $Clk^{mt/mt}$ mice (Fig. 8D). Similarly, several genes involved in triglyceride synthesis and secretion also showed increased expression at mealtime and these changes were significantly curtailed in $Clk^{mt/mt}$ mice (Figs. 8F, 9F). These data indicate that food entrainment up-regulates MTP at mealtime and this regulation requires normal Clock activity.

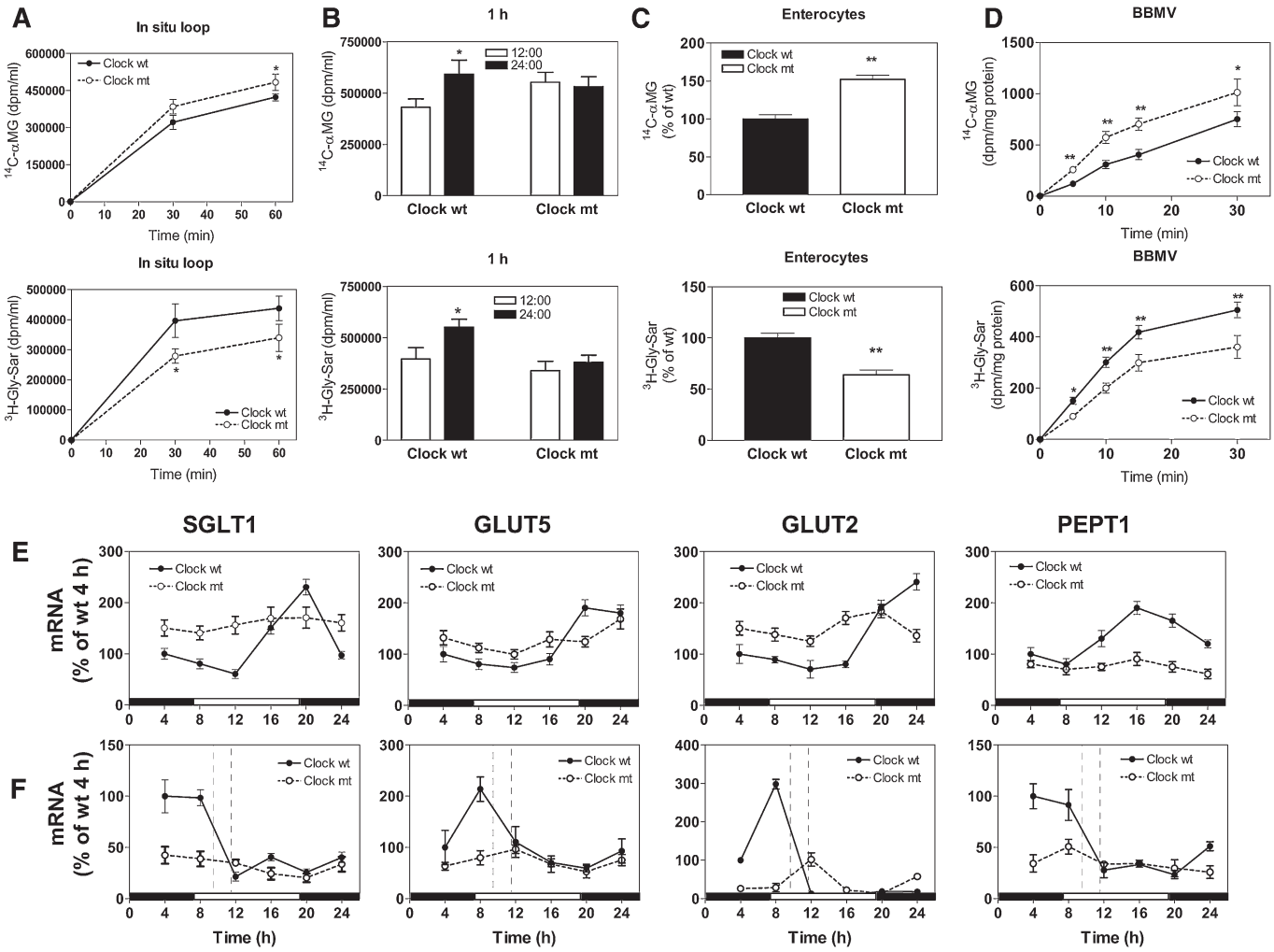

Fig. 5. Circadian and food-entrained regulation of glucose and peptide absorption. A: In situ loops were prepared at 1200 hours and injected with ^{14}C-αMG or ^{3}H-gly-sar. Appearance of radioactivity into portal vein was quantified. Mean ± SD, n = 3. B: In situ loops were prepared in WT and Clk$^{mt/mt}$ mice at 1200 hours and 2400 hours and injected with ^{14}C-αMG or ^{3}H-gly-sar. Radioactivity in plasma was quantified at 1 h. Mean ± SD, n = 3. C: Enterocytes were isolated from WT and Clk$^{mt/mt}$ mice at 1200 hours and incubated with ^{14}C-αMG or ^{3}H-gly-sar, After 10 min, enterocytes were centrifuged, washed, and counted. D: Brush border membrane vesicles (BBMV) were prepared from WT and Clk$^{mt/mt}$ mice at 1200 h incubated with ^{14}C-αMG or ^{3}H-gly-sar. At times, vesicles were washed and used to measure uptake. Mean ± SD. * $P < 0.5$, ** $P < 0.05$, n = 5–6. E: Jejunal mucosa were obtained from ad libitum-fed WT and Clk$^{mt/mt}$ mice at times and used to measure Na^{+}/glucose cotransporter 1 (SGLT1), glucose transporter (GLUT)2, GLUT5, and proton-coupled, oligopeptide transporter 1 (PEPT1) mRNA as in Fig 2. Mean ± SD, n = 5–6. F: WT and Clk$^{mt/mt}$ mice were subjected to 2 h food entrainment for 10 days, jejunal mucosa were obtained for mRNA quantifications. Mean ± SD, n = 5–6.

DISCUSSION

Light- and food-entrained regulation of clock genes in the gut requires Clock

Our data show that clock genes are expressed and exhibit circadian oscillations in the small intestine showing peak expressions at different times of the day and are consistent with their expression in enterocytes and Paneth cells (13). However, we noticed for the first time that circadian expression of the clock genes was dampened or lost in Clk$^{mt/mt}$ mice. In addition, we recognized that expression of the clock genes was significantly altered in mice subjected to food entrainment. Now, their peak expression occurred at or soon after mealtime, similar to that seen in the liver (8, 17, 18, 54) and adipose tissue (9, 10, 14). However, it is known that clock genes in the lung do not respond to food (18). Therefore, the intestine, in addition to the liver and fat tissue, is a target tissue of the FEO. In Clk$^{mt/mt}$ mice, however, the expressions of clock genes were resistant to food entrainment. Hence, we propose that Clock is important for light and food entrainment of intestinal clock genes.

Macronutrient absorption is altered in Clk$^{mt/mt}$ mice

The major function of the intestine is digestion and absorption of food. In this study, we provide the first evidence for the need of a circadian clock in the regulation of intestinal absorptive functions. Not surprisingly, nutrient absorption in WT mice was high at night and low during the day. However, Clk$^{mt/mt}$ mice absorbed similar amounts of nutrients at all times indicating no rhythmic variations. Amounts of lipids and carbohydrates absorbed

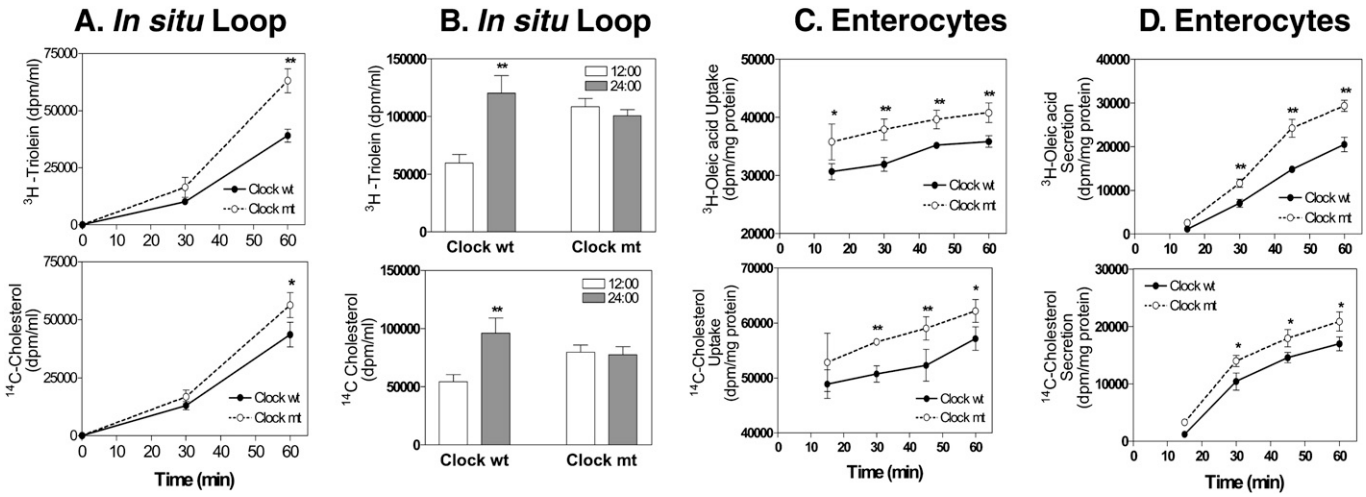

Fig. 6. Lipid absorption in WT and Clk$^{mt/mt}$ mice. A: Lipid absorption from in situ loops. Loops were prepared in WT and Clk$^{mt/mt}$ mice (n = 3) at 1200 hours and injected with ^{3}H-triolein or ^{14}Cholesterol. Radioactivity in plasma was quantified at 30 and 60 min. * $P = 0.05$. B: Absorption of lipids from in situ loops. Jejunal loops were prepared in WT and Clk$^{mt/mt}$ mice at 1200 hours and 2400 hours and injected with ^{3}H-triolein or ^{14}Cholesterol. Radioactivity in plasma was measured at 1 h. ** $P = 0.01$. C: Uptake of lipids by enterocytes. Enterocytes were isolated from WT and Clk$^{mt/mt}$ mice at 1200 hours and incubated in triplicate with either ^{3}H-triolein or ^{14}Cholesterol. At times, enterocytes were centrifuged, washed, and counted. * $P = 0.05$, ** $P = 0.01$. D: Secretion of lipids by enterocytes. Enterocytes were isolated from WT and Clk$^{mt/mt}$ mice at 1200 hours, incubated with either ^{3}H-triolein or ^{14}Cholesterol for 1 h, washed, and incubated in fresh media. At times, enterocytes were centrifuged, washed, and radioactivity in media was determined. Mean ± SD. * $P < 0.5$, ** $P < 0.05$, n = 5–6.

by mutant mice were similar to those absorbed by normal mice at night. In contrast, amounts of peptides absorbed by mutant mice were low, similar to those absorbed by WT mice during the day. Therefore, Clk$^{mt/mt}$ mice absorbed more lipids and carbohydrates and less peptides in a day compared with their normal siblings. We suggest that excess caloric intake due to increased carbohydrate and lipid absorption might contribute to hyperlipidemia, hyperglycemia, obesity, and metabolic syndrome in Clk$^{mt/mt}$ mice.

Food entrainment shifted the expression of different transport proteins to just before or at mealtime. Proteins involved in carbohydrates and peptide transport showed peak expression before meals, whereas lipid absorption genes showed peak expression at mealtime, indicating

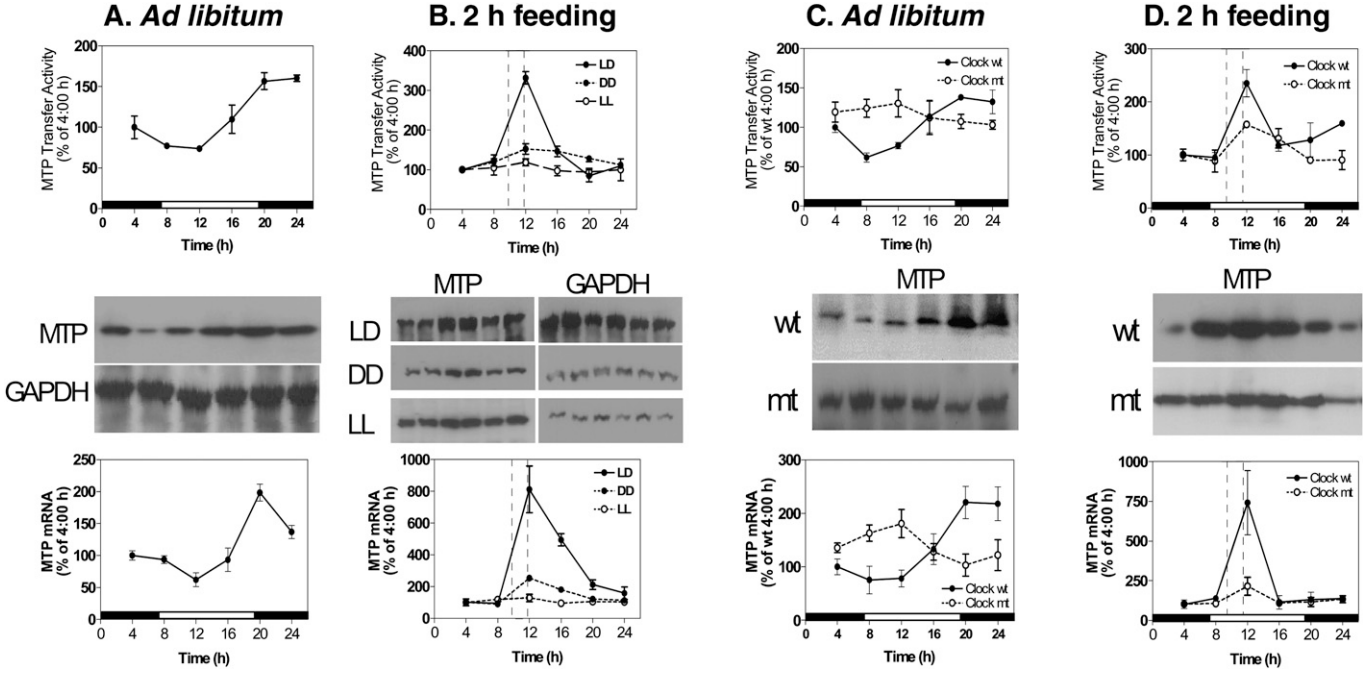

Fig. 7. Microsomal triglyceride transfer protein (MTP) expression in WT and Clk$^{mt/mt}$ mice. A: Changes in MTP activity, protein, and mRNA levels in the gut of mice kept in 12 h LD cycle and fed ad libitum. B: Changes in MTP activity, protein, and mRNA levels in mice fed 2 h for 10 days at 12 h LD cycle (LD), or switched to all dark (DD) or light (LL) for an additional 5 days. C, D: Changes in MTP activity, protein, and mRNA levels in WT and Clk$^{mt/mt}$ mice fed ad libitum (C) or 2 h feeding for 10 days (D). Mean ± SD, n = 6.

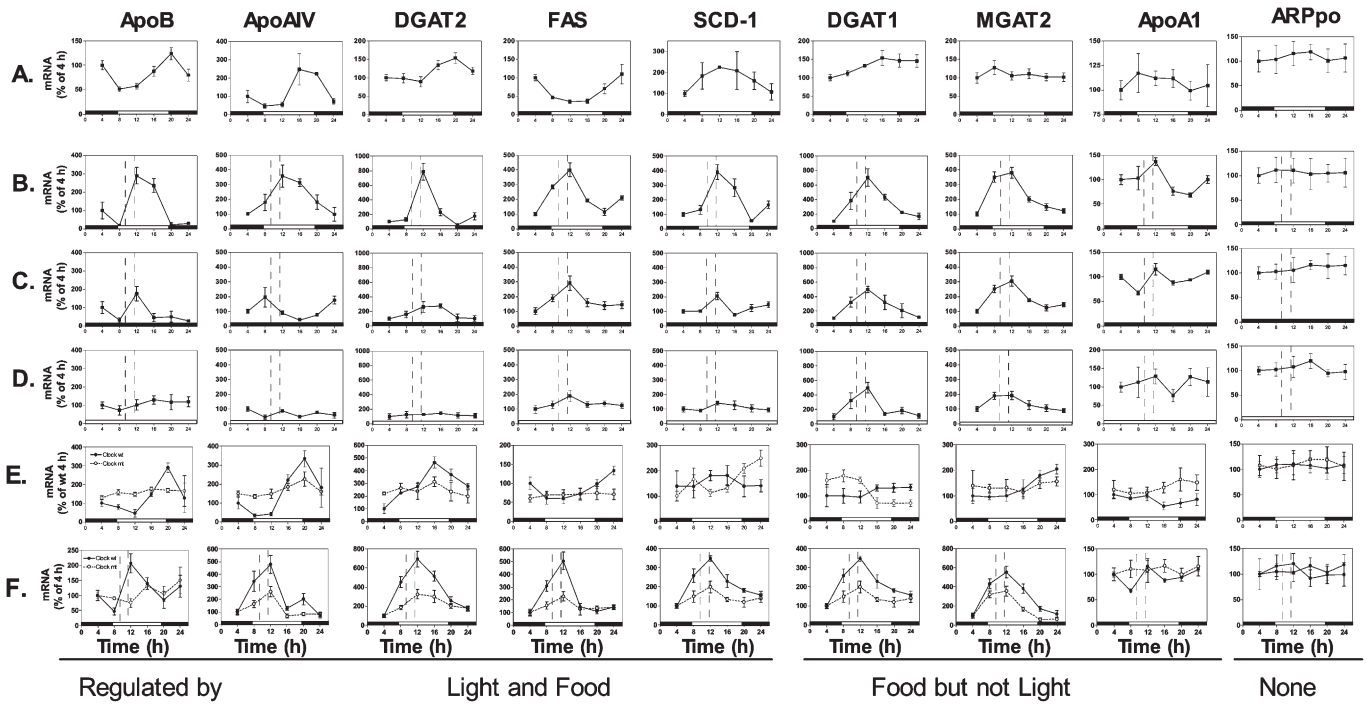

Fig. 8. Diurnal and food-entrained regulation of triglyceride synthesis and transport genes A: Ad libitum fed mice kept in 12 h LD cycle. B: Mice subjected to 2 h feeding for 10 days in 12 h LD cycle. C, D: Mice subjected to 2 h feeding for 10 days in 12 h LD cycle and the final 5 days in total dark (C), or the final 5 days in light (D). E: WT (solid circles) and $Clk^{mt/mt}$ (open circles) mice were fed ad libitum and kept in 12 h LD cycle. F: WT and $Clk^{mt/mt}$ mice were kept in 12 h LD cycle and had access to food for 2 h for 10 days. Intestines were used to measure mRNA levels as in Fig 2. Mean ± SD, n = 5–6.

differential regulation of these transport pathways by food. The reprogramming of nutrient transport proteins did not occur in $Clk^{mt/mt}$ mice indicating that food entrainment of nutrient transporters requires Clock and is probably dependent on normal expression of clock genes. From these studies, it is not clear whether the observed effects are a direct consequence of the expression of the $Clk^{mt/mt}$ protein in the intestine or a secondary response to the systemic expression of the mutant protein in the whole body. Nevertheless, these studies point out that deregulation of circadian control mechanisms might affect intestinal physiology.

Clock modulates macronutrient absorption by regulating transport proteins

How does Clock regulate nutrient absorption? We showed that Clock regulates the expression of critical nutrient transport proteins in the intestine and regulates nutrient absorption. Intestinal MTP, SGLT1, GLUT2, GLUT5, and PEPT1 mRNA in ad libitum-fed WT mice showed diurnal rhythms with peak expressions occurring at night. Several theories, including lumenal signals, vagal communications, and humoral regulations, have been proposed to explain diurnal regulation of intestinal transporters (5). Previously, we showed that diurnal regulation of the SGLT1 and PEPT1 mRNA is maintained in rats food-deprived for 4 days (33). Here, we observed that several nutrient transport proteins do not show circadian expressions in $Clk^{mt/mt}$ mice. Therefore, Clock is more important than the presence of food in the intestinal lumen in the regulation of intestinal transport proteins.

It is noteworthy that several nutrient transporters are affected by the expression of a dominant negative Clock. It is likely that the independent or combined regulation by Clock of different clock-controlled genes, which transmit circadian signals into physiological events, is involved in the control of protein, carbohydrate, and lipid absorption. For example, there is evidence to suggest that Dbp (37) and Pgc1α (55) regulate Pept1 and MTP, respectively. In addition, high concentrations of different hormones, such as leptin, in $Clk^{mt/mt}$ mice might affect the expression of different transporters. It is known that chronic leptinemia increases MTP expression (56, 57) and decreases PEPT1 (58) expression. More studies are needed to determine the role of clock-controlled genes and different hormones in the deregulation of macronutrient absorption in $Clk^{mt/mt}$ mice.

Clock is critical for food-consuming, but not for food-anticipatory, activities associated with food entrainment

Food entrainment is accompanied with noticeable changes in locomotor activities that represent anticipation and an urge to find food. Food-entrained $Clk^{mt/mt}$ mice show normal food anticipatory response as measured by changes in locomotor activity (26). Besides anticipatory response, food entrainment is also associated with changes in the expression of proteins involved in food consumption (40), e.g., MTP, SGLT1, and PEPT1 show altered expression when subjected to food entrainment. In the present study, we noticed that the expression of different transport proteins does not change in $Clk^{mt/mt}$ mice after

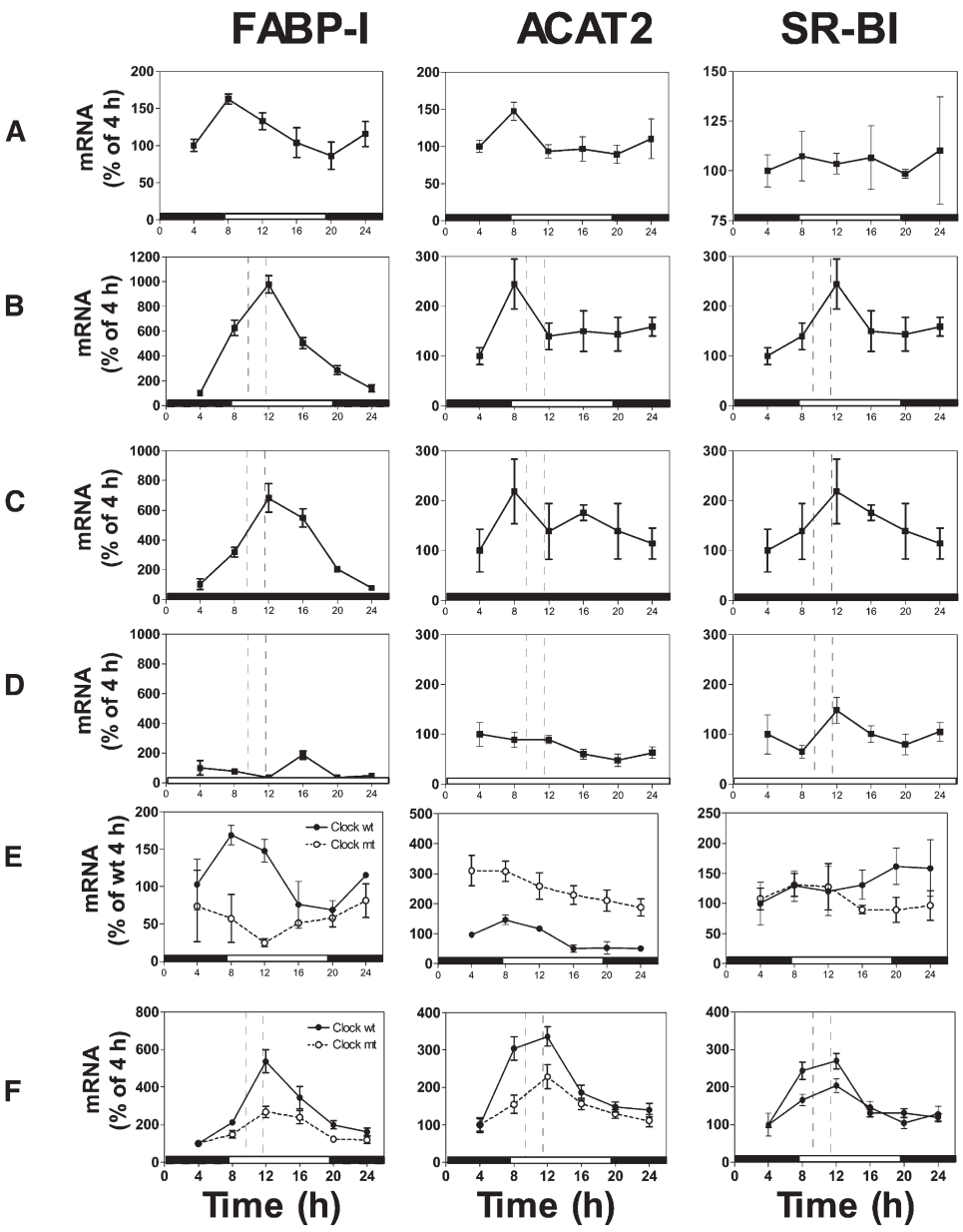

Fig. 9. Changes in mRNA levels of several candidate genes involved in lipid metabolism in mice fed ad libitum or subjected to food entrainment. A: Mice kept in 12 h LD cycle were fed ad libitum. B: Mice kept in 12 h LD cycle were fed from 0930 hours to 1130 hours for 10 days. C: Mice kept in 12 h LD cycle were fed from 0930 hours to 1130 hours for 10 days. They were also fed for additional 5 days between 0930 hours to 1130 hours. During these 5 days, mice were in constant dark. D: Mice kept in 12 h LD cycle were fed from 930 hours to 1130 hours for 10 days. They were also fed for an additional 5 days between 0930 hours and 1130 hours. During these 5 days, mice were in constant light. E: WT and $Clk^{mt/mt}$ siblings were kept in 12 h LD cycle and had free access to food. F: WT and $Clk^{mt/mt}$ siblings were kept in 12 h LD cycle and fed from 0930 hours to 1130 hours for 10 days. Intestines were used to measure mRNA levels as in Fig 2. Mean ± SD, n = 5–6.

food entrainment. Therefore, we propose that Clock might be critical for the entrainment of food-consuming behavior.

Increased susceptibility of intestinal functions to alterations in circadian rhythms

Microarray and proteomics studies have shown that >20% of the genes in the liver exhibit circadian variations (6, 59–61) indicating a small but substantial portion of the genome exhibits diurnal expression. In intestine, all the cryptidins (62) and most of the toll-like receptors (13) show circadian oscillations in the jejunum. Although we did not do a comprehensive systemic study, our sampling of critical intestinal functional proteins surprisingly revealed that several nutrient transport proteins show circadian expression and their expression is absent in $Clk^{mt/mt}$. Hence, it appears that a significantly higher number of intestinal genes might exhibit circadian expression. This might be a reason for the common complaints about gastrointestinal disturbances in shift workers.

Unique markers of food entrainment

Analysis of a significant number of intestinal genes involved in triglyceride synthesis and secretion revealed that all the genes respond to food entrainment. On the other hand, only a subset of these genes showed circadian rhythms. These data indicate that food entrainment might be a more dominant regulator of intestinal genes compared with circadian regulation. Nonetheless, genes involved in diurnal regulation are important for eliciting food entrainment. Therefore, we suggest that food entrainment coopts at least some of the circadian genes in exhibiting food entrainment.

In summary, our data show that the gut expresses clock genes and they show rhythmic expression. In addition, key proteins involved in macronutrient absorption show circadian expression. These genes respond to food entrainment and are expressed before or at mealtime. Therefore, food is an important regulator of intestinal gene expression. The circadian and food-entrained regulation of different genes was abolished in mice expressing a dominant negative Clock protein. Therefore, light-and food-entrained regulation of intestinal genes requires normal Clock activity.

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