The Transcriptional Repressor STRA13 Regulates a Subset of Peripheral Circadian Outputs

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Central and peripheral mammalian circadian clocks regulate a variety of behavioral and physiological processes through the rhythmic transcription of hundreds of clock-controlled genes. The circadian expression of many transcriptional regulators suggests that a major part of this circadian gene network is indirectly regulated by clock genes. Here we show that the basic helix-loop-helix transcriptional repressor Stra13 is rhythmically expressed in mouse peripheral organs. The circadian transcription of Stra13 is mediated by a response element recognized by the CLOCK-BMAL1 heterodimer and located in the proximal promoter region. CLOCK-BMAL1-dependent activation of Stra13 is strongly repressed by Cry1 and also by Stra13 itself. To determine putative Stra13 output genes, we performed microarray analyses of differential gene expression in the liver between wild type and Stra13−/− mice and identified 42 target genes including a subset of 20 previously known as clock-controlled genes. Importantly, we demonstrate that circadian gene expression of the serum protein insulin-like growth factor-binding protein 1 and of the NKG2D receptor ligand retinoic acid early transcript was suppressed in Stra13−/− mice. These biochemical and genetic data establish a role for the basic helix-loop-helix repressor STRA13 as a circadian output regulator in the periphery.

Circadian rhythms in physiology and behavior are observed in most organisms. They are generated by a self-sustained endogenous circadian clock that is reset by external time cues such as light and temperature (1). This mechanism is believed to provide organisms with an anticipatory adaptive mechanism to the daily predictable changes in their environment. Biochemical and genetic studies in various model systems have identified a molecular oscillator generated by transcriptional/translational feedback loops. In mammals, the main loop involves the E box-mediated transcriptional activation of the Per1, Per2, Per3, Cry1, and Cry2 clock genes by the CLOCK-BMAL1 heterodimer. Then PER and CRY proteins form complexes that enter into the nucleus in a phosphorylation-dependent manner to repress the CLOCK-BMAL1-dependent transcription of their own genes, thereby generating a ~24-h period molecular oscillator (2). This loop controls also the rhythmic expression of the repressor REV-ERBα, which is required for rhythmic Bmal1 transcription, comprising a second loop thought to be important for the overall robustness of the oscillator (3). In mammals, the master oscillator is present in the suprachiasmatic nuclei (SCN) of the hypothalamus, which orchestrates autonomous oscillators in peripheral organs. Surprisingly, this oscillator can be observed in synchronized cultured cells ex vivo. The SCN oscillator is directly reset by light perceived and transmitted via the retinohypothalamic tract and is believed to entrain peripheral oscillators via ill-defined neuroendocrine pathways. Peripheral oscillators also appear to be reset by hormonal signals and the feeding schedule (4, 5). The mechanisms by which central and peripheral oscillators regulate physiological and behavioral output pathways has remained poorly understood. To address this issue, genome-wide analyses of circadian clock-controlled gene (CCG) expression in the SCN, peripheral organs, and cultured cells have recently been performed by several groups (6). Several hundred rhythmic transcripts were identified regulating a variety of key biological processes in a coordinated and tissue-specific manner (7–12). Interestingly, a significant proportion of transcriptional regulators have been found among these CCGs, suggesting that many physiological outputs may be indirectly or not exclusively controlled by the circadian oscillators in mammals as previously proposed in Drosophila (13). Accordingly, response elements for several rhythmically expressed transcription factors have been identified in the promoter region of CCGs (12). One of these clock-controlled transcription factors is Stra13 (also known as Dec1, Sharp2, or Bhlhb2), a member of the basic helix-loop-helix (bHLH) family of transcription factors, which are important regulators of cell growth, differentiation, and apoptosis. Stra13 is a transcriptional repressor that is up-
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MATERIALS AND METHODS

Animals—Eight-week-old C57BL/6J mice were purchased at IFFA-Credo (Lyon, France), housed in a 12 h light and 12 h of dark cycle (LD12:12) with the lights on (defined as Zeitgeber time (ZT) 0) at 7:00 a.m. in a temperature- and humidity-controlled environment, and fed ad libitum. After 2 weeks in LD 12:12, the mice were transferred to constant darkness (DD) and sacrificed at ZT0 or ZT12, according to the red dim light at various circadian times (CT). The tissues were collected, rapidly frozen in liquid nitrogen, and stored at −80 °C. Strain−/− mice in the C57BL/6J-Dv129 background have been described and were kept under the same conditions as above (16). Littermates were used as controls. The animal procedures were in accordance with Mount Sinai Institutional animal guidelines.

Plasmid Constructs—cDNA fragments for mouse StrA13 (NM_011498), Clock (NM_008341), Per1 (NM_011066), Per2 (NM_016974), Bmal1 (NM_008340), and Igfbp1 (NM_007489), containing pGL2 reporter plasmid. The following oligonucleotides were selected, rapidly frozen in liquid nitrogen, and stored at −80 °C. Strain−/− mice in the C57BL/6J-Dv129 background have been described and were kept under the same conditions as above (16). Littermates were used as controls. The animal procedures were in accordance with Mount Sinai Institutional animal guidelines.

 Constitutive expression vectors for CLOCK and BMAL1, the respective open reading frames were PCR amplified from pBKS-Clock (17) and pCR2-Bmal1 (18) using the following primers: 5′-TAATCCACCATGGTGTTTAC-3′ and 5′-GATCTC-GTGGCACTGCTTCC-3′ (Clock); 5′-GATGGTGAGTATGAGAAGGGAC-3′ and 5′-GATACCTTTCTCGGAGATGGATGTTTAC-3′ (Bmal1); 5′-GCCGGCTCACTGCTCAGGACCA-3′ and 5′-GATGGTCCTGCGAGACTGAGAAGGGAC-3′ (Clock); 5′-GATCTGGTGAGTATGAGAAGGGAC-3′ and 5′-GATACCTTTCTCGGAGATGGATGTTTAC-3′.

To construct expression vectors for STRA13 and STRA13, the respective open reading frames were amplified from pBKS-Clock (17) and pCR2-Bmal1 (18) using the following primers: 5′-TAATCCACCATGGTGTTTAC-3′ and 5′-GATCTC-GTGGCACTGCTTCC-3′ (Clock); 5′-GATGGTGAGTATGAGAAGGGAC-3′ and 5′-GATACCTTTCTCGGAGATGGATGTTTAC-3′ (Bmal1); 5′-GCCGGCTCACTGCTCAGGACCA-3′ and 5′-GATGGTCCTGCGAGACTGAGAAGGGAC-3′ (Clock); 5′-GATCTGGTGAGTATGAGAAGGGAC-3′ and 5′-GATACCTTTCTCGGAGATGGATGTTTAC-3′.

Western Blotting—Western blot analysis was performed according to standard procedures using 50 µg of liver nuclear extract from mice kept in DD conditions. STRA13 was detected using a specific affinity-purified rabbit polyclonal antibody (Sigma GenoSys) that was raised against a C-terminal peptide2 and used at a dilution of 1:100 followed by chemiluminescence detection according to the manufacturer’s recommendations (Amersham Biosciences). Equal loading was checked by reprobing the membrane with an EF-1α polyclonal antibody (Upstate Biotechnology Inc.). The signals were quantified with ImageQuant software (Molecular Dynamics).

Cell Culture and Transient Transfections—NIH 3T3 cells and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37 °C in 5% CO2. NIH 3T3 cells were serum-starved as described (20). COS-7 cells were seeded at a density of 105 cells/well in 12-well multidishes. On the following day, the cells were cotransfected with reporter vector (40 ng) and expression vector (200 ng) for ligand (for luciferase assays or for an epitope tag and used at a dilution of 1:100 followed by chemiluminescence detection according to the manufacturer’s recommendations (Amersham Biosciences). The cells were then incubated for 48 h in fresh medium and lysed in 50 mM Tris, pH 7.8, 1 mM dithiothreitol, 0.1% Triton X-100 lysis buffer, and luciferase assays were performed using a Trilux luminometer (Wallac). The activities were normalized to the total protein concentrations determined with the Bradford method.

DNA Microarrays Experiments—For each genotype, the livers from two (C57BL/6) or three (C121) animals were dissected, and total RNA was prepared. cDNA synthesis, biotin labeling of cDNA, and hybridization to murine U74Av2 Genechip (one chip/sample) were performed according to the recommendations of the manufacturer (Affymetrix). The .cel files were censored using the robust multiarray average algorithm (21). To identify differentially expressed genes, we used the significance analysis of microarrays (SAM) method with a maximal false discovery rate of 20% and a fold change of at least 1.5 for genes called significant by SAM (22). Fold change was calculated as the mean from two (C57BL/6) or three (C121) knockout animals/mean from two (C57BL/6) or three (C121) wild type animals. Functional annotation was done using NetAffx (www.affymetrix.com/index/index.affx; released August 2003). The resulting data sets were compared with publicly available liver data sets of rhythmic transcripts (7, 9–12).

RESULTS

Circadian Expression of StrA13 in Peripheral Tissues—The StrA13 gene is expressed in many embryonic and adult tissues with high levels in the liver, kidney, and lung (14). Here we show using a quantitative RNA protection assay that StrA13 mRNA exhibits a robust rhythmic expression pattern in the liver of mice kept in a LD12:12 cycle, with a −5-fold amplitude and peak level at ZT12 and ZT0, respectively (Fig. 1A). Interestingly, this rhythmic expression pattern was also observed in zebrafish liver, suggesting that StrA13 oscillating expression plays a physiologically important role in the vertebrate circadian system (data not shown). Under constant darkness conditions a similar circadian expression pattern was observed in liver as well as in several other peripheral tissues.
including the heart, kidney, and lung, indicating that Stra13 cyclic expression is driven by endogenous circadian oscillators in these organs (Fig. 1B). A 2-h serum shock of cultured cells followed by serum starvation has previously been shown to trigger circadian gene expression in vitro (20). This treatment was also able to induce Stra13 oscillating expression in mouse NIH 3T3 fibroblasts (Fig. 1C). Western blot analysis of STRA13 protein expression in mouse liver nuclear extracts showed a circadian pattern consistent with the mRNA expression profile with a peak at CT12 (Fig. 1D). Together these expression data
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indicate that Stra13 is under a circadian regulation in mouse peripheral tissues, in accordance with two recent microarray analyses of the liver gene expression (10, 12). Notably the peak of Stra13 mRNA expression corresponds to the known maximal activity of the CLOCK-BMAL1 heterodimer in liver, and Stra13 mRNA levels are depressed in the livers of Clock/Clock mice (10).

The CLOCK-BMAL1 Heterodimer Activates the Stra13 Promoter—The components of the positive limb of the main feedback loop of the circadian oscillator, CLOCK and BMAL1, have been shown to directly regulate the expression of several clock genes and CCGs through E box elements generally localized in the proximal promoter region or the first intron (23–25). Four such E box sequences were identified within the proximal 5′-flanking region of the mouse Stra13 gene, two of them (E3 and E4) being also conserved in the puffer fish (fugu) and human promoters (Fig. 2A). When a luciferase reporter construct containing the mouse Stra13 proximal promoter region (Sstra13Δ595::Luc construct) and expression vectors for CLOCK and BMAL1 were cotransfected in COS-7 cells, a significant (∼3-fold) induction over basal activity level was observed (Fig. 2B). Deletion constructs lacking either part of the E box element E4 (Sstra13Δ540::Luc construct) or both E box elements E3 and E4 (Sstra13Δ312::Luc construct) were unresponsive to CLOCK-BMAL1 (Fig. 2B), demonstrating that element E4 is critical for CLOCK-BMAL1 responsiveness. Interestingly the region including elements E3 and E4 had a configuration very close to that recently defined as being optimal for mediating rhythmic transcription, a perfect class B E box followed by a divergent E box (Fig. 2A) (26). To test whether this composite element was sufficient to confer responsiveness to the CLOCK-BMAL1 heterodimer, we cloned this sequence in triplicate in front of minimal promoter driving the luciferase reporter gene. This construct was strongly stimulated by the CLOCK-BMAL1 heterodimer, whereas a mutated version was inactive (Fig. 2C). As expected this activation could be totally inhibited by coexpressing CRY1, a strong repressor of the negative limb of the circadian oscillator (27). Interestingly, cotransfection of a STRA13 expression vector also resulted in a potent repression of CLOCK-BMAL1-dependent activation as previously described for the Per1 promoter (28). These functional data provide a direct and simple mechanism for the rhythmic transcriptional regulation of Stra13 by circadian oscillators and furthermore suggest a role for Stra13 in negative autoregulation of this mechanism.

Stra13 Is Not Required for Normal Circadian Oscillator Activity—The transcriptional activators CLOCK and BMAL1 contain bHLH DNA-binding domains also present in the STRA13 repressor protein. Consequently, the role of STRA13 in the circadian system could be to negatively regulate the circadian oscillator through DNA binding interference with the CLOCK-BMAL1 heterodimer as previously suggested from in vitro studies (28). To genetically test this assumption, we analyzed wild type and STRA13-deficient mice kept in constant darkness for the expression of Per2, Per3, Rev-erba, and Dbp whose transcription is known to be under the control of the CLOCK-BMAL1 heterodimer (3, 25, 29). The liver circadian expression profiles of these four genes were nearly identical in mice from both genotypes (Fig. 3A). Consistently, expression patterns of Bmal1 and Clock, which are targets of Rev-erba, were not altered in Stra13−/− mutant mice. These data suggest that Stra13 is not a critical regulator of peripheral circadian oscillators. Alternatively the disruption of Stra13 in mutant mice may be compensated by its paralogs Sharp-1 (also known as Dec2), which exhibits a robust circadian expression pattern in the rat SCN and whose promoter is down-regulated in vitro by STRA13 in human cells (28, 30). Analysis of Sharp-1 mRNA expression in the liver from wild type mice kept in DD showed weaker expression, oscillating with a peak at CT8–CT12 (Fig. 3B and C). Expression levels of Sharp-1 were increased by ∼2-fold at all time points in Stra13−/− mutant animals, consistent with a role for Stra13 in Sharp-1 expression (30). We conclude from these observations that Sharp-1 is a Stra13 target gene in vivo and that Stra13 is not required for circadian oscillator activity in the presence of Sharp-1.

STRA13 Regulates a Subset of CCGs in Liver—To further investigate the physiological role of Stra13 in the mammalian circadian clock, we sought to identify circadianly expressed STRA13 target genes. To this end, we performed a microarray analysis of liver gene expression at CT4 and CT12 in wild type and Stra13−/− mice using DNA GeneChips (Affymetrix) and compared the resulting data sets with previously established lists of rhythmic transcripts in liver. The CT4 time point was selected to minimize the possible redundant activity of Sharp-1 as Sharp-1 expression level remained low while the STRA13 protein was present at CT4 (Figs. 1D and 3C). CT12 was analyzed as the point of maximum STRA13 protein accumulation (Fig. 1D). Using the recently developed robust multarray average algorithm for normalization followed by a SAM statistical procedure for the detection of differentially expressed genes in mutant versus wild type mice, we identified 42 STRA13 target genes showing at least a 1.5-fold change in expression, of which 20 were known as CCGs in liver (Table I) (7, 9–12). Notably a majority (66%) of the identified targets were down-regulated in Stra13−/− mice, suggesting that indirect mechanisms are often involved in STRA13-dependent transcriptional regulation. Another general observation is that no clock component was present in both data sets, confirming our RPA analysis data (Fig. 3A). Putative STRA13 target genes (26 genes) are involved in several key biological processes including metabolism, detoxification, serum protein production, cell growth, immunity, and proteolysis. With no exception, each of these functional clusters contained one or several CCGs.

De novo cholesterol biosynthesis is a key liver metabolic function, and the rate-limiting step in this pathway is controlled by Hmgcr (3-hydroxy-3-methylglutaryl-CoA reductase) that converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate. Hmgcr activity and gene expression are clock-regulated (10, 51 1). Hmgcr was found to be down-regulated in Stra13−/− mice, suggesting a role for Stra13 in cholesterol metabolism. Regulating the balance between branched chain amino acid catabolism and utilization for protein synthesis as essential amino acids is another important liver metabolic function. We found that the E1β subunit (Bckdhb) of the large mitochondrial multimeric enzymatic complex regulating this process is clock-controlled and up-regulated in Stra13−/− mice.

Detoxification and steroid catabolism are essential hepatic functions that are under circadian control through the rhythmic transcription of cytochromes P-450 and phase II enzymes (UDP-glucuronosyl transferases and glutathione S-transferases) (7, 9–12). STRA13 was found to regulate three circadianly expressed genes coding for such detoxifying enzymes at CT4, Cyp2c70 (cytochrome 2c70), Cyp2a4 (steroid 15 α-hydroxylase), and Gstt2 (glutathione S-transferase theta 2). Interestingly, Alas1 (δ-aminolevulinic acid synthase 1), which catalyzes the rate-limiting step in the biosynthesis of heme that is required for cytochrome P-450 function, is also a CCG that was down-regulated at CT4 in Stra13−/− mice.

The liver produces many different serum proteins, some of which are under circadian regulation such as Igfbp1 (insulin-like growth factor-binding protein 1), which plays a role in cell growth and metabolism (32). We observed that Igfbp1 was
**FIG. 2.** The *Stra13* promoter is regulated by circadian oscillator components. A, schematic of the mouse *Stra13* proximal promoter region. Boxes E1–E4 denote E box response elements. The black box denotes a perfect class b E box sequence (CACGTG), and the white boxes denote imperfect E box sequences. Sequences of the mouse, human, and puffer fish (fugu) E3 and E4 elements illustrate the evolutionary conservation of these sequences in vertebrates. B, transient cotransfection of COS-7 cells with luciferase reporter constructs driven by either the full-length sequence (*Stra13Δ595::Luc*) or 5' deletions (*Stra13Δ540::Luc* and *Stra13Δ312::Luc*) of the *Stra13* proximal together with the empty pcDNA or Clock and Bmal1 expression vectors. C, transient cotransfection of COS-7 cells with luciferase reporter constructs containing a minimal promoter driven by either wild type (*Stra13(E3E4)3x::Luc*) or mutated (*Stra13(E3E4)m3x::Luc*) E3 and E4 box elements together with empty pcDNA or the indicated combination of Clock, Bmal1, Cry1, and *Stra13* expression vectors. The values are normalized as the means ± S.D. of at least three independent transfections.
significantly down-regulated in Stra13−/− mice at CT4, suggesting an indirect role for Stra13 for its normal expression.

The immune system is known to show circadian variations at different levels, and a number of CCGs involved in the immune response have been recently identified in Drosophila and mammals (9–12, 33, 34). Because STRA13 is required for normal immune function as a key regulator of T lymphocyte activation, we investigated the expression of several genes involved in immune function (16). Our data show that the CCG retinoic acid early transcript (Raet1c), which encodes a membrane bound protein that functions as a ligand for the activating NKG2D receptor from NK and T lymphocyte cells (35), was down-regulated in Stra13−/− mice at both CT4 and CT12. Interestingly, Ctss (cathepsin S), a cysteine protease recently shown to be involved in antigen processing (36), is clock-controlled with a phase similar to that of Raet1c and down-regulated in Stra13−/− mice at CT4, suggesting a coregulation of these two functionally related genes by STRA13.

A direct link between circadian gene regulation and cell division in liver has been recently provided by the observation that liver regeneration is retarded in cryptochrome-deficient mice (37). Because STRA13 is a known regulator of cell growth and differentiation (14, 15), we investigated the expression changes of genes involved in these processes in Stra13-deficient mice. This analysis revealed a down-regulation of the epidermal growth factor receptor Erbb3 in Stra13−/− mice, suggesting a pathway through which STRA13 may play a role in the clock-dependent control of cell growth.

Finally, expression of two CCG involved in the regulation of transmethylation (S-adenosylhomocysteine hydrolase) and in

![Figure 3](image.png)

**FIG. 3. Normal circadian oscillator function and up-regulation of Sharp-1 in Stra13−/− mice.** A, RPA analysis of Per2, Per3, Clock, Bmal1, Rev-erbα, and Dbp mRNA expression in the liver of wild type and Stra13−/− mice kept in DD conditions. A representative of experiment is shown. B, RPA analysis of Sharp-1 mRNA expression in the liver of wild type and Stra13−/− mice kept in DD conditions. C, quantification of the Sharp-1 expression data shown in B. The data are normalized as the means ± S.D. from two or three different animals of each genotype. The black and gray bars represent the subjective night and day, respectively. Yeast (Y) RNA was included as a negative control and the constitutively expressed 36B4 mRNA was used for normalization.
RNA binding (RNA-binding motif 3) was found to be lower in Stra13−/− mice, suggesting that these genes may be indirect targets of this transrepressor.

To independently determine the importance of STRA13 in the regulation of these subset of CCGs, we analyzed the liver targets of this transrepressor. A high amplitude oscillation of Alas1 and Cyp2a4 mRNA with peak and trough values at CT12–CT16 and CT0–CT4, respectively, was observed in control animals (Fig. 4, C and D). The lack of STRA13 in mutant mice resulted in elevated trough levels (CT0–CT4) and normal peak levels, indicating that STRA13 is a repressor of Alas1 and Cyp2a4, contributing to the high amplitude rhythmic expression of these two target genes (Fig. 4, C and D). Together these data show that STRA13 is an important regulator of a subset of CCGs in mouse liver.

DISCUSSION

The genetic control of circadian behavior and physiology in mammals involves the rhythmic transcription of hundreds of
circadian output genes in the SCN and peripheral organs (7, 10–12). How central and peripheral circadian oscillators orchestrate the regulation of this extensive gene network is therefore an important issue for the understanding of the mammalian circadian system. Recent work in Drosophila showed that only 9 of 128 CCGs were under the direct control of dCLOCK (13). Along this line, only a small number of CCGs in mammals were found to contain conserved canonical E box elements within their proximal promoter regions (10). This suggests that a majority of CCGs are indirectly regulated by circadian oscillators most likely via transcriptional cascades. This was further supported by the identification of more than 50 transcription factors with a circadian expression pattern in different mouse tissues (6). In a given tissue, these rhythmically expressed transcription factors oscillate with different phases, thus allowing clock genes to coordinate through these regulators a large repertoire of clock-controlled outputs throughout the 24-h cycle.

In this report, we have analyzed the mechanism and the physiological role of the circadian regulation of Strata13, a bHLH transcriptional repressor expressed in numerous peripheral tissues and playing a critical role in cell growth arrest (14–16). Our expression and biochemical data, together with the previous observation that Strata13 was down-regulated in Clock/Clock mutant mice, strongly suggest that Strata13 is a direct transcriptional output of peripheral circadian oscillators (10). Strata13 has also been shown to be up-regulated by retinoic acid, cAMP, transforming growth factor-β, prostaglandin E2, serum starvation, and hypoxia (14, 15, 38–41). Strata13 appears therefore to be the target of multiple signaling pathways that may cooperate or interfere depending on the physiological context and time. For instance, retinoic acid, which was recently shown to reset peripheral oscillators in the vascular system (42), could also modulate specific CCGs through its potent stimulatory effect on Strata13 expression.

To get insight into the physiological role of STRA13, we hypothesized that this bHLH transcription factor could regulate either the molecular oscillator itself or downstream physiological outputs or both. Our extensive analysis of clock gene expression in Strata13−/− mouse liver demonstrated that STRA13 was dispensable for normal circadian clock function. Consistent with this observation, no significant behavioral or SCN gene expression phenotypes were observed in mutant mice.3 Based on expression in the rat SCN, biochemical, and light response experiments, Strata13 and its paralog Sharp-1 were recently proposed to be core components of circadian oscillators, and both genes were also shown to be mutually regulated in vitro (28, 30, 43). Our expression data showing that Sharp-1 is a target of Strata13 in vivo suggest that the absence of phenotype at the peripheral oscillator level may be caused by redundancy between Strata13 and Sharp-1. The development of double mutant mice should help to clarify this issue. Nevertheless, the identification in liver of CCGs involved in various physiological processes such as metabolism, detoxification, cell growth, immune response, and proteolysis or of

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**Fig. 4. Altered clock-controlled gene expression in Strata13−/− mice.** Analysis of Igfbp1 (A), Rae1c (B), Alas1 (C), and Cyp2a4 (D) mRNA circadian expression in the liver of wild type and Strata13−/− mice kept in DD conditions. RPA was used for Igfbp1 and Alas1 and real time PCR for Rae1c and Cyp2a4. The constitutively expressed 36B4 mRNA was used for normalization. The data are normalized as the means ± S.D. from two or three different animals of each genotype. The asterisk indicates statistically significant difference between mean values of Strata13−/− versus wild type mice, with a Student t test p value <0.05; some error bars are too small to be visible. The black and gray bars represent the subjective night and day, respectively.
unknown function among a total of 42 STRA13 target genes indicates that this redundancy may only be partial or specific to clock genes. Half of these putative STRA13 target genes are also cycling in liver, indicating that a principle function of STRA13 is circadian transcriptional regulation. Interestingly, STRA13-regulated CCGs did not cluster into a single functional group but were instead involved in different biological processes in analogy to what was observed at the genomic scale (10–12).

This diversity is exemplified by the finding that altered circadian expression was further confirmed by *Igfbp1*, *Raet1c*, *Alas1*, and *Cyp2a4*. Surprisingly, a majority of these targets were elevated in *stra13−/−* animals including *Igfbp1* and *Raet1c* were elevated in *stra13−/−*. Because STRA13 is a transcriptional repressor, we postulate that indirect mechanisms are likely responsible. For example, *stra13* may repress transcription of other repressor proteins such that its removal results in indirect transcriptional activation. Consistent with this hypothesis, we find *Sharp-1/DE2C2* mRNA levels elevated in *stra13−/−* mice. However, the similarity of phase and biochemical properties between *stra13* and *Sharp-1/DE2C2* suggests that this putative mechanism may operate through an unidentified factor.

Furthermore, our results suggest a role for *stra13* in mediating important circadian regulated physiological outputs in liver. For example, IGF-binding protein 1 is a liver-specific secreted protein that belongs to a family of six binding proteins that are important modulators of insulin-like growth factor I and II (IGF-1 and IGF-2) biological activity while having also intrinsic activities independent of IGF receptor signaling (32). Circadian and antiphase oscillations of serum-free IGF-1 and IGF-binding protein 1 concentrations have been described in humans, whereas the total IGF-1 level was found unchanged over time (44, 45). Notably, besides its effects on somatic growth, an important biological effect of IGF-1 is to decrease circulating glucose levels, suggesting a possible role for the STRA13-dependent up-regulation of *Igfbp1* at CT0–CT4 in the moderation of hypoglycemia during the resting phase by decreasing IGF-1 bioavailability. In addition, our data also show that STRA13 is a critical positive regulator of *Raet1c* circadian expression. *Raet1c* is a member of a family of five genes (*Raet1 a-e*) that encode stimulating NKG2D receptor ligands (35). These membrane proteins have been shown to be mainly expressed in tumor cells, and they are believed to play a major role in tumor surveillance (46). Our data provide experimental evidence that STRA13 is a potent regulator of important components of the innate immune system in the liver. We speculate that it may be advantageous for the liver to express NKG2D ligands to suppress the growth or survival of cells that may become tumoral as hepatocytes are regularly exposed to damaging agents during the feeding phase.

*Alas1* and *Cyp2a4* are two target genes whose circadian pattern of expression was specifically altered between the peak (CT12) and trough (CT4) levels. In terms of gene regulation, this suggests that these functionally related genes are regulated in a coordinated manner by several circadian transcription factors acting negatively and positively to achieve a high amplitude oscillation. This assumption is supported by recent data showing that *Alas1* and *Cyp2a4* are respectively the target genes whose combinatorial actions with positive regulators such as CAR and DBP determine the observed composite circadian expression profile. Physiologically, the requirement for a high amplitude circadian oscillation of *Alas1* may be a mechanism by which the cellular metabolism reduces the deleterious effects of β-aminolevulinic acid and heme during the resting phase while providing higher levels when liver metabolism is fully activated. In line with this hypothesis, *Alas1* was also found to oscillate in *Drosophila*, a diurnal species, with a peak preceding the activity phase (34). Because of the potential toxicity of β-aminolevulinic acid and heme as a pro-oxidant and iron chelator, respectively, we hypothesize that the up-regulation of *Alas1* exclusively at CT0–CT4 in *stra13−/−* deficient liver suggests that it may be advantageous for the liver to express NKG2D receptor ligands to suppress the growth or survival of cells that may repress transcription of NKG2D receptor ligands while maximizing the bioavailability for these steroid hormones during the resting phase.

In conclusion this present work shows that the bHLH transcriptional repressor *stra13* is a transcriptional output of peripheral circadian oscillators regulating a subset of CCGs. Previously, *Dbp*, a leucine zipper transcription factor, was shown to be an output of circadian oscillators and to control the circadian regulation of three liver P-450 cytochromes *Cyp7a* (cholesterol 7-α-hydroxylase), *Cyp2a4* (steroid 15 α-hydroxylase), and *Cyp2a5* (coumarin 7-hydroxylase) (25, 48, 50). Collectively, these observations support a circadian gene regulatory network in which clock-controlled genes rather than bona fide clock components initiate transcriptional cascades that underlie observed circadian physiological outputs.

Acknowledgments—We are grateful to Drs. P. Chamblon, M. Ikeda, J. S. Takahashi, and A. Yasiu for the generous gift of reagents, to Dr. F. Girardot for help with bioinformatics, and to Dr. B. Rayet for critical reading.

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