Gene Model 129 (Gm129) Encodes a Novel Transcriptional Repressor That Modulates Circadian Gene Expression*

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Background: Circadian gene expression in mammals is driven by rhythmic CLOCK-BMAL1 activities.

Results: Gm129 binds to the CLOCK-BMAL1 complex on DNA and represses its transcriptional activator activity to regulate the circadian gene expression phase.

Conclusion: Gm129 is a novel circadian clock modulator.

Significance: The discovery of Gm129 reveals a new regulatory component of circadian gene expression.

The mammalian circadian clock is a molecular oscillator composed of a feedback loop that involves transcriptional activators CLOCK and BMAL1, and repressors Cryptochrome (CRY) and Period (PER). Here we show that a direct CLOCK-BMAL1 target gene, Gm129, is a novel regulator of the feedback loop. ChIP analysis revealed that the CLOCK-BMAL1-CRY1 complex strongly occupies the promoter region of Gm129. Both mRNA and protein levels of GM129 exhibit high amplitude circadian oscillations in mouse liver, and Gm129 gene encodes a nuclear-localized protein that directly interacts with BMAL1 and represses CLOCK-BMAL1 activity. In vitro and in vivo protein-DNA interaction results demonstrate that, like CRY1, GM129 functions as a repressor by binding to the CLOCK-BMAL1 complex on DNA. Although Gm129−/− or Cry1−/− Gm129−/− mice retain a robust circadian rhythm, the peaks of Nr1d1 and Dbp mRNAs in liver exhibit a significant phase delay compared with control. Our results suggest that, in addition to CRYs and PERs, the GM129 protein contributes to the transcriptional feedback loop by modulating CLOCK-BMAL1 activity as a transcriptional repressor.

Circadian clocks are endogenous timekeeping systems that govern the daily cycles of behavior and physiology in a variety of organisms (1, 2). In mammals, the 24-h circadian oscillator operates as a transcription-translation feedback loop involving both positive (transcriptional activators) and negative (transcriptional repressors) components. The transcriptional activators are CLOCK/NPAS2 and BMAL1, whereas Cryptochrome (CRY)3 (CRY1 and CRY2) and Period (PER1 and PER2) act as transcriptional repressors. In the transcription-translation feedback loop model, CLOCK-BMAL1 heterodimers bind to the promoter region of Per and Cry genes through E-box DNA regulatory sites and activate transcription. Upon transcriptional activation, gradually accumulated CRY and PER proteins abrogate their own transcription by repressing the activity of CLOCK-BMAL1 (3, 4). This feedback loop repeats every ~24 h and results in rhythmic activities of the CLOCK-BMAL1 complex. Besides the promoters of Cry and Per genes, CLOCK-BMAL1 also binds to E-box elements in the promoter regions of thousands of clock-controlled genes, including transcription factors, such as the nuclear receptor Rev-erba (Nr1d1), and the PAR domain bZIP transcription factors Dbp (12). These CLOCK-BMAL1 controlled transcription factors also exhibit high amplitude oscillation at the protein level in tissues. Thus, the CLOCK-BMAL1 complex and clock-controlled transcription factors drive massive transcriptional oscillation in various tissues in mammals (5).

As described above, the interactions between CLOCK-BMAL1 and E-box elements play a major role in circadian transcriptional regulation. To understand the direct targets of the CLOCK-BMAL1 complex and the molecular architecture of the circadian timing system, several studies have been conducted to identify the CLOCK-BMAL1 DNA-binding sites in vivo using ChIP-Seq analysis (6, 7). It was reported that, in liver, BMAL1 rhythmically bound to ~2000 genomic targets (7). Based on these ChIP-Seq results, direct CLOCK-BMAL1 target genes were predicted and were ranked according to their binding strength. Interestingly, among the target genes with the highest BMAL1 chromatin binding strength, most are well studied circadian genes. The top 10 CLOCK-BMAL1 target genes include three core clock gene Per1, Per2, and Cry2 (8, 9); three nuclear receptors Rorc, Nr1d1, and Nr1d2 involved in the core clock regulation (10, 11), and three PAR domain bZIP transcription factors Dbp, Hlf, and Tef, which function as key genes in the output pathways (12). Only one gene remains uncharacterized, gene model 129 (Gm129), which encodes a novel protein with no predicted functional domains. Gm129 is also implicated as a direct CLOCK-BMAL1 target gene based upon its strong transcriptional oscillation in liver in a additional independent BMAL1 ChIP-seq analysis using NIH3T3 and WI38 cell lines (6).

Coincidently, a ChIP-seq and gene expression study in our group also found that Gm129 is a direct target gene of the
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BMAL1-CLOCK-CRY1 complex, and Gm129 exhibits high amplitude protein oscillation in mouse liver. These properties suggest that Gm129 might be an important factor in the molecular clock. Thus, we have conducted detailed molecular, biochemical, and genetic analysis to characterize the role of Gm129 in circadian clock regulation. In this study, we demonstrate that GM129 can directly interact with core clock proteins BMAL1 and PER2. Gm129 also localizes in the nucleus and strongly represses CLOCK-BMAL1 transcriptional activity in a reporter gene assay. Additional in vitro and in vivo assays suggest that the GM129 protein directly interacts with the CLOCK-BMAL1 complex on DNA. In mouse liver, the absence of Gm129 extends the peak expression of Nr1d1 and Dbp. More importantly, in the Cry1−/− background, the Gm129 knockout causes a significant phase shift of Nr1d1 and Dbp gene expression. We conclude that Gm129 functions as a novel transcriptional repressor in the mammalian clock transcription-translation feedback loop.

EXPERIMENTAL PROCEDURES

Mice—All animal procedures were in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill. Gm129 heterozygote mice were purchased from the University of California, Davis, KOMP repository and bred to obtain Gm129−/− mice. Primers were designed as indicated (Fig. 5A and Table 1) for genotyping. Cry1−/−, Gm129−/− double knock-out mice were generated by crossing Cry1−/− (9) and Gm129−/− mice. All mice were maintained on a 12-h light/dark schedule.

Plasmids—To generate pEGFPN1-Gm129, the Gm129 coding sequence was amplified by PCR from mouse fibroblast cDNA and inserted into the pEGFP-N1 vector. The pBABEpuro-Flag-HA-Gm129 plasmid was generated by inserting Gm129 and the FLAG tag sequence into the pBABEpuro vector backbone. The pFast-Flag-Gm129-Myc construct was generated by inserting the Gm129 coding sequence into the pFastbac1 (Invitrogen) vector.

Cell Lines—NIH3T3 cells expressing FLAG-GM129 were made by retrovirus infection. Briefly, the pBABEpuro-Flag-HA-Gm129 construct was co-transfected together with pVSVG and pCIHPZ into HEK293T to produce recombinant retrovirus particles. Then MEF cells were infected with the retrovirus and transfectants were selected in media containing puromycin for 2 weeks. Single colonies were picked and cultured for subsequent analysis. The Flp-In/FLAG-C1ORF51 cell line was generated according to the manufacturer’s protocols using the Flp-In T-REx-293 system (Invitrogen).

Immunoblotting and Antibodies—Both mouse liver lysates and liver nuclear extracts were prepared for immunoblotting. To make mouse liver lysate, mouse liver samples were homogenized and lysed with lysis buffer (15 mM HEPES, pH 7.8, 250 mM NaCl, 1% Nonidet P-40, 10% glycerol, protease inhibitor mixture, from Roche Applied Science). Proteins from mouse liver nuclei were prepared according to the NU procedure described previously (14). Anti-FLAG antibody was purchased from Sigma. Anti-CLOCK, anti-BMAL1 antibodies (Bethyl Lab), and anti-mPER2 antibody (ADL and anti-Gm129 (Santa Cruz Biotechnology) were used in the immunoblotting assays. Anti-mCRY antibody was produced in our laboratory (13).

Immunoprecipitation—Insect cells or mammalian cells were collected and lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Tween-20, 0.1% Nonidet P-40, protease inhibitor mixture, Roche Applied Science). Lysates were incubated with anti-FLAG M2-agarose beads (Sigma) for 4 h at 4 °C. Beads were washed 4 times with lysis buffer; bound proteins were eluted in 2× SDS-sample buffer and analyzed by immunoblotting.

Fluorescence Microscopy—For GFP fluorescence microscopy, HEK293 T cells were cultured on poly-d-lysine 12-mm coverslips (BD Biosciences) placed in 6-well plates. Cells were transfected with pEGFP-N1 and pEGFPN1-Gm129 plasmids using Lipofectamine 2000 reagent (Invitrogen) at around 50% confluence. 48 h after transfection, cells were washed twice with PBS, fixed immediately with 3.7% methanol-free formaldehyde in PBS for 15 min, and washed twice with PBS. Coverslips were mounted with SlowFade Gold Antifade Reagent with DAPI (Invitrogen). Images were captured using a LeicaSP2 confocal microscope.

Chromatin Immunoprecipitation (ChIP) and ChIP Sequencing—Primers used in ChIP-PCR are given in Table 1. ChIP-PCR assays using cell nuclear extracts were conducted as described previously (13). For ChIP-PCR and ChIP-Seq using mouse tissues, liver chromatin was prepared according to a previously published protocol (15) with minor modifications. Briefly, livers from mice were immediately homogenized and cross-linked in PBS buffer including 1% formaldehyde (5 ml/liver), and the homogenate was incubated for 15 min at 25 °C. Cross-linking reactions were stopped by the addition of 21 ml of ice-cold nuclei buffer (2.2 mM sucrose, 125 mM glycine, 10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT, 0.5 mM PMSF, and 10% glycerol). The homogenate was layered on top of 10 ml of cold nuclei buffer and centrifuged for 90 min at 24,000 rpm (100,000 × g) at 4 °C in a Beckmann SW27 rotor. The nuclei were washed with wash buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA) and stored at −80 °C. Cross-linked nuclei were then subjected to ChIP analysis as previously described (13). For ChIP-seq, ChIP DNA was ligated to single-end sequencing adapters and amplified. DNA fragments of 300–800 bp in size were purified and sequenced (50 base) on an Illumina Hi-seq2000 instrument (Illumina, CA). The reads were aligned to the mm9 mouse reference genome using Bowtie accepting up to 4 reportable alignments and two mismatches per read (16). In cases where more than a single alignment was possible, only the one with the highest mapping quality was reported using the “−best” option of Bowtie. To generate ChIP-seq signal tracks, reads were extended in silico to 150 bp, and the number of reads overlapping with each base in the genome were counted and divided by the chromosome-wide average of per-base read coverage. The average signal over 50 bp windows was calculated and viewed using the University of California Santa Cruz (UCSC) genome browser (Fig. 1).
Reporter Gene Assay—Firefly luciferase was used as a reporter controlled by the mPer1 promoter sequence (17). Renilla luciferase was used as a transfection control. HEK 293T cells were co-transfected with the indicated amounts of plasmids using Lipofectamine 2000 (Invitrogen). 36 h post-transfection, cells were collected, and luciferase activities were assayed using the Dual-Luciferase® Reporter Assay System according to the manufacturer’s protocols (Promega).

Pulldown Assay with Immobilized DNA—Biotin-labeled E-box DNA or mutated E-box DNA (Table 1) was immobilized on magnetic streptavidin beads. Immobilized DNA was washed twice in reaction buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl,

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**TABLE 1**

Primer sets used for ChIP assay

| Name            | Sequence                     | Reference               |
|-----------------|------------------------------|-------------------------|
| mPer2E2F        | GGTTCGCCCCCGCCAGTATGC       | Yoo SH et al. PNAS(2005) |
| mPer2E2R        | CCGTCAGGGTGGGCTGCGGC        |                         |
| mDp5F           | TGTGACAGCTCGGTCTCCTT        |                         |
| mDp5R           | ATATTGGCAATGGGAGGA          |                         |
| mNt1d1CPF       | GCTGCTGGAAGAATGTGTCA        |                         |
| mNt1d1CPR       | ATGGAGAAATGAGGACCCAGG       |                         |
| mBmal50F        | CCGATGGTGGGGAAGAGTGAG       |                         |
| mBmal50R        | AGCCATGCGCACACTCAGC         |                         |
| hPer2CPF        | ATTGAGGAACCGCAAGGTTGAG      |                         |
| hPer2CPR        | GCCCAACAGCTGTCAGTAT         |                         |
| Gm129CPF        | CACGGCTGGAGGTGACAGAG        |                         |
| Gm129CPR        | GAAAGAGTGCGGAGTGACAGAG      |                         |

Primer sets used for Genotyping.

| Name            | Sequence                     | Reference               |
|-----------------|------------------------------|-------------------------|
| CSD-Gm129-TTR1  | CAGAACCTGCAACTGAAAGCAAAGC   |                         |
| Common-3’F      | CACACCTCCCCCTGAACCTGAAA     |                         |
| Gm129FG5        | GCA GTG CTG TCC TAT TTA AGG CTA TGA |                         |

Primer sets used for RT-PCR.

| Gene Name | Forward primer (5’-3’) | Reverse Primer (5’-3’) |
|-----------|------------------------|------------------------|
| Gm129     | ACTCAAGATGGTGGTCTTGT   | GGGCAGCTATGTGAGAAAC    |
| mGapdh    | AACTTGTGGCATAGTGGAAGG  | ACACATGGGGGTGAGAAACA   |
| mDp5      | GACACCGTGGAGGTGCTAAT   | ACCTCCGGCTCCAGTACTTC   |
| mNrt1d1   | CCTGACTCAAGGTGTGCTCCCAT | CATGGCCACCTTGAGACTTCCTG |
| mPer1     | TGAGGAGGCACAGAGGGAAAG   | GGCTGAGAGGAGCGTGTAGG   |
| mBmal1    | TCATGTGCGCCAAGACTGGA   | CAAATAGCTGTGCTGAGA     |

Oligo sets used for EMSA and DNA pulldown assay.

| Name   | Sequence       | Reference               |
|--------|----------------|-------------------------|
| M34TOP | GGGACACGTGACC  | Hogenesch et al. PNAS(1998) |
| M34BMT | GGTCAGGTGTCAC  |                         |
| E-box  | AGCGCGCCGCGGTTCAGTCTTCCACTATGTG | Yoo et al. |
| Mutated E-box | AGCGCGCGCGGTTCAGTCTTCCACTATGTG | PNAS(2005) |
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FIGURE 1. Gm129 is a nuclear protein and oscillates in mouse liver. A, circadian oscillation of Gm129 mRNA in mouse liver. Mice were sacrificed at zeitgeber times (ZT) 4, 8, 12, 16, 20, and 24/0 h. The abundance of Gm129 mRNA was determined by quantitative RT-PCR and normalized to that of Gapdh. The maximal value was normalized to 1. Error bars represent S.D. of 2 biological repeats. The 0 to 24-h time point is plotted twice to emphasize the trend. B, oscillation of GM129 and core clock protein levels in liver nuclei. Mice were kept in constant dark for 24 h following 12-h dark/12-h light cycles. After 24 h in the dark, nuclear extracts were prepared from livers collected at 0, 4, 8, 12, 16, and 20 h in dim red light and GM129 was detected by Western blots. The XPB protein was used as a loading control. The GM129 signal is marked by a red asterisk. Note there is a nonspecific antibody cross-reactive band above the GM129 signal. The blot is representative of two experiments. C, subcellular localization of GM129-EGFP fusion protein. HEK293T cells were transfected with plasmid DNA expressing GM129-EGFP protein or EGFP alone. 48 h post-transfection, cells were fixed and visualized by fluorescence microscopy (GFP) and DAPI staining. Nuclear localization was confirmed by merging the GFP signal with the DAPI signal. EGFP alone is a control that localizes to both cytoplasm and nucleus. Bar = 10 μm.

5 mM dithiothreitol, 10% glycerol, 0.01% Nonidet P-40, and 10 μg/ml of BSA) before setting up binding reactions. Reactions (200 μl) were prepared in reaction buffer with the indicated combinations of CLOCK-BMAL1 (10 nM), GM129 (10 nM), PER2 (10 nM), and CRY1 (10 nM) and 500 ng of immobilized DNA. After incubation for 1 h at 4 °C, DNA-protein complexes were collected on a magnet and washed three times with reaction buffer. The proteins associated with the DNA were analyzed by SDS-PAGE and immunoblotting.

RNA Extraction, Reverse Transcription and Quantitative Real-time RT-PCR—Mice of each genotype housed in light/dark (12:12) were sacrificed every 2 h starting from ZT 0 and livers were collected, washed with PBS, and immediately frozen in dry ice and stored in −80 °C until further use. RNA samples were prepared by TRizol reagent (Invitrogen) and RNAeasy (Qiagen). Total RNA (500 ng) was reverse transcribed with oligo(dT)20 using SuperScript III (Invitrogen) according to the (Qiagen). Total RNA (500 ng) was reverse transcribed with oligo(dT)20 using SuperScript III (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time PCR assays were performed by using an ABI 7300 System (Applied Biosystems) and Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) was used. Primers are listed in Table 1.

RESULTS

The Gm129 Gene Is a Direct Target of CLOCK-BMAL1-CRY1 and Oscillates in Mouse Liver—Previously we reported that CRY1 binds to CLOCK-BMAL1 on DNA by both in vitro EMSA and in vivo ChIP assays (13). To systematically examine binding of the native CLOCK-BMAL1-CRY1 core clock complex to CCG (clock-controlled gene) promoters in mouse tissue, we conducted ChIP assays using the liver to map the chromatin-binding sites of CLOCK, BMAL1, and CRY1. As shown in Fig. 1A, in agreement with a previously published report (18), both BMAL1 and CRY1 rhythmically bind to the Dhp and Nr1d1 promoters and reach their peak affinities at ZT6-ZT10 (for BMAL1) and ZT20-ZT24 (for CRY1) in ChIP assays. To simplify the experimental design and achieve the best signal-to-

FIGURE 2. Gm129 is a nuclear protein and oscillates in mouse liver. A, circadian oscillation of Gm129 mRNA in mouse liver. Mice were sacrificed at zeitgeber times (ZT) 4, 8, 12, 16, 20, and 24/0 h. The abundance of Gm129 mRNA was determined by quantitative RT-PCR and normalized to that of Gapdh. The maximal value was normalized to 1. Error bars represent S.D. of 2 biological repeats. The 0 to 24-h time point is plotted twice to emphasize the trend. B, oscillation of GM129 and core clock protein levels in liver nuclei. Mice were kept in constant dark for 24 h following 12-h dark/12-h light cycles. After 24 h in the dark, nuclear extracts were prepared from livers collected at 0, 4, 8, 12, 16, and 20 h in dim red light and GM129 was detected by Western blots. The XPB protein was used as a loading control. The GM129 signal is marked by a red asterisk. Note there is a nonspecific antibody cross-reactive band above the GM129 signal. The blot is representative of two experiments. C, subcellular localization of GM129-EGFP fusion protein. HEK293T cells were transfected with plasmid DNA expressing GM129-EGFP protein or EGFP alone. 48 h post-transfection, cells were fixed and visualized by fluorescence microscopy (GFP) and DAPI staining. Nuclear localization was confirmed by merging the GFP signal with the DAPI signal. EGFP alone is a control that localizes to both cytoplasm and nucleus. Bar = 10 μm.
noise ratio for identifying chromatin-binding sites of the core clock protein complex, we conducted CLOCK and BMAL1 ChIP-seq using liver harvested at ZT8 and CRY1 ChIP-seq with liver harvested at ZT20. Fig. 1B and C, show the representative samples of ChIP-seq data we obtained using anti-CLOCK, anti-BMAL1, and anti-CRY1 antibodies. As shown in Fig. 1B, CLOCK, BMAL1, and CRY1 all bind to promoters of the core clock repressive factors: Cry1, Cry2, Per1, and Per2. Besides these core components, these positive (CLOCK and BMAL1) and negative (CRY1) regulators also bind to promoters of the secondary/consolidating loop component Rev-Erbα/Nr1d1 as well as to the promoter of the clock-controlled transcription factor Dbp1.

Consistent with other published studies (6, 7, 19), our ChIP-seq results showed that the Gm129 gene has high affinity binding sites for CLOCK, BMAL1, and CRY1 in its promoter region (Fig. 1C). To further characterize this binding, we carried out ChIP-PCR assays with livers harvested at different time points to examine circadian rhythmicity of binding of the CLOCK-BMAL1-CRY1 complex to the Gm129 promoter. Fig. 1D shows ChIP-PCR data over the course of a diurnal cycle for the Gm129 gene. Clearly, both BMAL1 and CRY1 rhythmically occupy E-boxes in the Gm129 promoter with different binding phases.

It was also reported that Gm129 exhibits circadian gene expression in the mouse liver (6). To confirm these results, time series quantitative PCR was conducted with mouse livers harvested over one diurnal cycle. Indeed, Gm129 mRNA levels show very high amplitude (≈ 17-fold difference between zenith and nadir) oscillation with a peak at ZT12 (Fig. 2A). Because in some cases a rhythmically expressed gene can produce a constitutively expressed protein (20), the GM129 protein level was also examined by time series immunoblotting of mouse liver extracts made over a circadian cycle (constant dark). As shown in Fig. 2B, GM129 protein also exhibits high amplitude oscillation in the mouse liver nucleus with a peak at CT12. Thus, all these results suggest that Gm129 is a direct target of the CLOCK-BMAL1-CRY1 complex and encodes a protein with high amplitude circadian oscillation.

GM129 Protein Localizes in the Nucleus and Interacts with PER2 and BMAL1—Gm129 is a 375-amino acid protein with no analogs in the mouse genome, and it has no known protein motifs or domains to guide for a potential functional assignment. Thus, we decided to determine its subcellular localization to obtain clues about its molecular functions. To this end, we constructed a GFP fusion construct of GM129 and transfected HEK293 cells with this plasmid. We found that the GFP-GM129 protein largely localizes in the nucleus (Fig. 2C). In contrast, GFP alone exhibits both nuclear and cytoplasmic localization. Interestingly, the top 10 direct CLOCK-BMAL1 target genes with known function all encode nuclear proteins that function as either core clock components (PERs and CRYs)
or circadian transcriptional factors that are responsible for output pathways (DBP, HLF, TEF, NR1D1, and NR1D2). Unlike circadian transcription factors in output pathways, core clock proteins normally interact with each other (21, 22). Thus, to further clarify the function of GM129, we decided to test protein-protein interactions between GM129 and core clock proteins using the baculovirus expression system. As shown in Fig. 3A, GM129 has a strong affinity to BMAL1 and PER2, but not CRY1. Additionally, GM129 binds to endogenous BMAL1 in a NIH3T3 cell line that stably expresses the FLAG-GM129 protein (Fig. 3B). Hence, nuclear localization and direct interaction with core clock proteins strongly support the hypothesis that GM129 is a novel circadian regulator.

GM129 Represses CLOCK and BMAL1-induced Transcription

Although GM129 strongly binds to BMAL1 and PER2 protein, it was not known whether this interaction can regulate the activ-
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It is of the core clock protein complex. Interestingly, like Gm129, Cry1 also binds to BMAL1 and Per2 and functions as a strong repressor of CLOCK-BMAL1. Thus, we reasoned that Gm129 might function as a transcriptional regulator by interacting with BMAL1. To investigate Gm129 function, a luciferase reporter gene assay was conducted in HEK293T cells using a Per1::Luc reporter (21). Coexpression of CLOCK and BMAL1 generate >6-fold induction of Luciferase activity compared with expressing BMAL1 alone (Fig. 3C). Cry1 is a positive control in this assay that efficiently represses CLOCK-BMAL1 activity. Importantly, the additional coexpression of either Cry1 or Gm129 reduced the luciferase activity to the basal level (Fig. 3C). As shown in Fig. 3D, the repressor function of Gm129 is also dose dependent, because as the amounts of Gm129 or Cry1 plasmids transfected were increased, there was increasing inhibition of CLOCK-BMAL1-activated transcription. In comparison with Cry1, it appears that Gm129 is nearly equally efficient in repressing CLOCK-BMAL1 activity (Fig. 3D). Together with nuclear localization and protein-protein interaction results, repression of CLOCK-BMAL1-activated transcription by Gm129 suggests a role in the negative arm of the transcription-translation feedback loop.

Gm129 Interacts with the CLOCK-BMAL1 Complex on DNA Both in Vivo and in Vitro—Although Gm129 protein represses activity of the CLOCK-BMAL1 in the reporter gene assay, the detailed molecular mechanism is unclear. Generally, a protein can function as a repressor by two mechanisms: (a) the repressor can bind to the activator complex on DNA and interfere with its interaction with co-activator or with RNA polymerase II complex or (b) the repressor can directly dissociate the activator from DNA. Previously, we have reported that Cry1 binds to the CLOCK-BMAL1 complex on DNA in vitro (13). Here, we have conducted parallel experiments to examine how Gm129 functions as a repressor of CLOCK-BMAL1. The in vivo system that we used employed an NIH3T3 cell line that stably expresses FLAG-tagged Gm129. A ChiP assay was conducted with these cells to measure the binding of Gm129 to the E-box elements in the Per2, Dbp, Nrd1d1, and Bmal1 promoters. As shown in Fig. 4A, FLAG-Gm129 showed significant recruitment to the Per2, Dbp, and Nrd1d1 E-box compared with the NIH3T3 controls. No significant recruitment was detected in the Bmal1 promoter that does not contain CLOCK-BMAL1-binding sites, which suggest the recruitment is specific and CLOCK-BMAL1 dependent. Equally important, FLAG-tagged C10RF51, a human homolog of Gm129, also interacts with hBMAL1 and binds to the hPer2 promoter in HEK293R cells that stably expresses the FLAG-C10RF51 protein, which suggests that the molecular function of the GM129 protein is evolutionarily conserved (Fig. 4, B and C).

The in vivo interactions between Gm129 and the Per2 promoter could be achieved either directly through the CLOCK-BMAL1 complex or through other proteins. Here, we used a defined in vitro system to elaborate on these protein-DNA interactions. The CLOCK-BMAL1 complex and FLAG-GM129 were purified using anti-FLAG affinity chromatography and baculovirus expression system as described previously (13). Purified Gm129 is shown in Fig. 4D. Then, we investigated the effects of Gm129 on the CLOCK-BMAL1-E-box ternary complex using an electromobility shift assay (EMSA). As shown in Fig. 4E, CLOCK-BMAL1 binds to the E-box specifically (lane 2), and addition of Gm129 protein retards the mobility of the CLOCK-BMAL1-E-box complex indicating the binding of Gm129 to the ternary complex (Fig. 4E, lanes 3–5). Cry1 is a positive control that can also shift the CLOCK-BMAL1-E-box complex (lane 7) (13). Gm129 on its own does not bind to E-box under the conditions used (lane 6), indicating its binding to the ternary CLOCK-BMAL1-E-box complex through interaction with CLOCK-BMAL1.

Next, the interactions of Gm129 with CLOCK-BMAL1-E-box was tested by an in vitro DNA pulldown assay to further confirm the conclusion from the EMSA experiments. In this assay, we used a 30-bp fragment encompassing a non-canonical E-box (CACGTT, Table 1) in the mouse Per2 promoter, which was shown to bind to the CLOCK-BMAL1 complex (14, 23). Purified Gm129 and other clock proteins were incubated with either wild-type or mutated E-box DNA immobilized on streptavidin-coupled magnetic beads. The beads were washed, and the DNA-bound proteins were analyzed by Western blot as shown in Fig. 4F. Clearly, Gm129 binds strongly to the CLOCK-BMAL1 complex on wild-type E-box DNA substrate (lane 2) but not to mutated E-box (lane 3). In agreement with previously published results (13), PER2 protein (negative control) does not bind to the CLOCK-BMAL1 complex on DNA (lane 5). Cry1 (positive control) also showed strong interaction with the CLOCK-BMAL1-E-box (lane 8). Overall, these data unambiguously show that Gm129 tightly binds to CLOCK-BMAL1 on E-box DNA. Together with the reporter gene assay, our biochemical analysis suggest that Gm129 appears to repress transcription as a consequence of binding stably to the CLOCK-BMAL1-E-box complex.

Gm129 Gene Deficiency Affects the Expression of Clock-controlled Genes—If the repressor function of Gm129 is essential and cannot be compensated by other genes, deletion of Gm129 in mice would affect the circadian rhythm rhythmicity. To test this, Gm129<sup>tm1e(KOMP)Wtsi</sup> heterozygous mice (hereafter Gm129<sup>+/−</sup>) were obtained from the NIH Knock-out Mouse Project (KOMP) Repository. The targeting strategy for the knock-out is summarized in Fig. 5A (24). Gm129<sup>−−</sup> homozygous mice were obtained by mating these heterozygotes. Genotyping was done by PCR as shown in Fig. 5B. Fig. 5, C and D, show that Gm129 mRNA and protein are not expressed in knock-out mice, thus the Gm129<sup>−−</sup> is a null allele. Heterozygote intercrosses produced progeny of each genotype in the expected Mendelian frequency, indicating that homozygous animals are viable. Overall, homozygous Gm129<sup>−−</sup> mice had no gross developmental abnormalities.

To determine the effects of Gm129 disruption on behavioral rhythmicity, we monitored wheel-running activity under constant darkness (DD) for 1 week, following an initial 14 days in a 12-h light/12-h dark (LD) cycle. Although the animals appear robustly rhythmic, we were not powered to detect minor period length differences (data not shown). The circadian oscillator controls not only behavior, but also gene transcription in the SCN and peripheral tissue. Hence, it was of interest to determine the effects of Gm129 deficiency on circadian regulation of gene transcription. Thus, we analyzed the expression pattern of...
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FIGURE 5. Generation of Gm129 knock-out mice. A, a schematic representation of the WT (Gm129+/−) allele and targeted allele (Gm129−/−). Homologous recombination leads to the insertion of a 7-kb LacZ and Neomycin cassette between exon 1 and exon 2 in the WT Gm129 allele. Black lines represent introns. Black and dashed rectangles represent non-coding and coding exons, respectively. Solid line A and dashed line B locate the products of PCR genotyping. Two small arrows show the location of the primers used for RT-PCR (Table 1). B, primers were designed as indicated in Fig. 5A and Table 1 for genotyping. 984- and 662-bp PCR products were amplified using WT, heterozygote or knock-out mouse tail genomic DNA, respectively. C, Gm129 gene expression (normalized to Gapdh) in WT and Gm129−/− mice. The value of Gm129 expression in knock-out mice was set to 1. Error bars represent S.D. of 3 biological repeats. D, GM129 protein deficiency in Gm129−/− mouse liver. Liver nuclear extracts were prepared from wild-type (Gm129+/−) or homozygous Gm129−/− mutant mice at ZT12 and analyzed by SDS-PAGE followed by immunoblotting. The GM129 signal is marked by a black asterisk. XPB is a loading control.

canonical clock genes Dbp, Nr1d1, Per1, and Bmal1 in mouse liver samples collected every 2 h for 24 h. In agreement with the behavioral analysis, the rhythmic expression of these clock genes was retained in Gm129−/− mice compared with wild-type (WT) mice (Fig. 6, A and B). However, the peaks of Dbp, Nr1d1, and Per1 gene expression persists longer in the Gm129−/− mouse liver between ZT10 and ZT14. Bmal1 expression is also slightly altered at ZT16. These effects are likely caused by absence of the GM129 transcriptional repressor that exhibits a peak expression level at ZT12.

Lack of Gm129 in Cry1 Knock-out Mice Delays the Phase of Nr1d1 and Dbp Expression—Because Gm129−/− mice do not show any significant alteration in their circadian rhythm, we suspect that GM129 functions in a redundant manner with another protein in mice. It was previously reported that, except for Bmal1 (25), deficiencies of single core clock regulator genes normally do not abolish circadian rhythms. For example, Cry1−/− and Cry2−/− single knock-out mice have stable circadian rhythms with altered periods (9), but Cry−/− double knock-out mice are totally arrhythmic. In addition, Per1 and Per2 genes also have redundant roles in circadian regulation (8, 26). Therefore, we decided to combine the Gm129 mutation with another core clock gene mutation that possibly compensates for GM129 function. Although Gm129 does not have analogs in the mouse genome, our protein–protein interaction assay and reporter gene assay suggest that the Gm129 gene has a functionally redundant role with Cryptochrome as a transcriptional repressor on CLOCK-BMAL1. Hence, we decided to make Cry1−/− Gm129−/− double knock-out mice to examine the roles of Gm129 in circadian regulation. Dbp, Nr1d1, Per1, and Bmal1 rhythmic gene expression in the liver was used as readouts of the circadian rhythm. Results are shown in Fig. 6, C and D, which is aligned under and may be compared with Fig. 6, A and B. In agreement with a previous report, the Cry1 mutation alone causes an advanced phase shift (~4 h) of Dbp (18), Per1 and Nr1d1 (black curve and blue curve). Most importantly, Cry−/− Gm129−/− mice exhibit robust, but significantly phase-delayed Dbp and Nr1d1 circadian gene expression (about 2–4 h phase shift) compared with Cry−/− mice (Fig. 6C). Per1 gene expression exhibits a broader peak between ZT4 and ZT10 compared with control (Fig. 6D, left panel). These results are consistent with the finding in Gm129−/− mice, in which peak transcription of Dbp, Nr1d1, and Per1 persists longer than in WT mice between ZT12 and ZT14. Equally important, rhythmic Bmal1 expression that is under control of the NR1D1 protein (3, 10) is also affected by the Gm129 knock-out mutation in Cry1−/− mice (Fig. 6D, right panel). Thus, we reasoned that in WT mice, the GM129 phase modulator function is largely masked by Cry1 that is a more potent repressor than GM129. However, in the absence of Cry1, GM129 has a stronger role in regulating CLOCK-BMAL1 activity. Thus, our genetic analyses suggest that GM129 is a circadian clock modulator rather than an essential core component.

DISCUSSION

In the circadian transcription-translation feedback loop model, CRY and PER transcriptional repressors are essential components of the feedback loop. Besides CRY and PER, there are additional non-essential components of the core clock machinery that can repress CLOCK-BMAL1 activity. These proteins include the helix-loop-helix transcription factors, such as Differentially Expressed in Chondorocytes-1 and -2 (DEC1 and DEC2) and Inhibitor of DNA Binding 2 (ID2). These proteins can inhibit CLOCK-BMAL1 activities possibly by interfering with formation of the CLOCK-BMAL1 complex (27–29). DEC1 protein was also reported to delay the phases of Per1, Dbp, and Nr1d1 in cells after serum synchronization (30). Another non-essential repressor is CLOCK-interacting Protein, Circadian (CIPC), which can interact with CLOCK and
inhibits CLOCK/BMAL1 transcriptional activity presumably by disrupting the binding between CLOCK and its transcriptional coactivators (31). Here, we demonstrated that similar to Cryptochrome, GM129 is a novel transcriptional repressor of the CLOCK/BMAL1 protein complex that interacts with CLOCK/BMAL1 on DNA.

It was surprising to find that the effect of GM129 on CLOCK-BMAL1 activity in the reporter gene assay is almost as strong as CRY1, and that the mechanism of GM129 is similar to that of the CRYs in that it binds to CLOCK-BMAL1 E-box complexes. However, important differences must exist between the function of CRYs and GM129 because GM129 is unable to compensate for the absence of CRYs in Cry1−/−Cry2−/− double knockout mice, which are arrhythmic (9). Our results also suggest that, unlike CRY1, GM129 is a non-essential CLOCK-BMAL1 repressor that is not required for circadian rhythm generation, but functions as a modulator to influence the phase of CLOCK-BMAL1 controlled transcriptional oscillation. We noticed that, in addition to BMAL1, GM129 also interacts with PER2 in our immunoprecipitation assays. Further investigation on these protein-protein interactions might help us to understand the differences between GM129 and Cryptochromes. It is also conceivable that Gm129 might be essential for the function of some peripheral clocks when isolated cells are analyzed.

Our finding of Gm129 was driven by the hypothesis that mining ChIP-seq data for genes with high affinity CLOCK-BMAL1-binding sites in their promoter will identify important factors in the circadian clock regulation. In addition to Gm129, other genes also contain high affinity CLOCK-BMAL1-binding sites in their promoter region with uncharacterized functions in circadian regulation.

**FIGURE 6. Effects of disruption of the Gm129 gene on circadian gene expression in mouse liver.** A and B, circadian expression of Dbp, Nr1d1, Per1, and Bmal1 in liver from wild-type (black curve) and Gm129−/− (gray curve) mice analyzed by quantitative RT-PCR. C and D, circadian expression of Dbp, Nr1d1, Per1, and Bmal1 in Cry1−/− (blue curve) and Cry1−/−/Gm129−/− (red curve) mouse livers analyzed by quantitative RT-PCR. Liver samples were collected every 2 h from mice that were housed under 12-h light/12-h dark conditions. Dbp and Nr1d1 mRNA levels were first normalized to Gapdh expression. Then, the maximal value of gene expression in the day/night cycle was normalized to 1. Error bars represent S.D. of two biological repeats. Red asterisk indicates significantly altered Dbp, Nr1d1, Per1, and Bmal1 transcription in livers from Gm129−/− and Cry1−/−/Gm129−/− mice compared with control (p < 0.05).
GM129 protein shows very strong oscillation with a peak at ZT12. Thus, GM129 and CRYs regulate the molecular clock in two separate time windows.

In conclusion, our findings indicate a physiological role for GM129 in the transcriptional regulation of clock-controlled genes. GM129 protein levels show very strong oscillation with a peak at ZT12, 8 h earlier than the peak of PER and CRY transcriptional repressors (peak CT20-CT24) (Fig. 2B). Thus, GM129 protein can repress CLOCK-BMAL1 activity in a different time window compared with CRY and PER (Fig. 7). Together with other essential and non-essential CLOCK-BMAL1 repressors, GM129 contributes to the precision of the molecular clock and fine-tunes regulation of rhythmic gene expression.

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