CHARACTERIZATION OF NEW MOUSE CIRCADIAN CLOCK GENE: GM129

Yunus Annayev

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry and Biophysics.

Chapel Hill

2014

Approved by:

Aziz Sancar

Stephen Crews

William Marzluff

Brian Strahl

Greg Wang

ABSTRACT

Yunus Annayev: Characterization of a new circadian clock gene Gm129
(Under the direction of Aziz Sancar)

Circadian clock is a 24hr rhythmicity that controls behavioral, metabolic events in most of the organisms. It has been reported that around 10% of all transcripts in mice show circadian expression pattern. The circadian clock is regulated by core clock which in most organisms is controlled by a transcription-translation feedback loop (TTFL). Transcriptional activators constitute positive arm of the TTFL and transcriptional repressors constitute the negative arm of the (TTFL).

In a ChIPseq analysis in mice liver we found that core clock genes bind strongly to the promoter of a previously uncharacterized gene called Gene Model 129 (Gm129). Gm129 transcript and the protein show a very high amplitude oscillation in mouse liver. Given these core clock gene like characteristics of Gm129, I analyzed its function in the core clock and found that it interacts with BMAL1 and PER2. Additionally, Gm129 binds to BMAL1/DNA complex both *in vitro* and *in vivo*. In the reporter gene assay it inhibits CLOCK/BMAL1 induced transcription suggesting that it functions as a repressor. Gm129 knock-out mice show altered circadian transcription of clock genes and this effect is more dramatic when Gm129 mutation was combined with Cry1 mutation.

Overall, I have demonstrated that Gm129 is novel circadian clock repressor. Characterization of Gm129 provides more insight on how the circadian clock machinery functions in mammalian organisms.

ACKNOWLEDGEMENTS

I would like to thank Dr. Aziz Sancar for welcoming me to his lab, first as a technician and then as a student. I am thankful for his support and encouragement during my graduate studies.

I would like to thank Dr. Rui Ye for his supervision and help in finishing my project.

I would like to thank Drs. Chris Selby and Nuri Ozturk for teaching and mentoring me when I first joined the lab.

I would like to thank current and former members of the Sancar lab in particular Drs. Laura Lindsey-Boltz, Michael Kemp, Joyce Reardon, Shobhan Gaddameedhi, Jin Hyup Lee, Yi-Ying Chiou, Ozdemirhan Sercin for their inputs during the group meetings and daily experiments.

Finally, I would like to thank my wife, Enesh, for her support and patience for the last 3 years.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

bHLH basic helix-loop-helix

bZIP basic leucine zipper

BMAL1 brain and muscle ARNT-like 1 protein

C1orf51 chromosome 1 open reading frame 51

CCGs clock controlled genes

ChIP chromatin immunoprecipitation

ChIPseq chromatin immunoprecipitation sequencing

CLOCK circadian locomotor output cycles kaput protein

CRY cryptochrome protein

DAPI 4',6-diamidino-2-phenylindole

DBP D site of albumin promoter binding protein

E-box enhancer box

EGFP enhanced green fluorescent protein

FASPS familial advanced sleep-phase syndrome

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GM129 gene model 129

HLF hepatic leukemia factor

KOMP knock-out mice project

NP40 nonylphenoxypolyethoxylethanol

NPAS2 neuronal PAS domain-containing protein 2

Nr1d1 nuclear receptor subfamily 1, group D, member 1

Nr1d2 nuclear Receptor Subfamily 1, Group D, Member 2

qPCR quantitative real time polymerase chain reaction

PAGE polyacrylamide gel electrophoresis

PCR polymerase chain reaction

PER period protein

RT-PCR reverse transcriptase PCR

RORC RAR-Related Orphan Receptor C

SCN suprachiasmatic nucleus

SDS sodium dodecyl sulfate

TEF thyrotrophic embryonic factor

TTFL transcription-translation feedback loop

μm micrometer

XPB Xeroderma Pigmentosum B

ZT zeitgeber time

CHAPTER 1: INTRODUCTION

Circadian Clock

The term "circadian" is derived from Latin words *circa* (about, approximate) and *diem* (day) and refers to a time period in an approximate length of the day. Circadian clocks are endogenous timekeeping systems that govern the daily cycles of behavior and physiology in a variety of organisms (2,3). The molecular clock that drives circadian expression of clock controlled genes (CCGs) has been well characterized in model organisms such as *Neurospora crassa, Arabidopsis thaliana, Drosophila melanogaster,* and *Mus musculus.*

Although core clock mechanisms in most organisms are conserved, the key players and functions of the genes has been diversified and became more complex from primitive organisms to mammals (4). One of the first core clock genes discovered is *Period* (Per) in *Drosophila* (5). Genetic studies in mice lead to discovery of additional genes that function in the regulation of the circadian clock (6,7). Classical model of mammalian core clock involves CLOCK and BMAL1 as transcription activators and *Cryptochrome* (CRY) and *Period* (PER) as transcription repressors. CLOCK and BMAL1 constitute the positive arm of the transcription-translation feedback loop (TTFL and CRY and PER constitute the negative arm of the transcription-translation feedback loop (TTFL (**Figure 1.1**) (1).

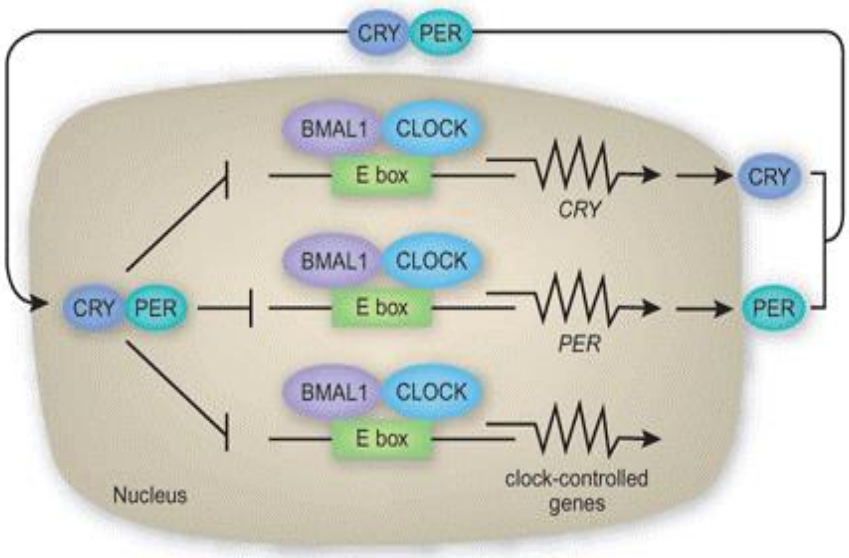

Figure 1.1: Core clock model. CLOCK and BMAL1 function in the positive arm of the TTFL by activating circadian transcription through binding to the E-Box enhancer elements in the promoters of clock controlled genes. CRY and PER function in the negative arm of the TTFL and inhibit CLOCK/BMAL1 induced transcription (1).

Each of the core clock genes are well characterized in mice. The effects of gene mutations on period length are analyzed by recording the wheel running activities of mice in which mice are kept in light/dark cycle for a certain period of time and then stitched to dark/dark cycle. Period length of wild-type mice in total darkness is about 23.8 hours. *Cry1* mutation in mice shortens the period by 1 hour and *Cry2* mutation lengthens it by 1 hour. Cry1/Cry2 double knock-out mice are arrhythmic (8). *Bmal1* knock-out mice are also arrhythmic (9). *Clock* knock-out mice show 0.5hr shorter periods (10) and *Per2* knock-out mice are arrhythmic after several days in constant darkness (11). **Table 1.1** summarizes the period changes in mice mutant to specific clock gene.

Gene	Allele	Mutant phenotypes in DD
Bmal1	Bmal1−/−	Arrhythmic
Clock	ClockΔ19/Δ19	4 h longer period/arrhythmic
	Clock−/−	0.5 h shorter period
Per1	Per1Brdm1	1 h shorter period
	Per1ldc	0.5 h shorter period
	Per1−/−	0.5 h shorter pd/arrhythmic
Per2	Per2Brdm1	1.5 shorter pd/arrhythmic
	Per2ldc	Arrhythmic
Per3	Per3−/−	0–0.5 h shorter period
Cry1	Cry1−/−	1 h shorter period
Cry2	Cry2−/−	1 h longer period
Rev-erbα	Rev-Erbα−/−	0.5 h shorter period
		Disrupted photic entrainment
Rorα	Staggerer	0.5 h shorter period
		Disrupted photic entrainment
Rorβ	Rorβ−/−	0.5 h longer period
Rorγ	Rorγ−/−	Unknown
NPAS2	NPAS2−/−	0.2 h shorter period
Bmal2		n/d
CK1ε	CK1εtau c	0.4 h shorter period
	CK1ε−/−	0.3 h longer period
CK1δ	Csnklδ−/+	0.5 h shorter period
Decl	Dec1−/−	No difference in period
	Dec1−/−	0.15 h longer period
	Sharp2−/−	No difference in period
Dec2	Sharp1−/−	No difference in period
Fbxl3	Fbxl3−/−	2–3 h longer period

Table 1.1 Effect of clock gene mutations on period lenght. Summary of period changes in mice deficient of core clock genes or secondary clock regulators. n/d- not determined, DD- dark-dark cycle. (Modified from (12)).

Since the establishment of core clock model, there were discoveries of other genes that play roles in the regulation of the circadian clock. Nuclear receptor proteins (*Nr1d1, Nr1d2,* and RORs (α, β, γ)) and D-box binding proteins (DBP and NFIL3) have been shown to function in the regulation of the circadian clock. Clock controlled genes have also been shown to be controlled by other additional response elements such as D-box (DBP response element TTATG(C/T)AA) and RRE (RevErbA response element, or ROR response element (RORE) (A/T)A(A/T)NT (A/G)GGTCA)) (13). Some of the core clock genes are regulated by these two regulatory sites. *Per (1, 2, and 3), Cry1, Nr1d1, Nr1d2, Rorα,* and *Rorβ* genes have D-boxes in their promoters. In contrast, *Bmal1*, *Clock, Npas2, Cry1, NFIL3,* and *Rorc* are regulated by RORE sites. **Figure 1.2** shows the most recent clock model in which the regulation of clock controlled genes with E-boxes, D-boxes and RORE sites are summarized. Nuclear receptors *Nr1d1* and *Nr1d2* (also called Rev-Erb α and β) act as repressors of clock controlled genes that have RORE sites in their promoters and *Bmal.* Opposed to *Nr1d1* and *Nr1d2,* RORs (α,β,γ) activated the transcription of genes with RORE sites (14). Latest promoter element that was characterized in clock controlled genes is he D-box. DBP, which itself is regulated by E-box promoter elements, acts as the activator of D-box genes which include *Per* genes. Repressor of D-box genes is NFIL3 (also known as E4BP4) which is regulated by the RORE in its promoter (13). Combination of these two and the classic E-box driven core clock mechanisms together with posttranslational modifications of core clock proteins regulate the circadian clock and achieve almost 24-hour periods in mice and other mammals.

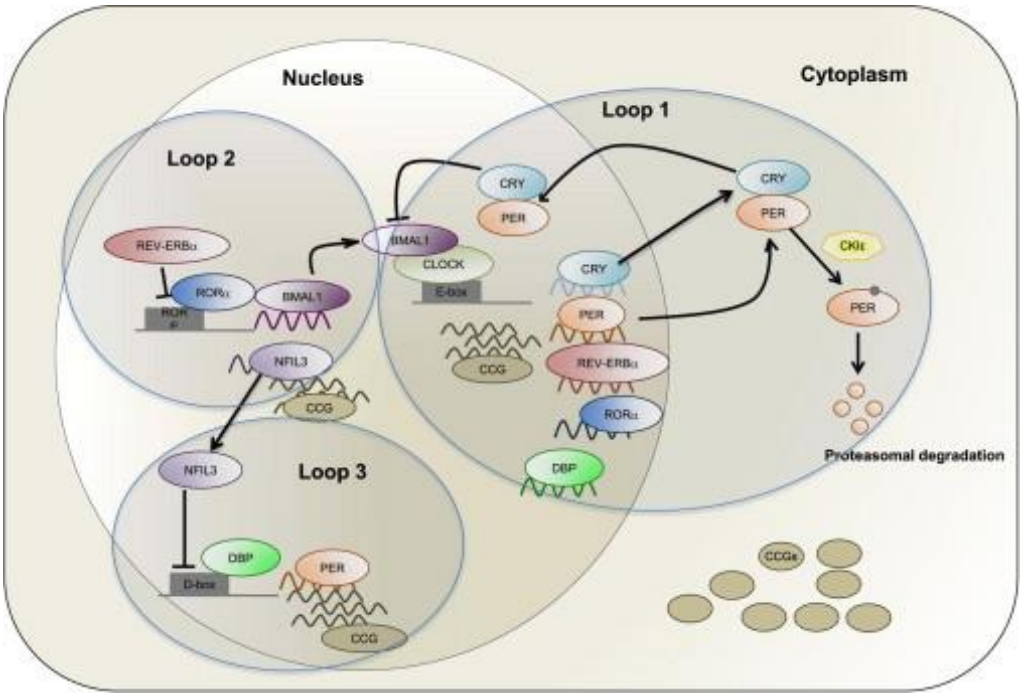

Figure 1.2 Core clock model summarizing secondary loops of circadian clock regulation. Loop 1 involves classic core clock proteins that regulate clock controlled genes by binding to the E-boxes. Loop 2 involves nuclear receptor proteins that regulate core clock genes by binding to the RORE sites. Loop 3 involves D-box binding protein DBP which activate the transcription and NFIL3 which represses the transcription of clock controlled genes regulated by the D-box elements. (Adapted from (15)).

The master regulator of the circadian clock in mammals is located in suprachiasmatic nucleus (SCN). However, it has been shown that peripheral tissues such as liver (16) have their own circadian clocks indicating presence of cell-autonomous oscillators. Individual tissues and organs rely on signals from the master regulator (SCN) to be entrained with the surrounding environment. SCN receives photic information from retina and keeps other parts of the body entrained by means of neuronal signals, endocrine signals and body temperature fluctuations. Genome-wide transcriptome analysis found that between %2-%10 of genes in the peripheral tissues show circadian oscillation (17-20).

Most of the oscillating genes in peripheral tissues function in key pathways such as metabolic pathways, signaling pathways etc. (21). Therefore, it is expected that the disruption of circadian rhythm can have serious consequences in the organism. One of the classic papers demonstrating a link between circadian clock disruption and health implications showed that there is increased risk of breast cancer in nurses working on rotating night shifts (22). In addition, circadian clock disruption has also been linked to sleep disorders. Familial advanced sleep-phase syndrome (FASPS) is a sleep disorder in which people have difficulty staying awake until desired time and staying asleep until desired time. S662G mutation in the *Per2* gene which increases PER2 turnover and leads to shorter periods was shown to be the primary cause of FASPS (23). Another mutation in core clock gene *Clock* ($CLOCK^{\Delta 19}$) was shown to make mice obese as a results of hypoglycemia and hypoinsulinaemia since key enzymes in the metabolic pathways are controlled by the circadian clock (24). There are some studies linking core clock gene mutation to predisposition to cancer but that area remains controversial because of several conflicting studies (25,26). *Bmal1* knock-out mice were shown to age early and the reasoning was the regulatory

pathways that Bmal1 is associated with such as the regulation of reactive oxygen species (ROS) homeostasis (27).

Overall, circadian clock is an important aspect of daily life in almost all organisms and its disruption has some serious implications for the organisms' physiology and well-being. There are still some unanswered questions regarding how each of the core clock genes function. It is possible that there remains more genes that play a role in circadian clock regulation to be discovered. This thesis is based on the work that characterizes a novel circadian clock gene called Gene Model 129 (Gm129). We show that Gm129 functions as a repressor in the negative arm of the TTFL.

CHAPTER 2:

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

Summary

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 significant phase delay compared to control. Our results suggest that, in addition to CRYs and PERs, GM129 protein contributes to the transcriptional feedback loop by modulating CLOCK:BMAL1 activity as a transcriptional repressor.

INTRODUCTION

Circadian clocks are endogenous timekeeping systems that govern the daily cycles of behavior and physiology in a variety of organisms (2,3). In mammals, the 24-hour circadian oscillator operates as a transcription-translation feedback loop (TTFL) involving both positive (transcriptional activators) and negative (transcriptional repressors) components. The transcriptional activators are CLOCK/NPAS2 and BMAL1, whereas Cryptochrome (CRY1 and CRY2), and Period (PER1 and PER2) act as transcriptional repressors. In the TTFL 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 CLOCK:BMAL1's activity (28,29). This feedback loop repeats every ~24 hours 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-erb alpha* (*Nr1d1),* and the PAR domain bZIP transcription factors *Dbp* (21). 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 (18).

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 (30,31). It was reported that, in liver, BMAL1 rhythmically bound to ~2000 genomic targets (31). Based on these ChIP-Seq results, direct CLOCK:BMAL1 target genes were predicted and were ranked according to binding strength. Interestingly, among the target genes with highest BMAL1 chromatin binding strength, most of them are well-studied circadian genes. The top 10 CLOCK:BMAL1 target genes include three core clock gene *Per1, Per2, Cry2* (8,32); three nuclear receptors *Rorc, Nr1d1* and *Nr1d2* involved in the core clock regulation (33,34), and three PAR domain bZIP transcription factors *Dbp, Hlf* and *Tef* which function as key genes in the output pathways (35). Only one gene remains uncharacterized, which is *gene model 129* (*Gm129*) that 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 line (30).

Coincidently, a ChIP-seq and gene expression study in our group also found that Gm129 is a direct target gene of the 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 *Gm129*'s roles 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 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.

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 both by *in vitro* EMSA assay and *in vivo* ChIP assay in mouse fibroblasts (36). 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 liver to map the chromatin binding sites of CLOCK, BMAL1 and CRY1. As shown in **Figure 2.1A,** in agreement with a previously published report (37), both BMAL1 and CRY1 rhythmically bind to *Dbp* and *Nr1d1* promoter and reach their peak affinities at ZT6-ZT10 (for BMAL1) and ZT20-ZT24 (for CRY1) in ChIP assays. To simplify the experiment design and achieve *best* signal-to-noise ratio for identifying chromatin binding sites of core clock protein complex, we conducted our CLOCK and BMAL1 ChIP-seq using liver harvested at ZT8 and CRY1 ChIP-seq with liver harvested at ZT20. **Figures 2.1B and 2.1C** show the representative samples of ChIP-seq data we obtained using anti-CLOCK, anti-BMAL1, and anti-CRY1 antibodies. As shown in **Figure 2.1B,** CLOCK, BMAL1 and CRY1 all bind to the 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 the promoters of the secondary/consolidating loop component *Rev-Erb alpha/Nr1d1* as well as to the promoter of the clock-controlled transcription factor *Dbp1*.

Consistent with other published studies (30,31,38), our ChIP-seq results showed that the *Gm129* gene has high affinity binding sites for CLOCK, BMAL1 and CRY1 in its promoter region (**Figure 2.1C**). 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 CLOCK:BMAL1:CRY1 complex to the *Gm129* promoter. **Figure 2.1D** 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 mouse liver (30). 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 (**Figure 2.2A**). Because in some cases, a rhythmically expressed gene can produce a constitutively expressed protein (39), 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 **Figure 2.2B**, 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 CLOCK:BMAL1:CRY1 complex and encodes a protein with high amplitude circadian oscillation.

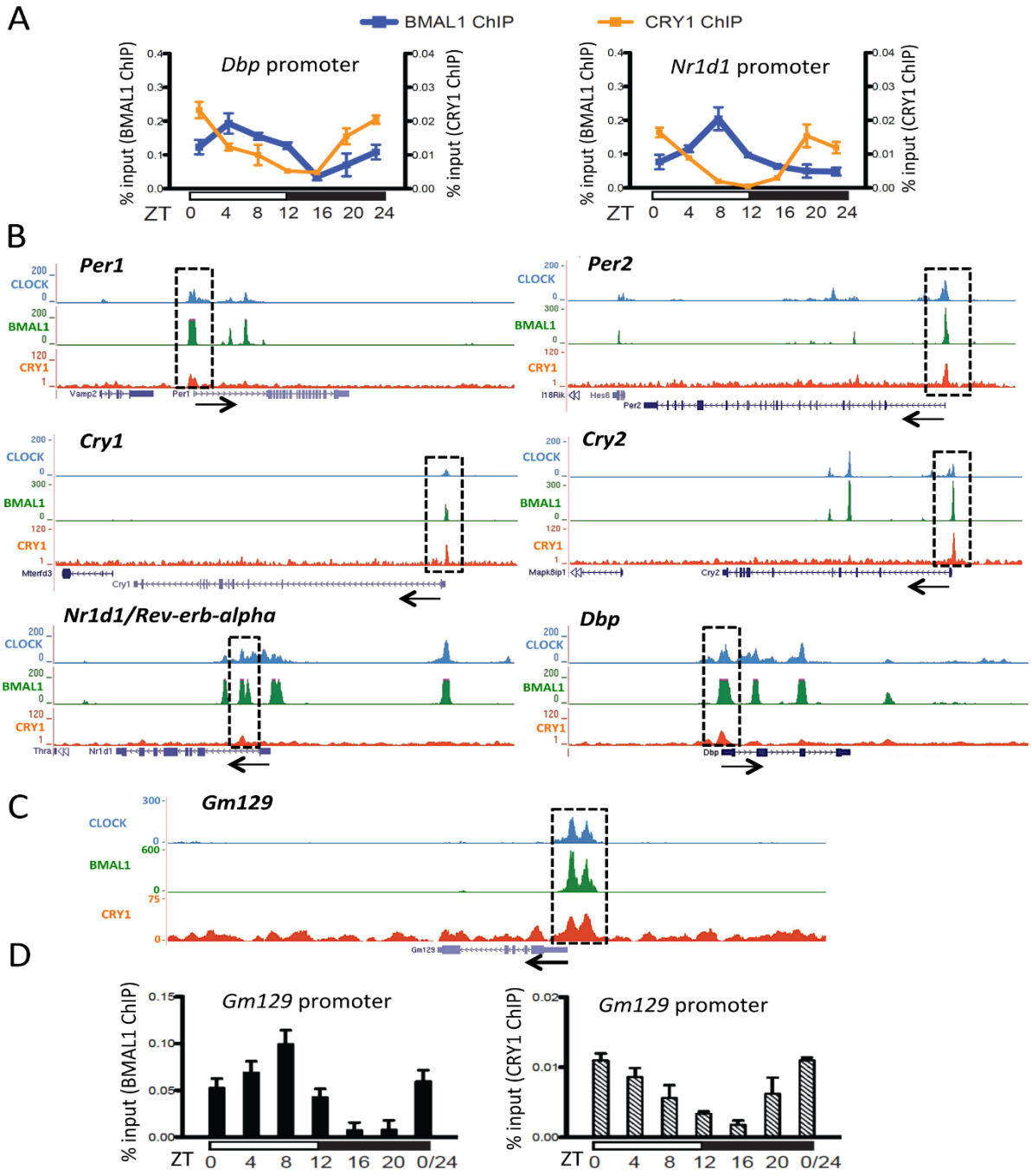

Figure 2.1: *Gm129* is a direct target of the CLOCK: BMAL1: CRY complex.
(A) Rhythmic Binding of BMAL1 (blue curve) and CRY1 (orange curve) to the *Dbp* and *Nr1d1* promoter region in mouse liver over one 12h light:12h dark cycle. Chromatin immunoprecipitation analysis was conducted using antibodies against BMAL1 and CRY1. Cross-linked chromatins

were prepared from mouse liver tissues taken at 4-h intervals in a 24- hour cycle. Error bars represent standard deviation of 3 biological repeats.
(B) Highly overlapping CLOCK, BMAL1 and CRY1 binding to the *Per1, Per2, Cry1, Nr1d1* and *Dbp* gene. Gene tracks represent binding of CLOCK (blue), BMAL1(green), and CRY1(red) to various genes. Gray rectangles highlight co-occupied sites. The scale of the *y* axis (peak height) is adjusted for individual traces for visual clarity. Beneath each set of gene tracks is a representation of the intron/exon structure. Black arrow indicates the orientation of transcription.
(C) Binding of CLOCK:BMAL1:CRY1 complex to the promoter region of the *Gm129* gene.
(D) Rhythmic binding of BMAL1 (black bars) and CRY1 (dashed bars) to the *Gm129* promoter region in mouse liver over one 12h light:12h dark cycle. Nuclear extracts were prepared from mouse liver tissues collected at 4-h intervals in a 24- hour cycle and subjected to chromatin immunoprecipitation analysis using antibodies against BMAL1 and CRY1. Error bars represent standard deviation of 3 biological repeats.

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 HEK293T cells with this plasmid. We found that the GFP-GM129 protein largely localizes in the nucleus (**Figure 2.2C**). 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 (40,41). 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 **Figure 2.3A**, GM129 has a strong affinity to BMAL1 and PER2, but not CRY1. Additionally, GM129 binds to endogenous BMAL1 in an NIH3T3 cell line that stably expresses Flag-GM129 protein (**Figure 2.3B**). Hence, the nuclear localization and direct interaction with core clock proteins strongly support the hypothesis that GM129 is a novel circadian regulator.

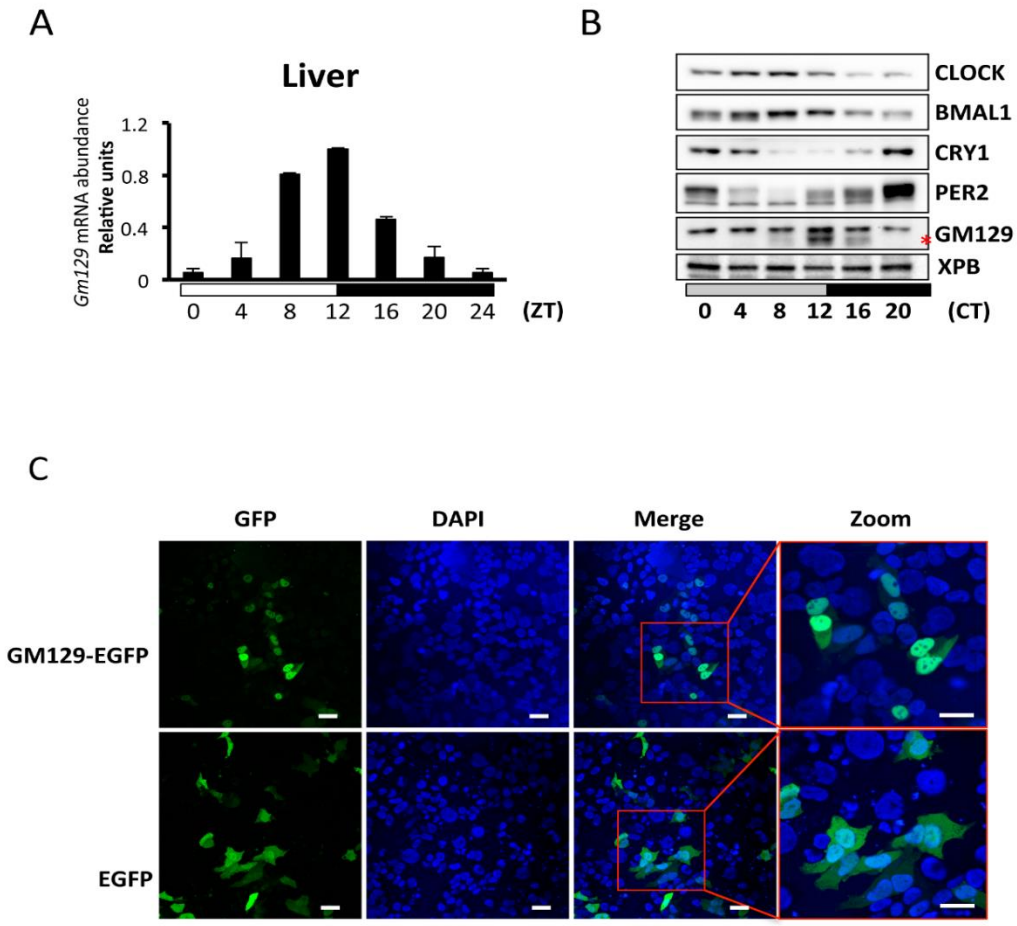

Figure 2.2: GM129 is a nuclear protein and oscillates in mouse liver

(A) Circadian oscillation of Gm129 mRNA in mouse liver. Mice were sacrificed at Zeitgeber times (ZT) 4, 8, 12, 16, 20 and 24/0 hours. 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 standard deviation of 2 biological repeats. The 0/24 hour 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 hours following 12hrs dark /12hrs light cycles. After 24 hours in the dark, nuclear extracts were prepared from livers collected at 0, 4, 8, 12, 16, and 20 hours 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 hours 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.

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 activity 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 (40). Co-expression of CLOCK and BMAL1 generate >6 fold induction of Luciferase activity compared to expressing BMAL1 alone (**Figure 2.3C**). CRY1 is a positive control in this assay that efficiently represses CLOCK:BMAL1 activity. Importantly, the additional co-expression of either CRY1 or GM129 reduced the Luciferase activity to the basal level (**Figure 2.3C**). As shown in **Figure 2.3D**, the repressor function of GM129 is also dose dependent, since 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 (**Figure 2.3D**). 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 (TTFL).

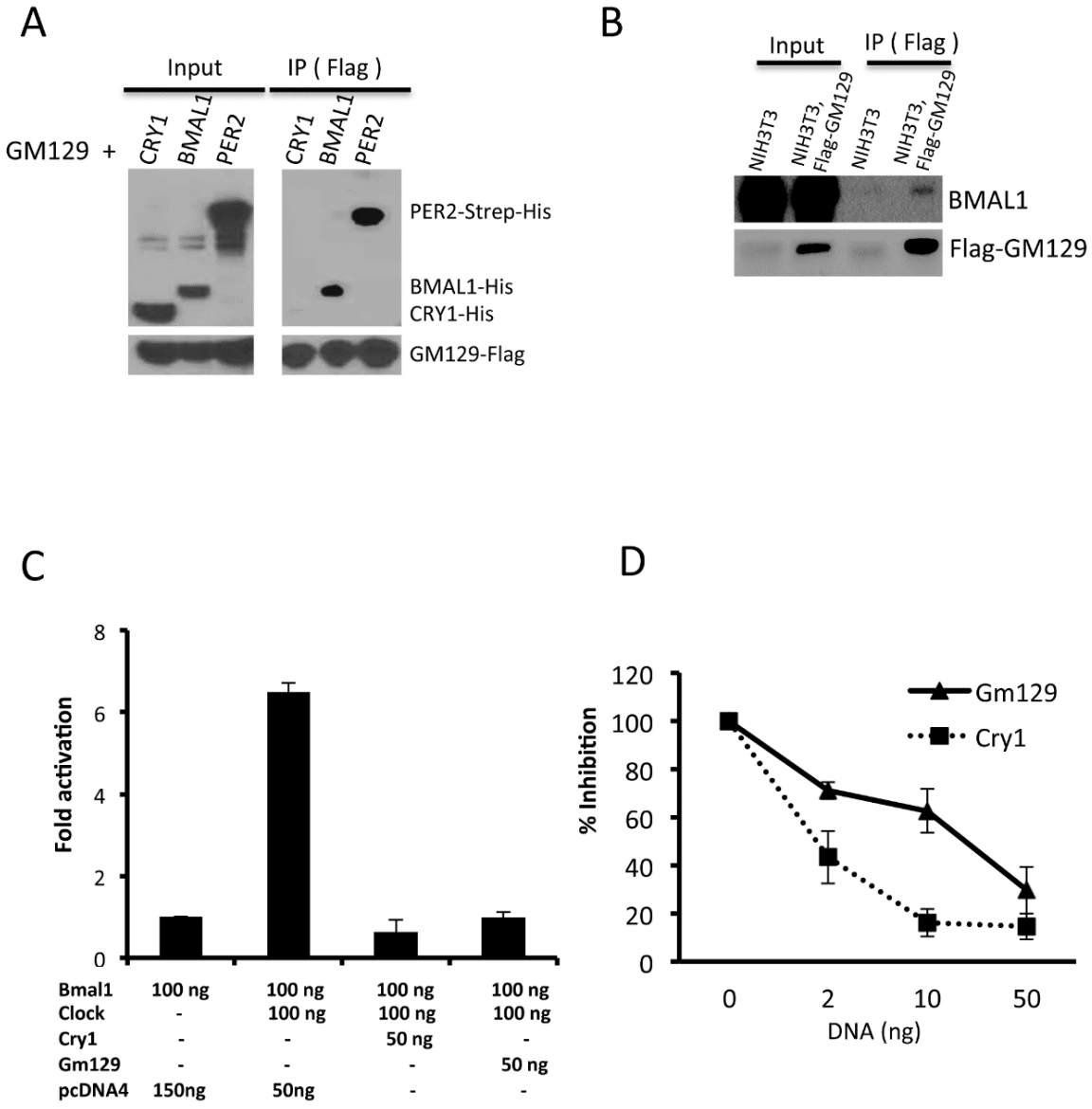

Figure 2.3: GM129 interacts with BMAL1 and PER2, and represses CLOCK/BMAL1 activated transcription.

(A) GM129 interacts with BMAL1 and PER2. Sf21 cells were co-infected with Flag-GM129 baculovirus together with either PER2-Strep-His, BMAL1-His, or CRY1-His baculovirus. Cell extracts were prepared 48 hours after infection and subjected to immunoprecipitation using FLAG resin. Immunoprecipitates were analyzed by western blot.

(B) GM129 interacts with endogenous BMAL1 in an NIH3T3 cell line stably expressing Flag-GM129. Immunoprecipitates obtained with FLAG resin from whole cell lysates were probed by

immunoblotting. GM129 and BMAL1 were detected by anti-FLAG anti-BMAL1 antibody, respectively.
(C) HEK293 cells were transiently transfected with 25 ng of *Per1* luciferase reporter construct, 2.5 ng of pBind (for normalization of transfection efficiency) and a total 250 ng of the indicated combinations of *Clock*, *Bmal1*, *Gm129*, and *Cry1* expressing constructs together with pcDNA4. Cells were collected 48 h after transfection. Error bars represent standard deviation from 3 experimental repeats.
(D) Dose–response studies of inhibition of CLOCK-BMAL1 induced transcription by the GM129 and CRY1 proteins. Three amounts (2, 10, 50 ng) of the Cry1 and Gm129 plasmid were used in the reporter gene assay. Experiments were done in triplicate and average values are plotted. Error bars represent standard deviation of 3 biological repeats. Note: X-axis is not set to scale.

GM129 interacts with the CLOCK/BMAL1 complex on DNA both *in vivo* and *in vitro*

Although GM129 protein represses CLOCK-BMAL1's activity in the reporter gene assay, the detailed molecular mechanism is unclear. Generally, a protein can function as a repressor by two mechanisms: (a) Repressor can bind to the activator complex on DNA and interfere its interaction with co-activator or with RNA polymerase II complex. (b) Repressor can directly dissociate the activator from DNA. Previously, we have reported that CRY1 binds to the CLOCK-BMAL1 complex on DNA *in vitro* and *in vivo* (36). 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*, *Nr1d1* and *Bmal1* promoters. As shown in **Figure 2.4A**, Flag-GM129 showed significant recruitment to the *Per2*, *Dbp*, and *Nr1d1* E-box compared to 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:BMLA1 dependent. Equally important, Flag tagged C1ORF51, a human homolog of *Gm129*, also interacts with hBMAL1 and binds to the *hPer2* promoter in HEK293R cells that stably expresses Flag-C1ORF51 protein, which suggests that the molecular function of GM129 protein is evolutionarily conserved (**Figures 2.4B and 2.4C**).

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 (36). Purified GM129 is shown in **Figure 2.4D**. Then, we investigated the effects of GM129 on the CLOCK:BMAL1:E-box ternary complex using electromobility shift assay (EMSA). As shown in **Figure 2.4E**, 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 (lanes 3,4,5). CRY1 is a positive control that can also shift the CLOCK:BMAL1:E-box complex (lane 7) (36). GM129 on its own does not bind to E-box under the conditions used (lane 6), indicating that 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 pull-down 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 2.1**) in the mouse *Per2* promoter, which was shown to bind to CLOCK:BMAL1 complex (42,43). 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 **Figure 2.4F**. 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 (36), PER2 protein (negative control) does not bind to the CLOCK:BMAL1 complex on DNA (lane5). 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.

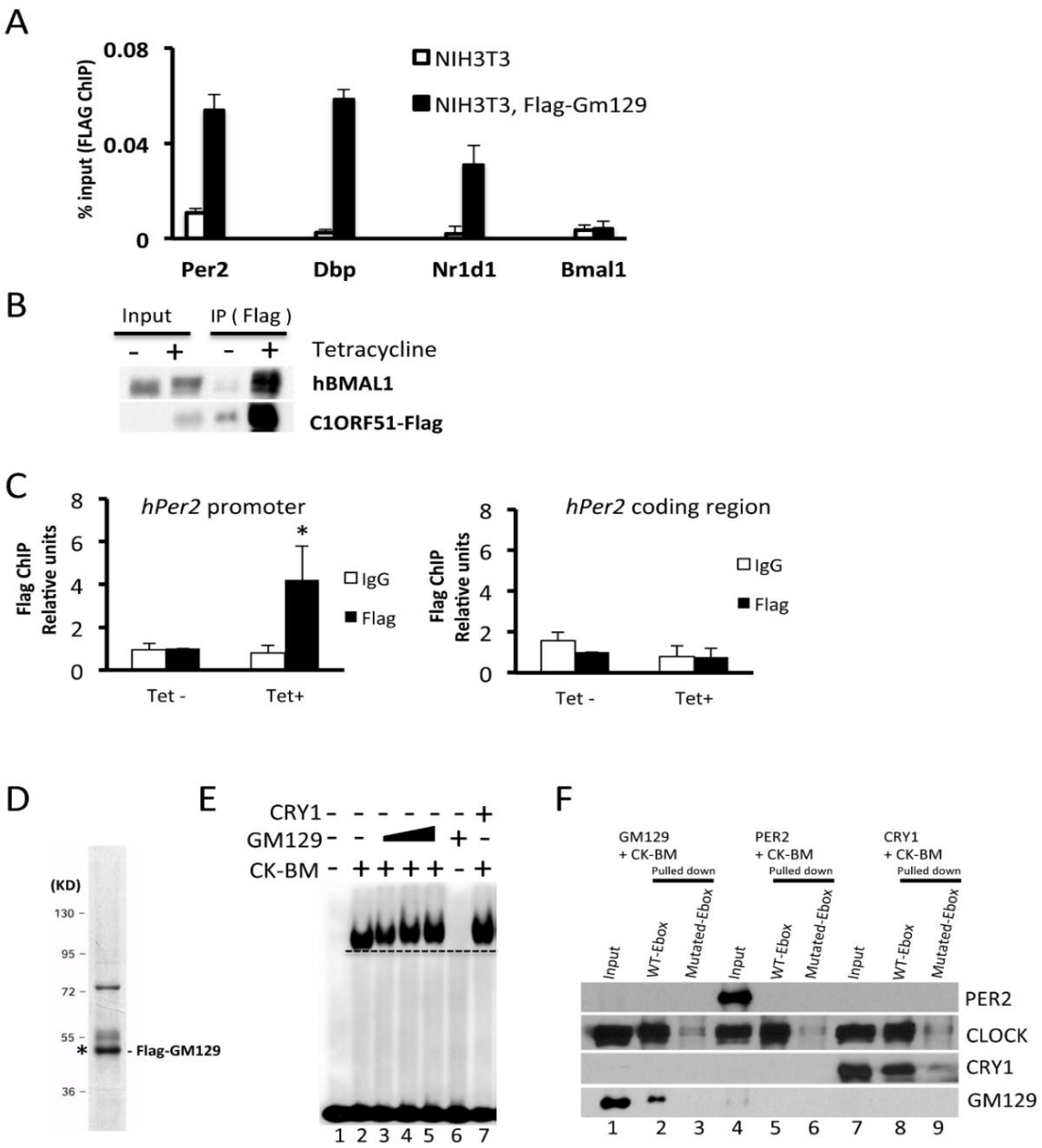

Figure 2.4: GM129 binds to CLOCK/BMAL1 on DNA both *in vitro* and *in vivo.*
(A) Flag-GM129 binding to E-box DNA sequence in the *Per2*, *Dbp* and *Nr1d1* promoters was determined by ChIP assays followed by quantitative PCR. Crosslinked nuclear extracts were isolated from cells and subjected to ChIP assay with FLAG antibodies against Flag-GM129. NIH3T3 cells and *Bmal1* promoter region primers were used as controls for immunoprecipitation

and PCR, respectively. Data are expressed as percentage of input. Error bars represent standard deviation of 3 experiments.
(B) Interaction of human homolog of GM129 (C1ORF51) with endogenous hBMAL1 in extracts made from a 293R cell line stably expressing Flag-C1ORF51 under the control of a tetracycline inducible promoter (Flp-In/FLAG-C1ORF51 cells). FLAG resin was used to pull down Flag-C1ORF51, and immunoprecipitates were analyzed by western blot. Note that Flag-C1ORF51 protein is also enriched by flag resin without tetracycline due to the minor leaky expression.
(C) C1ORF51 binding to the E-box DNA sequence in h*Per2* promoter was determined by ChIP assays followed by quantitative PCR. Crosslinked nuclear extracts were isolated after 24 hours tetracycline induction and subjected to ChIP assay with FLAG antibodies against Flag-C1ORF51 or IgG. Upon tetracycline treatment, Flag-C1ORF51 showed significant recruitment to the *hPer2* E-box compared to the IgG control and non-tetracycline controls (Left panel, black asterisk: $p<0.05$). No significant recruitment was detected in the *hPer2* coding region (Right panel). Data are expressed as percentage of input. Error bars represent standard deviation of 3 experiments.
(D) Flag-GM129 protein was expressed using the Sf21/baculovirus system. After affinity chromatography purification, protein was analyzed by SDS-PAGE/Commassie blue staining. GM129 protein is marked with asterisk confirmed by immunoblotting.
(E) Effects of GM129 on the mobility of the CLOCK:BMAL1:E-box complex analyzed by electromobility shift assay (EMSA). A radiolabeled 14mer E-box duplex (1.5nM) (M34, Table 2.1) (44) was incubated with purified BMAL1-CLOCK heterodimer (0.5nM), and then increasing amounts of GM129 proteins (0.1, 0.5, 1nM) were added. Note the addition of GM129 results in slower migration of the protein-DNA complex (lanes 3, 4 and 5). GM129 alone (1 nM) does not bind to DNA (lane 6). CRY1 (1 nM) was used as a positive control to show the shifted CLOCK:BMAL1:CRY1 complex on DNA. A dashed line parallel to the leading edge of the CLOCK:BMAL1:E-box complex is drawn to indicate the change in mobility of the protein-DNA bands upon addition of GM129 and CRY1.
(F) GM129 binds to CLOCK:BMAL1:E-box determined by *in vitro* pull down assays. The CLOCK-BMAL1 complex (10 nM) was mixed with 10 nM GM129, PER2, or CRY1 protein, then 500 ng immobilized 30-mer WT E-box or mutated E-box duplex DNA was added for an additional 60 minutes incubation in binding buffer. Immobilized complexes were washed and then protein-DNA complexes were eluted and analyzed by western blot. Proteins were detected by anti-CLOCK or anti-Flag (GM129, PER2, and CRY1) antibodies.

Gm129 gene deficiency affects the expression of clock-controlled genes

If GM129's repressor function is essential and cannot be compensated by other genes, depletion of *Gm129* in mice would affect circadian rhythm rhythmicity. To test this, *Gm129*$^{tm1e(KOMP)Wtsi}$ heterozygous mice (hereafter *Gm129*$^{+/-}$) were obtained from the NIH Knockout Mouse Project (KOMP) Repository. The targeting strategy for the knockout is summarized in **Figure 2.5A** (45). *Gm129*$^{-/-}$ homozygous mice were obtained by mating these heterozygotes. Genotyping was done by PCR as shown in **Figure 2.5B**. **Figures 2.5C and 2.5D** show that *Gm129* RNA and protein are not expressed in knock-out mice, thus the *Gm129*$^{-/-}$ 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*$^{-/-}$ 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 hr light/ 12 hr 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 canonical clock genes *Dbp*, *Nr1d1*,*Per1* and *Bmal1* in mouse liver samples collected every 2 hours for 24 hours. In agreement with the behavioral analysis, the rhythmic expression of these clock genes retain in *Gm129*$^{-/-}$ mice compared to wild-type (WT) mice (**Figure 2.6A and 2.6B**). However, the peak of *Dbp*, *Nr1d1* and *Per1* gene

expression persists longer in *Gm129*$^{-/-}$ mouse liver between ZT10-ZT14. *Bmal1* expression is also slightly altered at ZT16. These effects are likely caused by the absence of the GM129 transcriptional repressor that exhibits a peak expression level at ZT12.

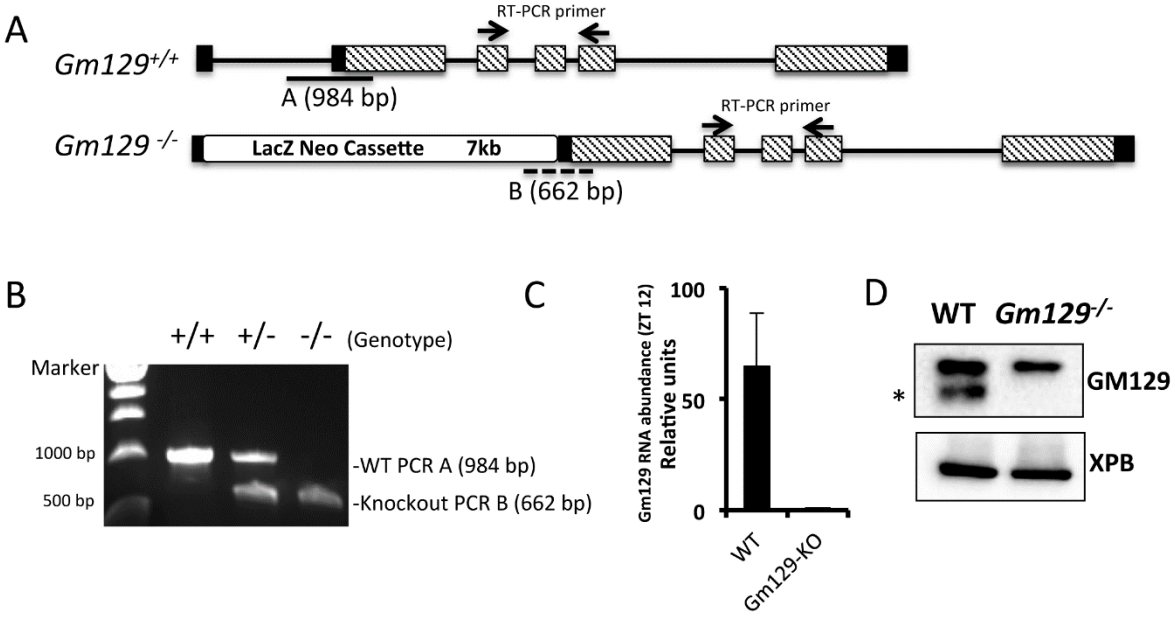

Figure 2.5: Generation of *Gm129* knock-out mice

(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 2.1).

(B) Primers were designed as indicated in Figure5A and Table 2.1 for genotyping. 984bp and 662bp PCR products were amplified using WT, heterozygote or knock-out mouse tail genomic DNA, respectively.

(C) *Gm129* gene expression (normalized to *Gapdh*) in the WT mice and *Gm129*$^{-/-}$ mice. The value of *Gm129* expression in Knock-out mice was set to 1. Error bars represent standard deviation 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.

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* (9), 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 period (8), but *Cry*$^{-/-}$ double knock-out mice are totally arrhythmic. In addition, *Per1* and *Per2* genes also have redundant roles in circadian regulation (11,32). Therefore, we decided to combine the *Gm129* mutation with another core clock gene mutation that possibly compensates GM129's function. Although *Gm129* does not have analogs in the mouse genome, our protein-protein interaction assay and reporter gene assay suggest *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 role of *Gm129* in circadian regulation. *Dbp, Nr1d1, Per1* and *Bmal1* rhythmic gene expression in liver were used as readouts of the circadian rhythm. Results are shown in **Figure 2.6C and 2.6D**, which is aligned under and may be compared with **Figure 2.6A and 2.6B**. In agreement with a previous report, the *Cry1* mutation alone causes an advanced phase shift (~ 4 hours) of *Dbp* (37), *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 hours phase shift) compared to *Cry*$^{-/-}$ mice (**Figure 2.6C**). *Per1* gene expression exhibit a broader peak between ZT4-ZT10 compared to control (**Figure 2.6D, 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-ZT14. Equally important, rhythmic *Bmal1* expression that is under the control of NR1D1 protein (3,10), is also affected by the *Gm129* knock-out mutation

in *Cry1*$^{-/-}$ mice (**Figure 2.6D, right panel**). Thus, we reasoned that in WT mice, GM129's 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.

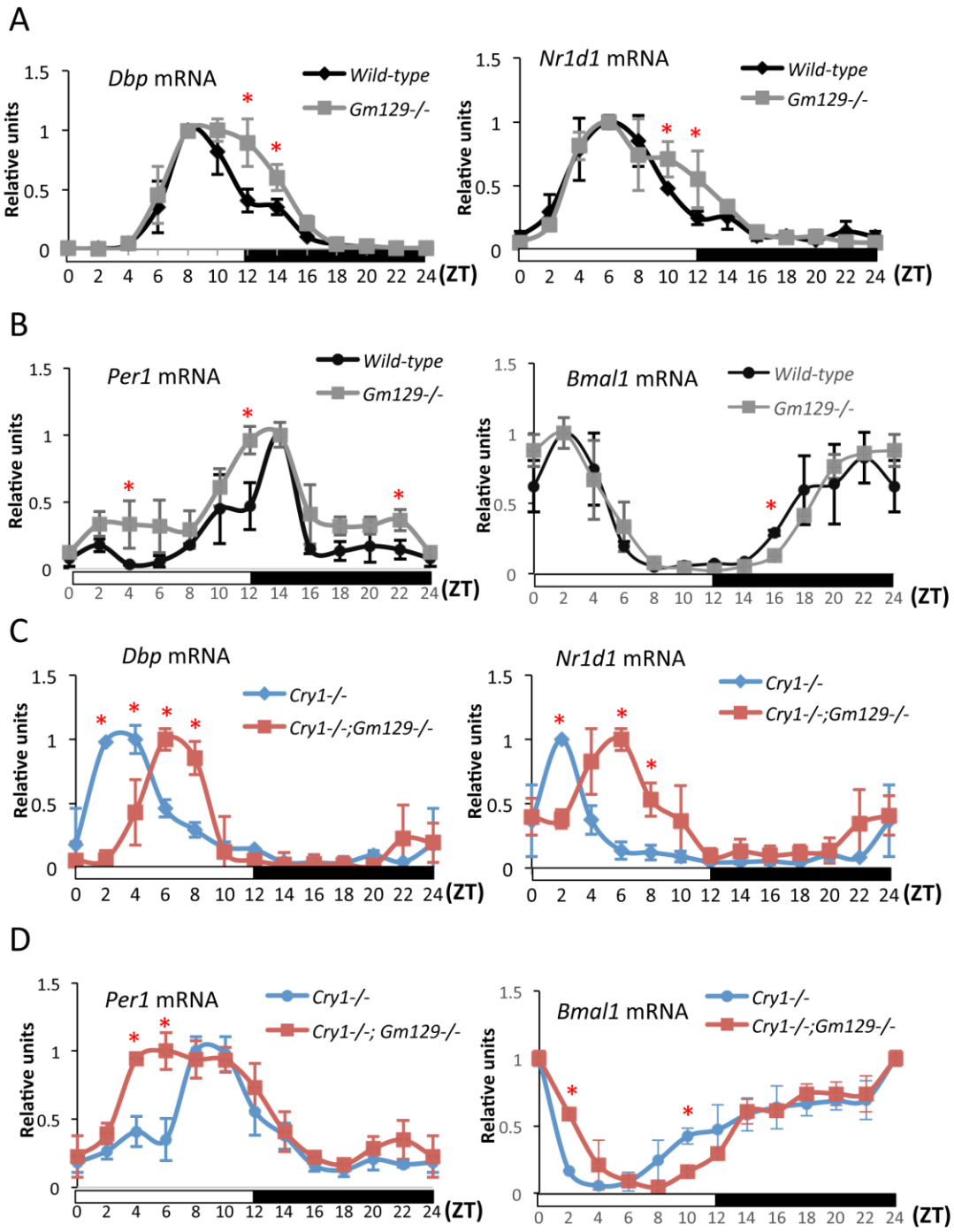

Figure 2.6: Effects of the disruption of *Gm129* gene on circadian gene expression in mouse liver.

(A and B) Circadian expression of *Dbp*, *Nr1d1*, *Per1*, and *Bmal1* in wild-type (black curve) and *Gm129*$^{-/-}$ (Grey curve) mouse liver analyzed by quantitative RT-PCR.

(C and D) Circadian expression of *Dbp, Nr1d1, Per1* and *Bmal1* in *Cry*$^{-/-}$ (blue curve) and *Cry*$^{-/-}$ *Gm129*$^{-/-}$ (red curve) mouse livers analyzed by quantitative RT-PCR.
Liver samples were collected every 2 hours from mice that were housed under 12 hours light:12 hour 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 standard deviation of two biological repeats. Red asterisk indicates significantly altered *Dbp, Nr1d1, Per1*, and *Bmal1* transcription in *Gm129*$^{-/-}$ and *Cry*$^{-/-}$*Gm129*$^{-/-}$ mouse liver compared to control ($p<0.05$).

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 UC Davis KOMP repository and bred to obtain *Gm129*$^{-/-}$ mice. Primers were designed as indicated (**Figure 2.5A and Table 2.1**) for genotyping. *Cry1*$^{-/-}$, *Gm129*$^{-/-}$ double knock-out mice were generated by crossing *Cry1*$^{-/-}$ (8) and *Gm129*$^{-/-}$ mice. All mice were maintained on a 12 hour light, 12 hour 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 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 with the Flag tag sequence at the forward oligo and Myc tag sequence at the reverse oligo. Constructs for expressing mCLOCK, mBMAL1, mCRY1, and mPER2 were described previously (36).

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 (15mM HEPES pH 7.8, 250mM NaCl, 1% NP40, 10% Glycerol, protease inhibitor cocktail from Roche). Proteins from mouse liver nuclei were prepared according to the NUN procedure described previously (43). ANTI-FLAG antibody was purchased from Sigma-Aldrich. Anti-CLOCK, anti-BMAL1 antibodies (Bethyl Lab), anti-mPER2 antibody (ADI) and anti-Gm129 (Santa Cruz Biotechnology) were used in the immunoblotting assays. Anti-mCRY antibody was produced in our laboratory (36).

Immunoprecipitation

Insect cells or mammalian cells were collected and lysed in lysis buffer (50 mM TrisCl pH 7.5, 150mM NaCl, 10% Glycerol, 1%Tween-20, 0.1 % NP40, protease inhibitor cocktail from Roche). Lysates were incubated with anti-FLAG M2-agarose beads (Sigma) for 4 hours at 4 °C. Beads were washed 4 times with lysis buffer; bound proteins were eluted in 2X SDS-sample buffer and analyzed by immunoblotting.

Fluorescence Microscopy

For GFP fluorescence microscopy, HEK293T 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 hours 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 captures using a LeicaSP2 confocal microscope.

Chromatin Immunoprecipitation (ChIP) and ChIP-Sequencing

Primers used in ChIP-PCR are given in **Table 2.1**. ChIP-PCR assays using cell nuclear extracts were conducted as described previously (36). For ChIP-PCR and ChIP-Seq using mouse tissues, liver chromatin was prepared according to a previously published protocol (46) with minor modification. Briefly, livers from mice were immediately homogenized and cross-linked in PBS buffer including 1% formaldehyde (5 ml per 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 M 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 according the procedures described previously (36). 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, California, USA). The reads were aligned to the mm9 mouse reference genome using Bowtie accepting up to 4 reportable alignments and two mismatches per read (47). In cases where more than a single alignment was possible, only the one with the highest mapping quality was reported using bowtie's "--best" option. To generate ChIP-seq signal tracks, reads were extended *in-silico* to 150bp, and the number of reads overlapping with each base in the genome was counted and divided by the chromosome-wide average of per-base read coverage. The average signal over 50bp windows was calculated and viewed using the UCSC genome browser (**Figure 2.1**).

Reporter gene assay

Firefly luciferase was used as a reporter that is controlled by the *mPer1* promoter sequence (48). Renilla luciferase was used as a transfection control. HEK 293T cells were co-transfected with indicated amounts of plasmids using Lipofectamine® 2000 (Invitrogen). 36 hours post transfection, cells were collected, and luciferase activities were assayed using Dual-Luciferase® Reporter Assay System according to the manufacturer's protocols (Promogea).

Pulldown Assay with Immobilized DNA

Biotin labeled E-box DNA or mutated E-box DNA (**Table 2.1**) was immobilized on magnetic streptavidin beads. Immobilized DNA was washed twice in reaction buffer (10 mm Tris-HCl, pH 7.9, 50 mm NaCl, 5 mM dithiothreitol, 10% glycerol, 0.01% Nonidet P-40 and 10 **μ**g/ml BSA) before setting up binding reactions. Reactions (200μl) 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 associating 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 LD12:12 were sacrificed every 2 hours starting from ZT 0 and livers were collected, washed with PBS and immediately frozen in dry ice and stored in -80 ºC 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 manufacturer's instructions. Quantitative Real-time PCR assays were performed by using an ABI 7300 System (Applied Biosystems) and MaximaTM SYBR Green/ROX qPCR Master Mix (Fermentas) was used. Primers are listed in **Table 2.1**.

Table 2.1

Primer sets used for ChIP assay.

Name	Sequence	Reference
mPer2E2F	GGTTCCGCCCCGCCAGTATGC	Yoo SH et al. PNAS(2005)
mPer2E2R	CCGTCACTTGGTGCGCTCGGC	
mDbp5F	TGTGAACACTCGGCTCCTTT	This study
mDbp5R	ATATTTGGCCAATGGGAGGA	
mNr1d1CPF	GCTGCTGGAAAAGTGTGTCA	This study
mNr1d1CPR	ATGGAGAAATGAGGCACCAG	
mBmal50F	CGGATTGGTCGGAAAGTAGG	This study
mBmal50R	AGCCATGCCGACACTCAC	
hPer2CPF	ATTGAGGAACCGACGAGGT	This study
hPer2CPR	GCCCACAGCTGCACGTAT	
Gm129CPF	CACGGCTGGAGTGTACAGAG	This study
Gm129CPR	GAAAGAGTGGGGAGTCACGA	

Primer sets used for Genotyping.

Name	Sequence	Reference
CSD-Gm129-TTR1	CAGAACCTGCAACTGAAAAGCAAAGC	This study
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	ACTCAAGATGGGTCGCTTTG	GGGCAGCTATGTGAGGAAAC
mGapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
mDbp	GACACCGTGGAGGTGCTAAT	ACCTCCGGCTCCAGTACTTC
mNr1d1	CCTGACTCAAGGTTGTCCCACAT	CATGGCCACTTGTAGACTTCCTG
mPer1	TGAGGAGCCAGAGAGGAAAG	GGCTGAAGAGGCAGTGTAGG
mBmal1	TCATTGATGCCAAGACTGGA	CAAATAGCCTGTGCTGTGGA

Oligo sets used for EMSA and DNA pulldown assay.

Name	Sequence	Reference
M34TOP	GGGACACGTGACCC	Hogenesch et al. PNAS(1998)
M34BTM	GGGTCACGTGTCCC	
E-box	AGCGCGCGCGGTCACGTTTTCCACTATGTG	Yoo et al. PNAS(2005)
Mutated E-box	AGCGCGCGCGGTGCTAGTTTCCACTATGTG	

DISCUSSION

In the circadian TTFL 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 the formation of the CLOCK:BMAL1 complex (49-51). DEC1 protein was also reported to delay the phases of *Per1*, *Dbp* and *Nr1d1* in cells after serum synchronization (52). 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 (53). Here, we demonstrated that similar to Cryptochrome, GM129 is a novel transcriptional repressor of 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 since GM129 is unable to compensate for the absence of *Crys* in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double knock-out mice, which are arrhythmic (8). Our results

also suggest that, unlike CRY1, GM129 is an 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 contains high affinity CLOCK:BMAL1 binding sites in their promoter region with uncharacterized functions in circadian physiology. Investigations on these genes could introduce more new players in molecular clock regulation.

Gm129 may also contribute to non-clock pathways in addition to its function in the core clock machinery. Many circadian transcriptional regulators have roles in multiple pathways. It was previously reported that both PER and CRY can directly interact with nuclear receptors to influence related signaling pathways (54-56). Thus, in order to understand the function of GM129 protein in circadian physiology, it will be important to identify the GM129's binding partners and map its genome-wide binding sites in future studies.

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 CT12, 8 hours earlier than the peak of PER and CRY transcriptional repressors (peak CT20-CT24) (**Figure 2.2B**). Thus, GM129 protein can repress CLOCK:BMAL1 activity in a

different time window compared to CRY and PER (**Figure 2.7**). 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.

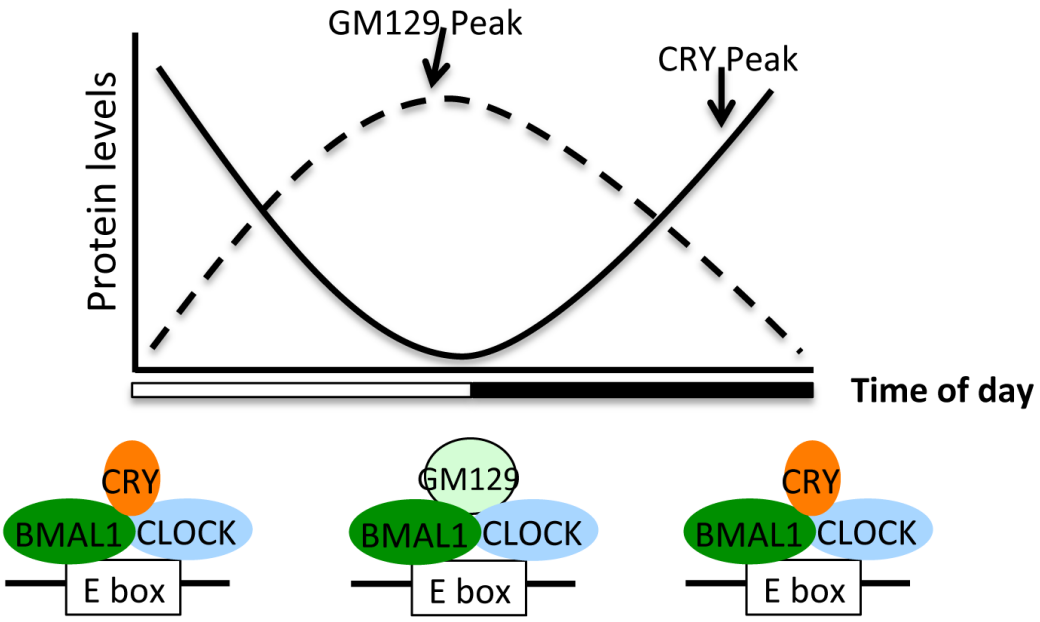

Figure 2.7: Model for the role of GM129 in modulating CLOCK:BMAL1 activities
Both GM129 and Cryptochromes could repress transcriptional activator CLOCK:BMAL by similar mechanisms but at different times. Cryptochromes reach their peak protein expression levels at ZT20-24, and GM129 protein has peak expression at ZT12. Thus, GM129 and CRYs regulate the molecular clock in two separate time windows.

ENDNOTES

[1]Chapter 2 was published as: Annayev Y, Adar S, Chiou YY, Lieb JD, Sancar A, Ye R. (2014) Gene model 129 (Gm129) encodes a novel transcriptional repressor that modulates circadian gene expression. *J Biol Chem.* 289(8):5013-24.

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

Daily rhythms in organismal behavioral, physiological, and metabolic events are controlled by circadian clock. Key components of the core clock components have been identified and it was shown that mutations in those genes have been implicated in sleep and metabolic disorders (23,24). There are also several non-essential clock genes that can influence circadian transcription but their deficiency does not have a major effect on the daily behavioral rhythms of mice (50-53). Here, I describe a novel gene called *Gm129* and show that it also modulates circadian transcription and functions as a repressor.

Biochemical and cellular assays show that GM129 is a nuclear protein, and it interacts with BMAL1 and PER2. Also, in the reporter gene assay it represses CLOCK/BMAL1 induced transcription of luciferase gene. Even though, Gm129 knock-out mice (Gm129-/-) don't show significant change in the circadian expression of clock genes, *Cry1* and *Gm129* double knock-out mice show about 4-6 hr phase advance in the circadian oscillation of clock genes analyzed indicating that Gm129 functions in the regulation of the circadian clock. However, there is still a lot of unknowns remain regarding the physiological role of Gm129.

We found that GM129 interacts with BMAL1 and PER2. It is reasonable to think that the GM129 interaction with BMAL1 is required for its repressor function since my *in vitro* data suggest that it binds to E-box DNA fragment only when CLOCK/BMAL1 complex is present in the reaction. On the other hand, the importance of the interaction with PER2 is unknown. In HEK-293T cells

recombinant GM129 is heavily phosphorylated when co-expressed with either BMAL1 or PER2 (data not shown) indicating an effect of PER2 interaction on GM129. Also, PER2 protein levels peak about 10 hours later compared to the peak of the GM129 protein which presents another challenge to understanding the significance of this interaction. Our analysis was mainly done in liver where PER2 and GM129 protein levels peak at the different times. Possible explanation maybe that the interaction of GM129 with PER2 may be important in some tissues where both proteins peak at around same time. Also, it is not necessary to have both proteins to peak at the same time, having enough of each proteins at some point in the circadian cycle so that they can function together maybe sufficient. GM129 and PER2 may function as repressors in small time frame before another repressor replaces either GM129 or PER2 (**Figure 3.1**). It is also possible that, these two proteins affect each other's stability and therefore regulate each other.

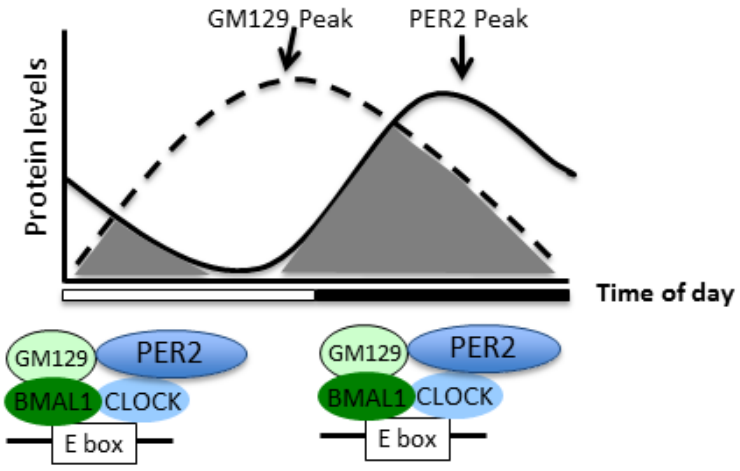

Figure 3.1: Possible importance of GM129 and PER2 complex.
GM129 and PER2 proteins peak at different time points. These proteins may form a complex for a short time period before other repressors take over. Gray areas under the plots represent those time points where they maybe small amounts of each protein.

An additional subject of investigation would be to identify other DNA sequences GM129 binds in the genome in addition to E-box enhancer elements. The fact that GM129 does not bind to BMAL1 promoter excludes the possibility of it binding to RORE (ROR response elements). However, it is still possible that GM129 can bind to the D-boxes. It is also possible that GM129 could bind to the promoters of certain group of genes not tested in our study and regulate specific pathways. Understanding of GM129 binding sites on the genome may provide wealth of evidence on its specific function in the regulation of the circadian clock and mammalian physiology.

Currently, we know that GM129 interacts with BMAL1 and PER2 but it is also possible that GM129 can interacts with other proteins. Specifically, it is not clear how GM129 represses CLOCK/BMAL1 controlled transcription. There may be other factors that function in transcription repression or another clock gene may be involved in that process.

Finally, Gm129-KO mice were not analyzed for any metabolic or physiological defects in this study. As stated in chapter 1, mutations in core clock genes results in metabolic and sleep disorders (23,24). Gm129-KO mice feed and breed well and did not have a distinct abnormal physical appearance. However, it would be interesting to study Gm129-KO mice for any abnormalities in metabolic, sleep or other physiological pathways. Moreover, Cry1/Gm129 double knock-out mice also could be analyzed to better define the function of *Gm129* because we see a more dramatic effect in circadian gene expression in this double knock-out compared to the single knock-out mice.

Recently, two separate groups published their studies on Gm129 (57,58). Both studies are in agreement with our findings. One of them discovered Gm129, similar to our discovery, by doing ChIP-seq on BMAL1 binding sites (58). In addition to GM129 interaction to BMAL1 and PER2 they also show that GM129 interacts with DEC2 and CRY2. They also show that repressor activity

of GM129 is dependent on histone deacetylases (HDACs). Repressor activity of GM129 was reversed when they treated the cells with HDAC inhibitor trichostatin A (TSA), an inhibitor of HDAC activity. In addition to interactions with the core clock proteins they show that GM129 interacts with HDAC1 and they conclude that GM129 mediated repression is HDAC dependent. They also analyzed GM129-KO mice and found that GM129-KO mice show a slight change in the period length. Similar to our study, they crossed GM129-KO mice with Cry1-KO mice to analyze wheel running activities. They were reasoning that Gm129 gene shows Cry2 gene like properties with respect to its interactions and effect on wheel running activities of mice. However, unlike Cry -/- double knockout mice, *Cry1*$^{-/-}$ *Gm129*$^{-/-}$ double knock-out mice weren't arrhythmic and had a 0.1hr difference compared to Cry1 -/- mice (58). The other group used computational tools to discover Gm129. They used machine learning to analyze and identify genes that resemble core clock genes from the genome-scale datasets. They tested the interaction of top candidate genes with the core clock proteins and found that GM129, similar to our finding, interacts with BMAL1 and PER2. They also show that GM129 abrogates BMAL1 interaction with the CBP and represses CLOCK/BMAL1 induced transcription (57). Overall, both studies show that GM129 is core clock gene and repress circadian transcription by several mechanisms.

In conclusion, I show that Gm129 is clock gene with repressor function and it interacts with the core clock genes. Gm129-KO mice show altered expression of clock genes and this effect is more dramatic when the Gm129 mutation is combined with Cry1 mutation. In addition, there are still some unanswered questions on the function of Gm129 regarding its binding sites in the genome, its interacting proteins and the phenotype of Gm129-KO mice.

REFERENCES

1. Sancar, A. (2008) The intelligent clock and the Rube Goldberg clock. *Nature structural & molecular biology* **15**, 23-24

2. Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., and Zoran, M. J. (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature reviews. Genetics* **6**, 544-556

3. Young, M. W., and Kay, S. A. (2001) Time zones: a comparative genetics of circadian clocks. *Nature reviews. Genetics* **2**, 702-715

4. Young, M. W., and Kay, S. A. (2001) Time zones: a comparative genetics of circadian clocks. *Nature reviews. Genetics* **2**, 702-715

5. Boothroyd, C. E., and Young, M. W. (2008) The in(put)s and out(put)s of the Drosophila circadian clock. *Annals of the New York Academy of Sciences* **1129**, 350-357

6. Antoch, M. P., Song, E. J., Chang, A. M., Vitaterna, M. H., Zhao, Y., Wilsbacher, L. D., Sangoram, A. M., King, D. P., Pinto, L. H., and Takahashi, J. S. (1997) Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell* **89**, 655-667

7. King, D. P., Zhao, Y., Sangoram, A. M., Wilsbacher, L. D., Tanaka, M., Antoch, M. P., Steeves, T. D., Vitaterna, M. H., Kornhauser, J. M., Lowrey, P. L., Turek, F. W., and Takahashi, J. S. (1997) Positional cloning of the mouse circadian clock gene. *Cell* **89**, 641-653

8. Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., Hitomi, K., Thresher, R. J., Ishikawa, T., Miyazaki, J., Takahashi, J. S., and Sancar, A. (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci U S A* **96**, 12114-12119

9. Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103**, 1009-1017

10. Debruyne, J. P., Noton, E., Lambert, C. M., Maywood, E. S., Weaver, D. R., and Reppert, S. M. (2006) A clock shock: mouse CLOCK is not required for circadian oscillator function. *Neuron* **50**, 465-477

11. Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., and Weaver, D. R. (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron* **30**, 525-536

12. Ripperger, J. A., Jud, C., and Albrecht, U. (2011) The daily rhythm of mice. *FEBS letters* **585**, 1384-1392

13. Ueda, H. R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., and Hashimoto, S. (2005) System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nature genetics* **37**, 187-192

14. Preitner, N., Damiola, F., Molina, L. L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002) The orphan nuclear receptor REV-ERB alpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**, 251-260

15. Curtis, A. M., Bellet, M. M., Sassone-Corsi, P., and O'Neill, L. A. (2014) Circadian clock proteins and immunity. *Immunity* **40**, 178-186

16. Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G. D., Sakaki, Y., Menaker, M., and Tei, H. (2000) Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288**, 682-685

17. Akhtar, R. A., Reddy, A. B., Maywood, E. S., Clayton, J. D., King, V. M., Smith, A. G., Gant, T. W., Hastings, M. H., and Kyriacou, C. P. (2002) Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Current biology : CB* **12**, 540-550

18. Hughes, M. E., DiTacchio, L., Hayes, K. R., Vollmers, C., Pulivarthy, S., Baggs, J. E., Panda, S., and Hogenesch, J. B. (2009) Harmonics of circadian gene transcription in mammals. *PLoS genetics* **5**, e1000442

19. McCarthy, J. J., Andrews, J. L., McDearmon, E. L., Campbell, K. S., Barber, B. K., Miller, B. H., Walker, J. R., Hogenesch, J. B., Takahashi, J. S., and Esser, K. A. (2007) Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiological genomics* **31**, 86-95

20. Oishi, K., Amagai, N., Shirai, H., Kadota, K., Ohkura, N., and Ishida, N. (2005) Genome-wide expression analysis reveals 100 adrenal gland-dependent circadian genes in the mouse liver. *DNA research : an international journal for rapid publication of reports on genes and genomes* **12**, 191-202

21. Gachon, F., Olela, F. F., Schaad, O., Descombes, P., and Schibler, U. (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell metabolism* **4**, 25-36

22. Schernhammer, E. S., Laden, F., Speizer, F. E., Willett, W. C., Hunter, D. J., Kawachi, I., and Colditz, G. A. (2001) Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. *Journal of the National Cancer Institute* **93**, 1563-1568

23. Vanselow, K., Vanselow, J. T., Westermark, P. O., Reischl, S., Maier, B., Korte, T., Herrmann, A., Herzel, H., Schlosser, A., and Kramer, A. (2006) Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS). *Genes & development* **20**, 2660-2672

24. Scott, E. M., Carter, A. M., and Grant, P. J. (2008) Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. *International journal of obesity* **32**, 658-662

25. Antoch, M. P., Toshkov, I., Kuropatwinski, K. K., and Jackson, M. (2013) Deficiency in PER proteins has no effect on the rate of spontaneous and radiation-induced carcinogenesis. *Cell cycle* **12**, 3673-3680

26. Fu, L., Pelicano, H., Liu, J., Huang, P., and Lee, C. (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* **111**, 41-50

27. Kondratov, R. V., Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V., and Antoch, M. P. (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes & development* **20**, 1868-1873

28. Takahashi, J. S., Hong, H. K., Ko, C. H., and McDearmon, E. L. (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nature reviews* **9**, 764-775

29. Reppert, S. M., and Weaver, D. R. (2002) Coordination of circadian timing in mammals. *Nature* **418**, 935-941

30. Hatanaka, F., Matsubara, C., Myung, J., Yoritaka, T., Kamimura, N., Tsutsumi, S., Kanai, A., Suzuki, Y., Sassone-Corsi, P., Aburatani, H., Sugano, S., and Takumi, T. (2010) Genome-wide profiling of the core clock protein BMAL1 targets reveals a strict relationship with metabolism. *Mol Cell Biol* **30**, 5636-5648

31. Rey, G., Cesbron, F., Rougemont, J., Reinke, H., Brunner, M., and Naef, F. (2011) Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver. *PLoS Biol* **9**, e1000595

32. Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z. S., Eichele, G., Bradley, A., and Lee, C. C. (2001) Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* **105**, 683-694

33. Cho, H., Zhao, X., Hatori, M., Yu, R. T., Barish, G. D., Lam, M. T., Chong, L. W., DiTacchio, L., Atkins, A. R., Glass, C. K., Liddle, C., Auwerx, J., Downes, M., Panda, S., and Evans, R. M. (2012) Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. *Nature* **485**, 123-127

34. Takeda, Y., Jothi, R., Birault, V., and Jetten, A. M. (2012) RORgamma directly regulates the circadian expression of clock genes and downstream targets in vivo. *Nucleic Acids Res* **40**, 8519-8535

35. Gachon, F., Olela, F. F., Schaad, O., Descombes, P., and Schibler, U. (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metab* **4**, 25-36

36. Ye, R., Selby, C. P., Ozturk, N., Annayev, Y., and Sancar, A. (2011) Biochemical analysis of the canonical model for the mammalian circadian clock. *J Biol Chem* **286**, 25891-25902

37. Stratmann, M., Stadler, F., Tamanini, F., van der Horst, G. T., and Ripperger, J. A. (2010) Flexible phase adjustment of circadian albumin D site-binding protein (DBP) gene expression by CRYPTOCHROME1. *Genes & development* **24**, 1317-1328

38. Koike, N., Yoo, S. H., Huang, H. C., Kumar, V., Lee, C., Kim, T. K., and Takahashi, J. S. (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* **338**, 349-354

39. Reddy, A. B., Karp, N. A., Maywood, E. S., Sage, E. A., Deery, M., O'Neill, J. S., Wong, G. K., Chesham, J., Odell, M., Lilley, K. S., Kyriacou, C. P., and Hastings, M. H. (2006) Circadian orchestration of the hepatic proteome. *Curr Biol* **16**, 1107-1115

40. Griffin, E. A., Jr., Staknis, D., and Weitz, C. J. (1999) Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. *Science* **286**, 768-771

41. Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H., and Reppert, S. M. (2000) Interacting molecular loops in the mammalian circadian clock. *Science* **288**, 1013-1019

42. Yoo, S. H., Ko, C. H., Lowrey, P. L., Buhr, E. D., Song, E. J., Chang, S., Yoo, O. J., Yamazaki, S., Lee, C., and Takahashi, J. S. (2005) A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. *Proc Natl Acad Sci U S A* **102**, 2608-2613

43. Asher, G., Reinke, H., Altmeyer, M., Gutierrez-Arcelus, M., Hottiger, M. O., and Schibler, U. (2010) Poly(ADP-ribose) polymerase 1 participates in the phase entrainment of circadian clocks to feeding. *Cell* **142**, 943-953

44. Hogenesch, J. B., Gu, Y. Z., Jain, S., and Bradfield, C. A. (1998) The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc Natl Acad Sci U S A* **95**, 5474-5479

45. Skarnes, W. C., Rosen, B., West, A. P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A. O., Thomas, M., Harrow, J., Cox, T., Jackson, D., Severin, J., Biggs, P., Fu, J., Nefedov, M., de Jong, P. J., Stewart, A. F., and Bradley, A. (2011) A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* **474**, 337-U361

46. Ripperger, J. A., and Schibler, U. (2006) Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat Genet* **38**, 369-374

47. Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10**, R25

48. Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280**, 1564-1569

49. Grechez-Cassiau, A., Panda, S., Lacoche, S., Teboul, M., Azmi, S., Laudet, V., Hogenesch, J. B., Taneja, R., and Delaunay, F. (2004) The transcriptional repressor STRA13 regulates a subset of peripheral circadian outputs. *J Biol Chem* **279**, 1141-1150

50. Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y., and Honma, K. (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature* **419**, 841-844

51. Hou, T. Y., Ward, S. M., Murad, J. M., Watson, N. P., Israel, M. A., and Duffield, G. E. (2009) ID2 (Inhibitor of DNA Binding 2) Is a Rhythmically Expressed Transcriptional Repressor Required for Circadian Clock Output in Mouse Liver. *Journal of Biological Chemistry* **284**, 31735-31745

52. Nakashima, A., Kawamoto, T., Honda, K. K., Ueshima, T., Noshiro, M., Iwata, T., Fujimoto, K., Kubo, H., Honma, S., Yorioka, N., Kohno, N., and Kato, Y. (2008) DEC1 modulates the circadian phase of clock gene expression. *Mol Cell Biol* **28**, 4080-4092

53. Zhao, W. N., Malinin, N., Yang, F. C., Staknis, D., Gekakis, N., Maier, B., Reischl, S., Kramer, A., and Weitz, C. J. (2007) CIPC is a mammalian circadian clock protein without invertebrate homologues. *Nature Cell Biology* **9**, 268-U253

54. Lamia, K. A., Papp, S. J., Yu, R. T., Barish, G. D., Uhlenhaut, N. H., Jonker, J. W., Downes, M., and Evans, R. M. (2011) Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature* **480**, 552-556

55. Schmutz, I., Ripperger, J. A., Baeriswyl-Aebischer, S., and Albrecht, U. (2010) The mammalian clock component PERIOD2 coordinates circadian output by interaction with nuclear receptors. *Genes & development* **24**, 345-357

56. Grimaldi, B., Bellet, M. M., Katada, S., Astarita, G., Hirayama, J., Amin, R. H., Granneman, J. G., Piomelli, D., Leff, T., and Sassone-Corsi, P. (2010) PER2 controls lipid metabolism by direct regulation of PPARgamma. *Cell Metab* **12**, 509-520

57. Anafi, R. C., Lee, Y., Sato, T. K., Venkataraman, A., Ramanathan, C., Kavakli, I. H., Hughes, M. E., Baggs, J. E., Growe, J., Liu, A. C., Kim, J., and Hogenesch, J. B. (2014) Machine Learning Helps Identify CHRONO as a Circadian Clock Component. *PLoS biology* **12**, e1001840

58. Goriki, A., Hatanaka, F., Myung, J., Kim, J. K., Yoritaka, T., Tanoue, S., Abe, T., Kiyonari, H., Fujimoto, K., Kato, Y., Todo, T., Matsubara, A., Forger, D., and Takumi, T. (2014) A Novel Protein, CHRONO, Functions as a Core Component of the Mammalian Circadian Clock. *PLoS biology* **12**, e1001839