Identification of Novel Components of the Mammalian Circadian Clock

by

Gülsüm Elif Muku

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Gülsüm Elif Muku

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examining committee have been made.

Committee Members:

_____

İ. Halil Kavaklı, Ph. D. (Advisor)

_____

Tuğba Bağcı Önder, Ph. D.

_____

Füsun Can, M.D. , Ph. D.

Date: _____

to my family…

ABSTRACT

The circadian clock is a biological timing system that oscillates with a period of 24 hours and is coordinated with the day-night cycle. Since a wide array of physiological and behavioral processes are regulated by the circadian clock, understanding the molecular clockwork will help develop treatments against various clock-related health problems including obesity, jetlag, insomnia, and psychological disorders. The molecular mechanism of the circadian clock has been extensively studied during the last decade, and consequently, numerous core clock components and clock modifiers have been identified so far. Yet, the clock mechanism is not entirely solved. There is a great competition among researchers for discovery of novel clock components, and one of the mostly commonly used methods on this purpose is the naive Bayesian strategy.

In this thesis work, high probability clock genes identified by the naive Bayesian strategy were experimentally screened through mammalian two-hybrid assay system for likely interactions with the core clock components. The entire screen yielded two hits, leptin receptor overlapping transcript-like 1 (*Leprotl1*) and sterol O-acyltransferase 1 (*SOAT1*), and both of them have metabolic functions. LEPROTL1 appears to interact with both mCRY1 and mCRY2, while SOAT1 interacts with only mCRY1. Herein, the involvement of *Leprotl1* in the circadian clockwork was investigated. It was shown that depletion of *Leprotl1* resulted in damped amplitude, long-period and phase advance phenotypes of the oscillations in cell lines, suggesting a role of *Leprotl1* in the proper functioning of the timing system.

ÖZET

Sirkadiyen saat bir biyolojik zamanlama sistemi olup 24 saatlik periyoda sahiptir ve işleyişi gece-gündüz döngüsüne göre düzenlenir. Birçok fizyolojik ve davranışsal işlem sirkadiyen saatin kontrolü altındadır, bu yüzden sirkadiyen saatlerin çalışma mekanizmasını tamamen çözmek sirkadiyen saatin bozulmasından kaynaklanan bazı sağlık problemlerine karşı tedavi geliştirmeye yardımcı olacaktır. Bu sağlık sorunlarının arasında obezite, jetlag, insomni ve psikolojik rahatsızlıklar yer alır. Geçtiğimiz on yıl içerisinde sirkadiyen saatin moleküler mekanizması birçok grup tarafından araştırılmış olup pek çok saat bileşeni ve düzenleyicisi saptanmıştır. Buna rağmen moleküler mekanizma bütünüyle çözülmüş değildir. Araştırmacılar arasında yeni bileşenler keşfetmek için ciddi bir rekabet söz konusudur ve bu uğurda sıklıkla kullanılan yöntemlerden biri naif Bayesian stratejisidir.

Bu tez çalışmasında, naif Bayesian stratejisiyle tanımlanmış yüksek ihtimalli aday saat genleri bilinen saat bileşenleriyle olası bir etkileşim bulmak amacıyla memeli ikili melez yöntemiyle deneysel olarak taranmıştır. Bütün taramanın sonucunda iki protein bulunmuştur: leptin reseptörü ile örtüşen transktript-benzeri 1 (*Leprotl1*) ve sterol O-asetiltransferaz 1 (*SOAT1*), bu iki proteinin de metabolik fonksiyonları vardır. LEPROTL1 proteininin hem mCRY1 hem de mCRY2 ile etkileştiği görülürken, SOAT1 proteininin yalnızca mCRY1 ile etkileştiği saptanmıştır. Bu çalışmada Leprotl1 geninin sirkadiyen saat mekanizmasındaki rolü araştırılmıştır. *Leprotl1* geni susturulduğunda hücrelerde salınım şiddeti azalırken süre fazının arttığı ve geciktiği görülmüştür, bu da *Leprotl1* geninin sirkadiyen saatin düzgün işleyişinde bir görevi olduğunu gösterir.

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NOMENCLATURE

AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BMAL1	Aryl hydrocarbon receptor nuclear translocator-like
CaMK	Calcium/calmodulin-dependent protein kinase
CBP	CREB binding protein
CK2	Casein kinase 2
CK2-α	Casein kinase 2 alpha
CKI-δ	Casein kinase 1 delta
CKI-ε	Casein kinase 1 epsilon
CLK	Clock
CLOCK	Circadian locomotor output cycles kaput
CRE	Calcium/cAMP response element
CREB	Ca^{+2}/cAMP-responsive element-binding protein
CRY	Cryptochrome
CYC	Cycle
DBP	D-box binding protein
DMEM	Dulbecco's modified Eagle's Medium
E4BP4	E4 promoter-binding protein 4
EE	evening element
ELF	E74-like factor
FAA	Food anticipatory activity
FBXL3	F-box and leucine-rich repeat protein 3
FOXO1	Forkhead box protein O1

FRQ	Frequency
G6Pase	Glucose 6-phosphatase
GABA	α-aminobutyric acid
GPCR	G-protein coupled receptor
GSK-3	glycogen synthase kinase-3 homolog
H3	Histone 3
H4	Histone 4
HBS	Hepes buffered saline
HDAC3	Histone deacetylase 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLF	Hepatic leukemia factor
HNF4α	Hepatocyte nuclear factor 4 alpha
HSF1	Heat shock factor 1
ipRGCs	Intrinsically photosensitive retinal ganglion cells
LB	Luria broth
LEPROTL1	Leptin receptor overlapping transcript-like 1
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NAMPT	Nicotinamide phosphoribosyltransferase
NCoR	Nuclear receptor corepressor
NMDA	N-methyl-D-aspartate
NPAS2	Neuronal PAS domain-containing protein 2
NRE	Nuclear receptor element
OB-R	Leptin receptor
OD	Optical density
PACAP	Pituitary adenylate cyclase-activating peptide
PARP1	Poly-ADP-ribosyl transferase 1

PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate carboxylase
PER	Period
PGC-1α	Proliferator-activated receptor-γ coactivator
PKA	Protein kinase A
PML	Promyelocytic leukemia
PMSF	Phenylmethanesulfonylfluoride
PP5	Protein phosphatase 5
PPARα	Peroxisome proliferative activated receptor alpha
PRR	Pseudo-response regulator
PTR	Posttranslational regulation loop
REV-ERBα	Nuclear receptor subfamily 1, group D, member 1
RevRE	REV-ERB response element
RHT	Retino-hypothalamic tract
RORE	ROR-responsive element
RORα	RAR-related orphan receptor A
SCN	Suprachiasmatic nucleus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SOAT1	Sterol O-acyltransferase 1
SREBP1c	Sterol element binding protein 1c
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-related modifier protein
TEF	Thyrotroph embryonic factor
TGN	Trans-Golgi network
TIM	Timeless
TOC1	Timing of CAB expression 1 protein

TTO	Transcription/translation oscillator model
TTR	Transcriptional-translational feedback regulation loop
USP8	Ubiquitin carboxyl-terminal hydrolase 8
VIP	Vasoactive intestinal polypeptide
VPAC2	Vasoactive intestinal peptide receptor 2
WC-1	White collar 1
WC-2	White collar 2
WCC	White collar complex
βTrCP	β-transducin repeat-containing protein

Chapter 1

INTRODUCTION

The circadian clock is a biological timing system that organizes daily incidents and physiological rhythmic alterations to anticipate 24-h environmental light/dark cycle and adapt to the external conditions that come with the Earth's daily rotation around its axis. Numerous physiological and behavioral processes including energy metabolism, body temperature, sleep-wake cycles, locomotor activity, and mental alertness are under the control of the circadian clock in almost all kingdoms of life. Therefore, perturbation of circadian rhythms is expectedly behind basically all human diseases. The mammalian circadian clock is hierarchically organized into the central and the peripheral clocks. The central oscillator is located as a small cluster of neurons named suprachiasmatic nucleus in a subregion of hypothalamus. Light serves as the most powerful input to entrain the central oscillator to the environmental light-dark cycle. The central pacemaker then transmits the external signal to synchronize the peripheral clocks that exist in both neural and non-neural tissues.

Molecular architecture of the mammalian circadian clock comprises a network of transcriptional-translational feedback loops with both positive and negative limbs. BMAL1/CLOCK heterodimer acting as positive elements activates the transcription of PER, CRY and other clock-controlled genes through binding to E-box regions of target genes. Accumulating PER and CRY eventually represses their own transcription by inhibiting the activity of BMAL1/CLOCK complex. As PER and CRY levels decrease

by proteosome-mediated degradation, inhibition on BMAL1/CLOCK is relieved, and this cycle repeats every 24 h.

Although abundant number of core clock components and clock regulators were identified, there are still many more to be discovered. For discovery of novel clock components, the naive Bayesian integration strategy is a commonly used and advantageous method. In this thesis work, 55 high probability clock components were cloned and screened for possible interactions with known core clock components PER1, PER2, PER3, CRY1, CRY2, CLOCK, BMAL1, and NPAS2. Among all the screened proteins, LEPROTL1 was found to interact with CRY1 and CRY2, and SOAT1 interacted with mCRY1 in mammalian two-hybrid assay. It was also shown that depletion of *Leprotl1* by siRNA transfection resulted in either amplitude damping or phase shift of the circadian rhythms in different cell lines.

In Chapter 2, detailed information about circadian clocks, molecular mechanisms of the molecular clockwork in different organisms, and relationship of circadian clocks with metabolism are introduced.

In Chapter 3, used materials and experimental methods are explained in detail. Generation of constructs, mammalian two-hybrid assay, and biochemical validation of protein expression is included in this chapter.

Chapter 4 presents the obtained results in this thesis work, and the results are discussed with concluding remarks in Chapter 5.

Chapter 2

LITERATURE REVIEW

2.1. Overview of Biological Rhythms

All life forms have been subject to miscellaneous universal rhythms for ages. These rhythms have governed much of the living activities and have been subsequently internalized in physiological to molecular levels of organismal composition. There are numerous different geophysical cycles with various periods: daily (24 hours ± 30 seconds), seasonal (365.24 days), lunar (29.53 days) and tidal (12.8 hour) cycles. Most of the behaviors are coordinated in light of temporal cues which are provided by these rhythms. Among the behaviors synchronized by geophysical cycles are daily feeding patterns, daily and seasonal migration, growth, reproduction, and hibernation. Most of the non-equatorial animals breed at certain times of the year to save energy. The lives of most non-equatorial species are under the control of the seasonal changes as food availability fluctuates depending on the seasonal alteration in day length and temperature. For instance, the Palolo worms release sperm and eggs by detaching the terminal parts of their bodies during the last quarter of the moon in October and November. Although their reproduction period was shown to be independent of the direct effect of lunar illuminance, this incident indicates that the Palolos have an endogenous circa-lunar clock. Moreover, the female menstrual cycle of humans conceivably reflects the lunar cycle [1-5].

Aside from the geophysical cycles, biological rhythms constitute another class of regular quantitative changes. There are three families of biological rhythms with different endogenous periods: circadian, ultradian and infradian rhythms. Circadian rhythms are the cycles repeating every 24 h. Sleep-wake cycles, body temperature, metabolic processes, feeding–fasting, hormonal release, renal activity, and locomotor activity are several examples of circadian rhythms. Ultradian rhythms, on the other hand, correspond to cycles that occur with a period shorter than the period of circadian rhythms, ranging from seconds to hours. Among numerous ultradian cycles are blood circulation, blinking, pulse, hormonal release, heart rate, thermoregulation, urination, bowel activity, nostril dilation, breath cycle, and appetite. In contrast to ultradian rhythms, infradian rhythms refer to the oscillations occurring with a lower frequency compared to circadian rhythms, extending from days to seasons. Some examples of ultradian rhythms include seasonal hibernation, annual migration or reproduction cycles of some animals, and menstrual cycle of humans [6-9]. Recent studies indicate that these rhythms are interdependent and they collaborate each other from plants [10] to man.

2.2. Daily Rhythms and the Circadian Clock

Circadian rhythms have mostly attracted the attention of scientists [1] as virtually all living entities have evolved a biological timing system to anticipate 24-h environmental light/dark cycle and adapt to the external conditions that come with the Earth's daily rotation around its axis [11-19]. The physiology and behavior of living systems operates in a circadian manner. Therefore, organisms have a supremely precise internal biological clock (i.e., circadian clock) that organizes daily incidents and synchronizes physiological rhythmic alterations with the course of a day [20-23]. Circadian clocks serve to engender self-sustaining, cell-autonomous rhythms with a frequency of approximately 24 hr [11]. Since organisms possessing circadian clock acquire a

predictive mechanism to anticipate recurring environmental alterations, the biological clock has been shown to provide an important selective advantage and confer fitness to living entities during the evolution [24-27]. For instance, *Arabidopsis thaliana* was demonstrated to produce biomass most efficiently only when the duration of environmental light/dark cycle corresponds to that of the innate clock [25]. In addition, some species of the cyanobacteria that are able to make use of varying light conditions by keeping themselves synchronized with the light/dark cycle surpassed incapable cyanobacteria species in terms of growth rate. [28]. Furthermore, animals encounter physiological course of actions at the correct time of day for hunting, bewaring of predators and finding partners, which correspondingly confers survival advantage [17, 29, 30].

2.2.1. History

The connection between geophysical cycles and the circadian clock was first identified in 1729 by the French scientist Jean-Jacques d'Ortous De Mairan. De Marian revealed that a heliotrope plant, *Mimosa pudica* has an internal clock based on the observation that opening and closing its leaves persisted in a daily manner even it was placed in a dark, sealed box. From that day forward, analogous circadian rhythms have been demonstrated to exist in virtually all living organisms [31, 32]. One of the core genetic components of the circadian clock, period (*per*) was discovered by Ron Konopka and Seymour Benzer in 1971. They demonstrated long-period, short-period, and arrhythmic mutant phenotypes that are distinguishing characteristics of circadian clock using *Drosophila melanogaster* as a model system [33, 34]. The endogenous period of long-period mutants is more than 24 hours in the absence of environmental cues like light and temperature, whereas short-period mutants have an endogenous period of less than 24 hours. On the other hand, arrhythmic mutant organisms do not display any apparent circadian pattern in the absence of external signals [35]. As techniques following

research tools in the field of molecular genetics have been developing since the mid-twentieth century, studies on the rhythm-generating circadian timing system have eminently expanded [36]. In 1994, another core clock component, the circadian locomotor output cycles kaput (*Clock*), was discovered by Takahashi group for the first time. The group initially described a mutation in mice, which later led to cloning of the mutated gene (i.e., *Clock*) [37, 38]. The discovery of the *Clock* gene is recognized as a critical breakthrough for circadian clock research since *Clock* mutant mice were found to have a lengthened activity period. *Period* and *Clock* genes were both identified by random mutagenesis in two different model organisms, and demonstrated to be the central players of the circadian clock mechanism [17, 39]. Researchers continued seeking further elements that take a role in the circadian clock machinery, and subsequently discovered plenty of new constituents in a short period [35]. In the last decade, circadian clock studies went through great advances in many aspects. Numerous novel components were discovered, through which mechanistic insights about oscillators were gained. Above all, the biological importance of clock was determined in medicine and agriculture [35, 36].

2.2.2. Biological importance

The circadian timing system functions to make various biological activities operate in the most efficient manner during the day by providing prediction mechanisms for environmental alterations. Furthermore, circadian oscillators serve to distinguish between conflicting biological incidents like sleeping and feeding [12]. Among the physiological and behavioral processes that are under the control of the circadian clock are energy metabolism, gastrointestinal tract motility, cardiovascular activity, endocrine secretion, body temperature, renal activity, locomotor activity (i.e., periodic moving behaviors of an animal) [14, 17, 29, 35], mental alertness, physical strength, blood pressure, blood viscosity [26, 40], sleep-wake cycles in animals, cell division and

conidiation in fungi, and leaf movement and photosynthesis in plants. Regulation of these processes is accomplished on a behavioral level or in individual tissues and cells [23]. As the circadian clock acts extensively on human physiology, perturbations of daily running oscillations have an expected impact on basically all human diseases [26]. Control of different biological processes by the circadian clock has been demonstrated by occurrences of several health problems including hypertensive crises, asthma and allergy attacks [41-43], metabolic and psychological disorders [11], myocardial infarction, fatigue, disorientation, insomnia and distorted hormone profiles as seen in jet-lagged travelers, and high morbidity as seen in shift workers and when synchronization between internal clock and external cues is disrupted [44-46]. Since the circadian timing system seems to be associated with various diseases, it stands to reason that treatments against these diseases might be developed by targeting circadian clock machinery [11]. Therefore, understanding the molecular mechanism of the circadian clock entirely is crucial for enlightening the connection between clock perturbation and human diseases [47].

2.2.3. Characteristics and entrainment

There are basically three characteristics to define an oscillation: periodicity, entrainment, and robustness. Periodicity refers to recurrence of a clock oscillation wave at regular intervals. As an attribute of the circadian clock, period length is persistent (free-running) and sustained under even constant conditions. Moreover, intrinsic rhythm of the circadian clock exists without any sensory input from the environment. Circadian oscillators are entrained by external cues such as light. In other words, period or phase of the endogenous clock machinery is reset to its equivalent of an external rhythm. In contrast, oscillations are resistant to broad variations in temperature, a phenomenon known as temperature compensation. Robustness is another characteristic of an oscillation and it refers to resilience of an oscillator to intrinsic or extrinsic

perturbations [26, 35, 48]. These main attributes of circadian clocks were established by research of Pittendrigh and Aschoff in the second half of twentieth century [32]. The parameters used to characterize the circadian clock are period, amplitude, and phase. Phase is the initial angle of a clock, period refers to the time difference between two successive peaks of a clock oscillation wave, and amplitude is the magnitude change between the peak and base points of a rhythmic wave [35].

Numerous factors, which are defined as *Zeitgebers* (cf German Zeit or "time," and Geber, or "giver"), shape the peak and trough points of circadian oscillation waves of physiological functions. These cues include feeding, emotional stress [26], exercise [49], social signals [50], temperature cycles [51], and sleep deprivation [52]. The most potent *Zeitgeber* that orchestrates the circadian phases to the environment, however, is the light [26]. Therefore, circadian timing system has been theoretically interpreted as a pacemaker that entrains to many different environmental signals through input pathways, and organizes various periodic outputs [19].

Circadian system is constituted by hierarchical organization of organs in multicellular organisms such as mammals. In this complex structure, a key role for orchestrating the whole circadian system is mainly served by the brain and the liver. As individual cells within tissues possess circadian clocks [53], their synchronization within the tissue is required for the circadian machinery to operate accurately. Consecutively, tissues are brought in harmony with each other, providing a stable phase-relationship among various tissues. To achieve such a systematic synchronization, individual cellular clocks are stimulated by inputs from other cells. After incorporating the phase information about the time of stimulus occurrence into their endogenous clock mechanism, individual oscillators pass the output signal regarding clock information to other cells. The same route is followed at the systems level, with different input and output pathways [11]. Light, for instance, is initially perceived by eyes as an input signal from the environment, and then relayed to the suprachiasmatic nuclei (SCN) where input

signals are deciphered to entrain to the environmental time zone [29, 54]. Beginning phases of the circadian outputs, that is, certain behaviors and physiological activities [35] described in the previous section are then adjusted according to these signals. Subsequently, tissues provide feedback information about regulated internal environment to the clock [55]. Therefore, the circadian timing system is conceptually regarded as a three-component complex organized into multiple interconnecting pathways: an input component, a clockwork component, and an output component (Figure 2.1) [26, 56]. This continuous synchronization of the circadian clock with environmental and internal body signals ensures the coordinate function of individual cellular clocks and the integration of tissue subnetworks into an ordered functional network system [11].

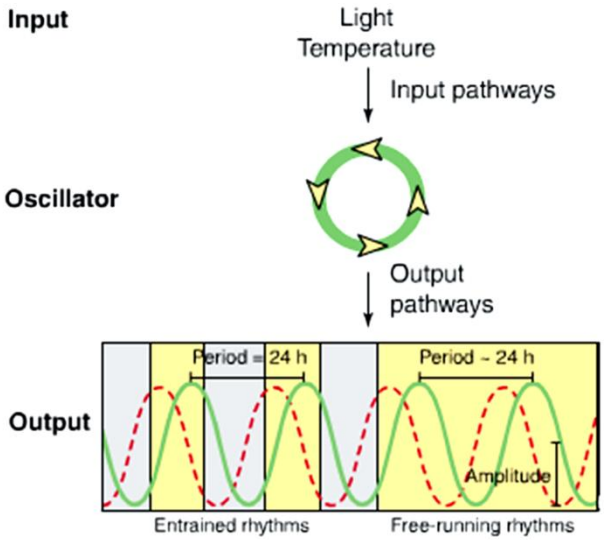


Figure 2. 1. Components of the circadian system. Adapted from *[57]*

2.3. Molecular Circadian Clockwork in Prokaryotes, Fungi, Plants, and Insects

The molecular machinery that operates in the absence of the external signals has been the primary focus of chronobiologists as most eukaryotes and some prokaryotes possess circadian clocks. In the last decade, significant contributions have been made to the understanding of the molecular bases of the circadian clocks in various organisms ranging from cyanobacteria to mammals. It has been found that the circadian clock components of these diverse organisms have similar roles in the molecular clock apparatus despite their structural differences. Central clock mechanisms, unlike the clock constituents, seem to be conserved among kingdoms [57]. Studies on the molecular bases of the circadian clocks using different model organisms established a common central mechanism of the circadian clocks [58]. All examined circadian clocks except for cyanobacterial clock have at least one self-governing internal oscillator that consists of autoregulatory feedback loops relying on positive and negative elements [34]. Negative elements refer to the proteins encoded by the clock genes. These clock proteins block the activity of positive elements that function as transcriptional activators of the clock genes, thereby suppressing their own expression. As the amount of the clock proteins decreases hindrance on positive elements is relieved, leading to re-initiation of the oscillator cycle (Figure 2.2) [58].

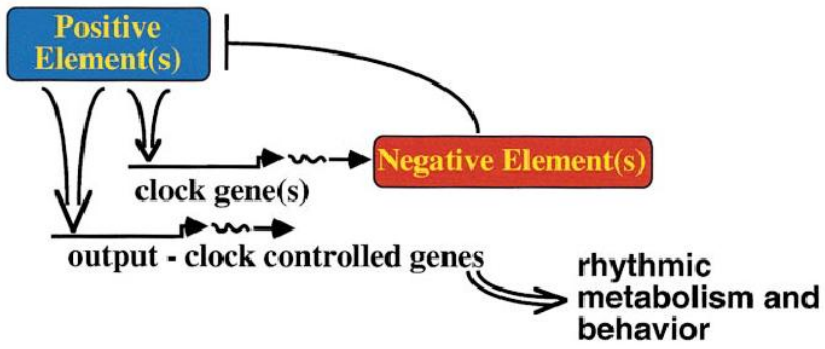


Figure 2. 2. Schematic illustration of the common mechanism of the circadian clocks. Adapted from *[13]*

2.3.1. *Synechococcus elongatus*

The simplest and the most ancient organism that have a circadian clock is a unicellular cyanobacterium, *Synechococcus elongatus*. Discovery of the circadian clock in cyanobacteria has shown for the first time that the circadian clocks exist in prokaryotes as well [13]. The cyanobacterial clock controls various activities such as cell division, amino acid uptake, nitrogen fixation, photosynthesis, and respiration [59]. There are mainly three proteins that regulate the core oscillator of the cyanobacterial clock: KaiA, KaiB, and KaiC [34, 60]. KaiC possesses autokinase, autophosphatase, and ATPase activities [61]. Moreover, both the abundance and the phosphorylation states of KaiC exhibit rhythmicity. KaiA stimulates the autophosphorylation of T432 residue of KaiC, and hyperphosphorylated state is reached after following autophosphorylation of S431 residue of KaiC. Hyperphosphorylated KaiC subsequently encounters spontaneous dephosphorylation [62] at T432, to which KaiB binds and inactivates KaiA, thereby enabling further dephosphorylation of KaiC [63-65]. *KaiA*, *kaiB*, and *kaiC* are periodically transcribed. Since *kaiB* and *kaiC* are co-transcribed from a single promoter, overexpression of *kaiC* represses the expression of both *kaiB* and *kaiC*. Thus, KaiC protein acts as a negative element that suppresses its own expression. In contrast, KaiA boosts the transcription of *kaiBC* when overexpressed, functioning as a positive element [57, 60]. There are two regulatory loops in the cyanobacterial timing system: a posttranslational regulation (PTR) loop, and a transcriptional-translational feedback regulation (TTR) loop. The output of PTR is the rhythmic phosphorylation circuit of KaiC [66], while TTR is involved in the control of *kaiBC* promoter activity [67]. Although oscillations are generated by PTR independently of TTR, cells lose synchrony in the absence of TTR circuit. Therefore, combination of the PTR pacemaker and the TTR regulator confers robustness and stability to the cyanobacterial circadian clock [68].

2.3.2. *Neurospora crassa*

The circadian clock of *Neurospora* consists of a positive element, the white collar complex (WCC), and a negative element, the frequency (FRQ) [34]. White collar complex is formed by the heterodimerization of two nuclear transcription factors, WC-1 and WC-2. Circadian oscillations are generated relying on the WCC-mediated periodic transcription of the *frq* clock gene [69, 70]. According to the proposed transcription/translation oscillator (TTO) model, the WCC is a transcriptional activator of the *frq*. The FRQ protein, on the other hand, translocates back into the nucleus and blocks the WCC activity to repress its own transcription, and promotes the WC-1 synthesis. This dual role of the FRQ protein results in the rhythmic levels of the FRQ [71-73].

2.3.3. *Arabidopsis thaliana*

The plant clock consists of interlocked morning and evening feedback loops that are formed by the interaction of transcription factors acting as repressors of gene expression [74-76]. Two morning-phased (i.e., proteins that are expressed during the day) transcription factors, CCA1 and LHY repress the expression of an evening-phased (i.e., proteins that are expressed during the night) pseudo-response regulator, timing of CAB expression 1 protein (TOC1) by binding to a cis-regulatory element named the evening element (EE) found in the promoter regions of most evening-phased clock genes [77, 78]. Conversely, TOC1 acts as a repressor of CCA1 and LHY during the night [79-82]. In another negative feedback loop, CCA1 and LHY activate the transcription of the pseudo-response regulator 7 and 9 (PRR7 and PRR9), which in turn repress CCA1 and LHY [83, 84]. Lastly, TOC1, GI, LUX, ELF3 and ELF4 act in double negative feedback loops with CCA1, LHY, PRR7 and PRR9 [85-88]. To sum up, most of the identified *Arabidopsis* clock components are repressors of other constituents of the

circadian clock. However, a recently identified protein, RVE8 has been shown to primarily activate the transcription of EE-containing clock and output genes. Therefore, the existing model of the plant clock has been revised to a great extent [89].

2.3.4. *Drosophila melanogaster*

As in other organisms, the *Drosophila* clock is composed of a core transcription/translation feedback loop in which the transcription factors clock (CLK) and cycle (CYC) function as transcriptional activators of period (*per*) and timeless (*tim*) genes, and other direct target genes during the day. At night, however, PER and TIM proteins dimerize and translocate to the nucleus where they repress their own transcription, acting as negative elements of the feedback loop [90]. Inhibition of *per* and *tim* transcription is achieved by a direct interaction between CLK/CYC and PER/TIM, as well as by posttranslational modifications [91, 92]. Different kinases including DOUBLETIME, casein kinase 2 (CK2), glycogen synthase kinase-3 homolog (GSK-3) and SHAGGY gradually hyperphosphorylate PER and TIM during the early night [93, 94]. Hyperphosphorylated proteins then inhibit the activity of the CLK/CYC complex by interacting with the complex [95]. Hindrance on the CLK/CYC complex is relieved in the morning when PER and TIM start to be degraded, for which specific E3 ubiquitin ligases are required. Specifically, SLIMB and JETLAG are responsible for the proteosome-mediated degradation of PER and TIM, respectively [96-98]. Recently, a deubiquitylating enzyme, ubiquitin carboxyl-terminal hydrolase 8 (USP8) has been shown to contribute to the rhythmic inhibition of the transcription by deubiquitylating the CLK in a circadian manner. The CLK/CYC complex directly activates the transcription of *usp8* as in the case of several other direct target genes. However, the transcriptional activity of the CLK/CYC complex is down-regulated after deubiquitylation of the CLK. In other words, USP8 represses its own transcription as well as transcription of other direct target genes by this means [99].

2.4. Molecular Mechanism of the Mammalian Circadian Clock

Early studies on the mammalian molecular clockwork demonstrated an evidently straightforward mechanism in which the basic helix–loop–helix transcription factors, BMAL1 and CLOCK heterodimerize and activate the transcription of period 1 (PER1), period 2 (PER2), cryptochrome 1 (CRY1) and cryptochrome 2, (CRY2) by binding to the E-box elements in their promoter regions (CACGTG) during the subjective day. In the brain tissue, CLOCK is functionally replaced by its homologue neuronal PAS domain-containing protein 2 (NPAS2) [100, 101]. As PER and CRY proteins accumulate, they dimerize and translocate back into the nucleus to repress their own transcription and that of other clock-controlled genes by inhibiting the activity of BMAL1/CLOCK heterodimer. PER/CRY complexes undergo proteosome-mediated degradation during the subjective night, thereby relieving the inhibition on the transcriptional activity of the BMAL1/CLOCK heterodimer, and allowing the initiation of a new cycle (Figure 2.3) [12, 22]. This transcriptional-translational feedback loop (TTL) that is composed of a positive and a negative limb takes approximately 24 hours [20], and transcription and translation of PERs, CRYs, and other clock-controlled genes exhibit an oscillatory pattern by this means. This model, however, has been later shown to be too simple to clarify the mammalian circadian clock [36].

According to the current model, a network of interconnected transcriptional-translational feedback loops with a number of supporting mechanisms including posttranslational modifications of proteins constitute the mammalian circadian oscillator [11, 21, 29, 35, 102]. One of these additional feedback loops named the stabilizing loop is intertwined with the core BMAL1/CLOCK-PER/CRY loop described above [12]. This secondary feedback loop is composed of the orphan nuclear receptors REV-ERBα and RAR-related orphan receptor A (RORα) [90]. *Rev-erbα* (*Nr1d1*) and *Rora* are the direct targets of the BMAL1/CLOCK heterodimer, thus their expression is under the control of this complex [20, 34]. There is a competition between REV-ERBα

and RORα for binding to the ROR-responsive element (RORE) located in the *Bmal1* promoter. RORα is a transcriptional activator of the *Bmal1*, whereas REV-ERBα represses the *Bmal1* trancription. Furthermore, physical interaction of PER2 with REV-ERBα fine-tunes the regulation of the *Bmal1* expression [103]. REV-ERBα also represses the transcription of the *Clock* by binding to the REV-ERB response element (RevRE) within the first intron of the *Clock* gene [104]. Thus, these two orphan nuclear receptors regulate their own activators [105-108]. Additional transcriptional circuits of the mammalian clock involve the nuclear receptor elements (NREs) to which nuclear receptors including REV-ERBα, peroxisome proliferative activated receptor alpha (PPARα), and glucocorticoid receptor bind through interacting with PER2 [106]. D-box elements have also important roles in the regulation of circadian transcription [102]. PAR-Zip family transcription factors such as D-box binding protein (DBP), hepatic leukemia factor (HLF), and thyrotroph embryonic factor (TEF) occupy the D-box elements and activate transcription of target clock genes, whereas E4 promoter-binding protein 4 (E4BP4) acts as a repressor [109]. As these transcription factors undergo E-box-mediated transcription, they indirectly control the expression of the clock-controlled genes in a circadian manner (Figure 2.4) [110].

Posttranslational modifications of proteins are indispensable elements of the circadian clockwork in addition the transcription-translation feedback loops. In the first place, phosphorylation of CRYs, CLOCK and BMAL1 by various kinases including casein kinase 1 epsilon (CKI-ε) and casein kinase 1 delta (CKI-δ) influences the subcellular distributions, stabilities and activities of these core clock proteins. Phosphorylation is essential for ubiquitin ligases to be recruited, eventually leading to proteosome-mediated degradation of ubiquitylated proteins. PERs that are phosphorylated by CKIs acquire binding sites for an E3 ubiquitin ligase, β-transducin repeat-containing protein (βTrCP) [111, 112]. CRYs are phosphorylated by AMP-activated protein kinase (AMPK), and then ubiquitylated by another enzyme, F-box and leucine-rich repeat protein 3 (FBXL3) [113]. Phosphorylation of BMAL1 by casein kinase 2 alpha (CK2-

α), on the other hand, has an important role in the nuclear accumulation of BMAL1 [114]. Phosphatases regulate the activity of kinases in a way that autophosphorylated CKI-ε, for instance, is incapable of phosphorylating PER2 unless the protein phosphatase 5 (PP5) removes the phosphate group from the kinase [115]. As distinct from PERs and CRYs, BMAL1 undergo a different posttranslational modification as well, SUMOylation, a process by which small ubiquitin-related modifier protein (SUMO) is added covalently to lysine residues. Proper SUMOylation of BMAL1 requires its interaction with CLOCK [116]. Moreover, it has been recently shown that SUMOylation of BMAL1 is directly correlated with the turnover of the BMAL1 protein [117]. SUMOylated BMAL1 is localized solely to promyelocytic leukemia (PML) nuclear bodies. Subsequently, its transactivation and ubiquitin-dependent degradation are simultaneously promoted [118]. A second modification of BMAL1 is acetylation which is a necessary process for sustainability of the circadian oscillations [119]. Moreover, transcription rates of the clock genes are controlled by the rhythmic acetylation/deacetylation and methylation/demethylation of the histones in the promoter regions of the clock genes [120]. Finally, the CLOCK protein has been reported to be a histone acetyltransferase. Transcription of the genes regulated by BMAL1/CLOCK heterodimer is activated upon acetylation of H3 and H4 in the promoter regions of these genes [121]. BMAL1 is also acetylated by CLOCK, which is required for CRYs to interact with the BMAL1/CLOCK complex, and exert its transcriptional repressor activity [122].

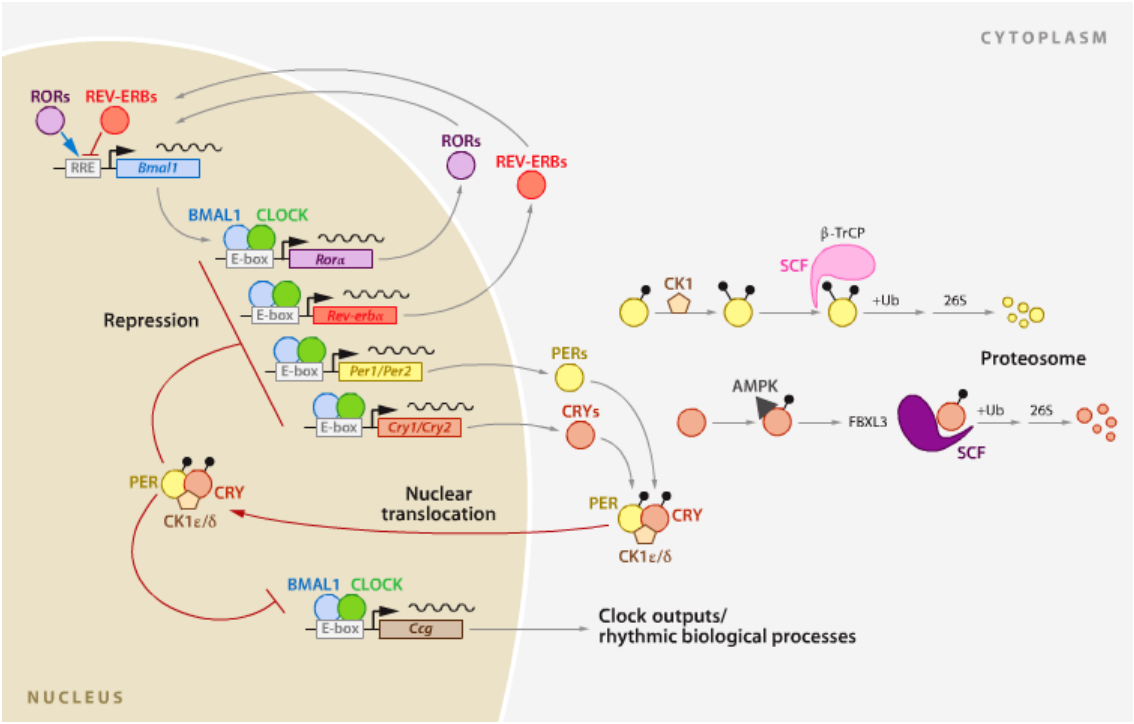


Figure 2. 3. Molecular mechanism of the mammalian clock. Adapted from *[20]*

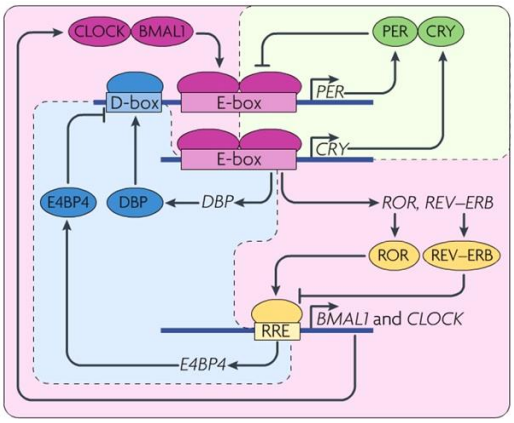


Figure 2. 4. Interlocking feedback loops of the mammalian molecular clockwork. Adapted from *[35]*

2.5. The Central Circadian Oscillator

In the mammalian circadian clock, the central circadian pacemaker that coordinate and keep all the circadian clocks in synchrony is the suprachiasmatic nucleus (SCN) located in the hypothalamus, just above the optic chiasm. Function of the SCN as the master circadian pacemaker was discovered after lesion and transplantation studies, in which researchers observed disruption of rhythmicity when the SCN was ablated [123]. The SCN is a bilaterally paired neuronal organization consisting of ~20,000 neurons that individually have cell-autonomous oscillators. These individual cells are synchronized and oscillate coherently. The oscillations engendered in the SCN turn to neuronal or humoral signals, by which the entire timing system is entrained to light/dark cycle throughout the body [124]. Therefore, the SCN acts as a transmitter between the external light/dark cycle and the internal circadian clocks [125] as it is a direct target of the retinal fibers [29].

2.5.1. Photoreception

Light input is sent to the SCN from intrinsically photosensitive retinal ganglion cells (ipRGCs). Intrinsic photosensitivity of ipRGCs to short-wavelength irradiation is solely conferred by melanopsin, a novel photosensitive G-protein coupled receptor (GPCR) expressed in the ipRGCs. [126]. Since melanopsin is primarily involved in the transduction of the light input to the SCN [127], melanopsin-mediated light entrainment of the circadian clock is achieved even in certain blind mammals who lose all the rod and cone cells that contain photosensory pigments, rhodopsin and color opsins, respectively. In individuals with a normal vision, input received from rods and cones is integrated with melanopsin signals by the ipRGCs, and then efferent projections are sent to the SCN via the retino-hypothalamic tract (RHT) that is formed by the ipRGCs (Figure 2.5) [128].

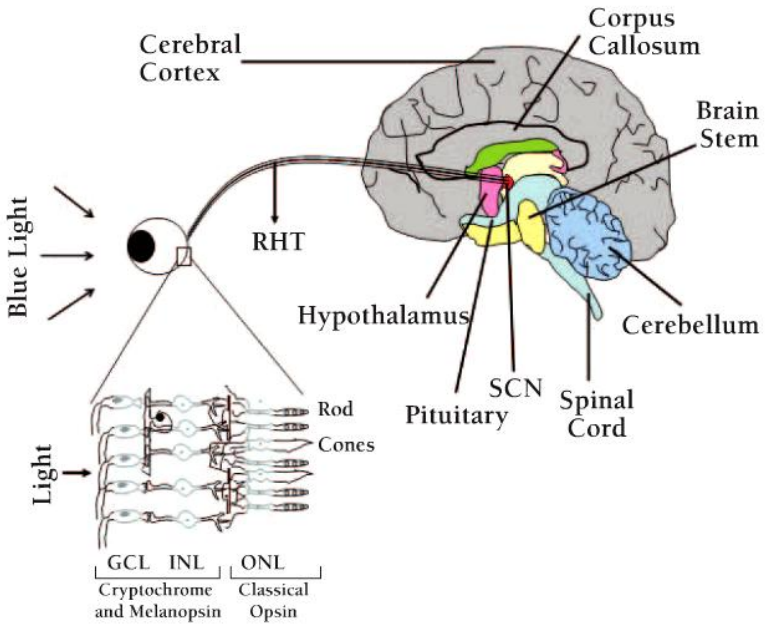


Figure 2. 5. Circadian photoreception by eyes and transmission of light input to the SCN. Adapted from *[26]*

2.5.2. Entrainment of the Central Pacemaker

Coupling and coordinated oscillations of individual neurons in the SCN are crucial for generation of a coherent circadian output. However, only a few molecules that act as synchronizer of the individual SCN cells were identified [34].

2.5.2.1. Light

ipRGCs upon light stimulation of retina during the subjective night release glutamate and pituitary adenylate cyclase-activating peptide (PACAP) at the terminal synapses of the RHT where they project their axons into the SCN [129]. The neuropeptide PACAP increases the effects of glutamate, acting as a modulator [130]. Glutamate, on the other hand, activates the glutamate receptors, NMDA and AMPA during the night, leading to membrane depolarization. Subsequently, calcium flows into the cytoplasm directly through NMDA receptors and indirectly through voltage-gated calcium channels [131]. Certain kinases including calcium/calmodulin-dependent protein kinase (CaMK), mitogen-activated protein kinase (MAPK), and protein kinase A (PKA) are activated by increasing amount of the intracellular calcium, which then phosphorylate and activate the transcription factor Ca^{+2}/cAMP-responsive element-binding protein (CREB) at Ser133 and Ser142 residues [132, 133]. Activated CREB subsequently binds to calcium/cAMP response elements (CREs) of genes in concert with auxiliary proteins such as CREB binding protein (CBP), and activates the expression of target genes including *Per1* and *Per2* in the SCN during the subjective night (Figure 2.6) [134]. Consequently, activation of *Per* expression during early night, when PER levels normally drop off causes delay in the oscillation, whereas phase of the oscillation is advanced when *Per* expression is induced during the late night as PER levels start to increase earlier than usual. Therefore, stimulation of *Per* transcription by the light is critical for the regulation of the endogenous circadian rhythm [135].

amplitude of oscillations generated by individual neurons as well as keeping them synchronized with one another. VIP was shown to be required for the SCN core to exert its synchronizing function as loss of either VIP or VPAC2R resulted in unstable, low amplitude and out of sync oscillations of individual neurons [15].

2.5.2.2. VIP

Vasoactive intestinal polypeptide (VIP) is the most abundant neuropeptide transmitter within the core region of the SCN, and release of VIP from the ventral part of the SCN oscillates [136, 137]. Acting at the VPAC2 receptors, VIP closes potassium channels leading to membrane depolarization of the SCN neurons[138], and induces *Per1* and *Per2* expression (Figure 2.7) [139]. Roles of the VIP in the circadian clocks include retaining both the oscillation amplitudes generated by individual neurons, and the harmony among them.

2.5.2.3. GABA

α-aminobutyric acid (GABA) is another common neurotransmitter released by nearly all the SCN neurons. Administration of GABA to the dissociated SCN neurons was shown to cause phase shifts through GABAA receptors of GABA, and neurons were synchronized when GABA is administered in a daily manner [140]. However, synchronization of the SCN neurons were not disrupted when GABAA and GABAB antagonists are applied [141]. Therefore, GABA appears to be responsible for SCN coupling although it is not necessarily involved in this process [22].

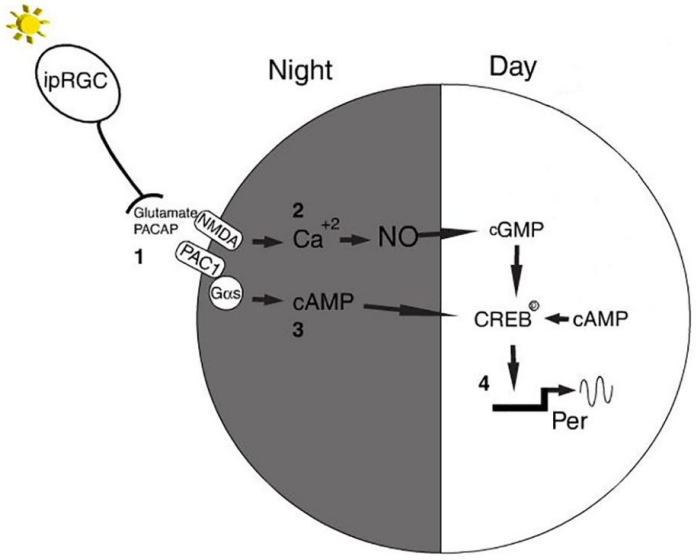


Figure 2. 6. Schematic illustration of the SCN synchronization to light/dark cycle. Adapted from *[15]*

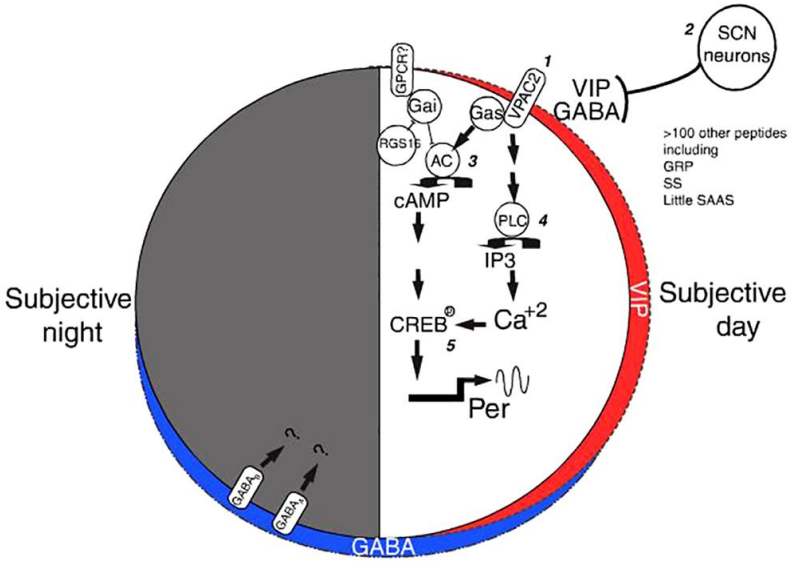


Figure 2. 7. Schematic illustration of how the SCN neurons are synchronized with one another. Adapted from *[15]*

2.6. Peripheral Clocks

There are multiple additional circadian clocks in peripheral tissues such as the liver, heart, lung and muscle besides the central pacemaker located in the SCN [34]. The core clocwork is preserved in these peripheral clocks, yet their entrainment mechanisms and the output pathways that they govern significantly diverge. 3 – 10% of all mRNAs in a given tissue oscillates in a circadian manner, and transcription of the corresponding genes that mostly differ in each tissue is driven by these peripheral clocks [142].

2.6.1. Resetting of the Peripheral Clocks

As biological clocks require entrainment to external cues for proper functioning of the entire system, peripheral oscillators lose synchronization with one another and with the central clock when the SCN is disrupted [143]. Therefore, the circadian system requires the control of peripheral clocks by the SCN to operate accurately. However, local mechanisms also regulate the tissue-specific gene expression patterns in addition to the central regulation. These local signals include food availability, hormone metabolites, and temperature that act to reset the peripheral clock phases (Figure 2.8). Since the SCN is not entrained to feeding, changing the time of food intake uncouples the peripheral clocks from the phase of the SCN [11, 144].

2.6.1.1. Temperature

Unlike the central pacemaker, peripheral oscillators are very sensitive to external temperature fluctuations, and reset by even low-amplitude rhythms of temperature that are in the circadian range [144, 145]. Possible mechanism of entrainment of peripheral

clocks by temperature includes the transcription factor heat shock factor 1 (HSF1) as temperature cycles can drive the transcription of *Hsf1* that has an oscillatory pattern [146]. At the organismal level, humoral and neural pathways between the hypothalamic thermal center and the body may have roles in the temperature entrainment [147].

2.6.1.2. Hormonal control

Glucocorticoid hormone mostly attracted the attention as an entraining signal for peripheral clocks. The core clock genes, *Bmal1*, *Cry1*, *Per1*, and *Per2* have glucocorticoid-response elements in their regulatory regions to which glucocorticoid binds and activates the transcription of clock genes as well as a number of clock-controlled genes. Glucocorticoids are able to orchestrate the rhythmic expression of the most clock genes in the liver even in the presence of a lesioned SCN by inducing the nuclear receptors including peroxisome proliferator-activated receptor (PPARα), and hepatic transcription factor HNF4α. BMAL1/CLOCK controls the transcription of *Hnf4α* through its E-boxes. Glucocorticoid receptors act as transcriptional activators of *Per2*.[148-150]. PPARα also responds to glucocorticoid and positively regulates the transcription of *Bmal1* [151].

2.6.1.3. Feeding/fasting and nutrient sensors

Peripheral clocks, particularly liver clocks are dominantly reset by the feeding/fasting behaviors. Oscillation phases of the clock gene and protein expression in the liver are adjusted according to the scheduled mealtime. Restricted feeding during the subjective day has been shown to shift the liver clock phase to the opposing phase in mice, while AMP-activated protein kinase (AMPK) is stimulated by fasting, exercise or hypoxia

[152] resulting in the phosphorylation, followed by degradation of CRY1 [153]. Circadian clocks might be affected by food availability through nutrient sensing pathways, in which cellular NAD^+ and AMP levels that are modulated by feeding/fasting behavior serve as nutrient sensors [154]. As the AMP/ATP ratio increases, cells tend to stimulate ATP-producing pathways, and lessen the use of ATP-consuming pathways. Major AMP/ATP sensor AMPK is activated by a conformational change elicited to its catalytic a-subunit upon binding of AMP to its g-subunit. In this form, AMPK becomes a substrate for the liver kinase B1 (LKB1), and catalytic a-subunit gets phosphorylated [155]. In addition to direct regulation of CRY1 degradation, active AMPK also acts indirectly on PER2 by phosporylating CK1ε. Phosphorylated CK1ε then effectively phosphorylates PER2, resulting in accelerated degradation of PER2 [156]. AMPK exhibits a robust periodicity in the mouse liver, and has a significant role in coupling between metabolism and the circadian clock [11].

Another nutrient sensor is the ratio of oxidized to reduced forms of nicotinamide adenine dinucleotide (phosphate) ($NAD(P)^+$/NAD(P)H) which affects the binding of BMAL1/CLOCK and BMAL1/NPAS2 complexes to the E-boxes of target genes such that NADH and NADPH induces their binding, whereas NAD^+ and $NADP^+$ suppresses their activity on clock-controlled genes [157]. The link between the nutrient sensors and the circadian clock is in fact bidirectional since NAD^+ salvage pathway is mediated by the enzyme nicotinamide phosphoribosyltransferase (NAMPT), transcription of which is under the control of the clock machinery. Therefore, $NAD(P)^+$/NAD(P)H ratio oscillates daily in the liver [158, 159]. Behavioral and metabolic circadian oscillations have been shown to shift when NAD^+ rhythmicity is disrupted [160], highlighting the importance of nutrient sensors in the clock mechanism. NAD^+ is involved as a cofactor in numerous cellular enzymatic reactions including sirtuins-mediated protein deacetylation that is driven by the enzyme deacetylase sirtuin 1 (SIRT1) that is stimulated by acute nutrient restriction [161]. SIRT1 also shows circadian rhythmicity,

and takes part in the both positive and negative limbs of the feedback regulation of the circadian clock. SIRT1 drives rhythmic deacetylation of BMAL1, histone H3, and PER2 upon directly binding to BMAL1/CLOCK heterodimer. In this way, SIRT1 promotes degradation of PER2. Deacetylation of BMAL1, on the other hand, results in reduced *Per2* and *Dbp* expression since BMAL1/CLOCK transcriptional activity is suppressed [162, 163]. In addition, the feeding-dependent signaling is mediated by NAD^+-sensitive poly-ADP-ribosyl transferase 1 (PARP1) which also interacts with the clock machinery [164]. PARP1 poly-ADP-ribosylates CLOCK in a periodic manner, preventing the BMAL1/CLOCK complex from binding to its target genes, and induces PER2 expression [164]. Scheduled meals have been shown to entrain the oscillations of PARP1 activity [20]. Nuclear receptors including REV-ERB, ROR and PPAR, as well as several other metabolic transcription factors and transcriptional activators such as DBP and PGC1α are also involved in the metabolic regulation of the peripheral clocks. These proteins exhibit circadian oscillations in peripheral tissues [165]. REV-ERBα has significant roles in the regulation of hepatic gluconeogenesis, adipocyte differentiation, and lipid metabolism in addition to inhibiting the transcription of *Bmal1* [166]. Lipid metabolism, on the other hand, is regulated by RORα, a competitor of REV-ERBα [167]. PPARs are activated by fatty acids and their intermediates. Among the physiological processes that are modulated by PPARs are insulin sensitivity (PPARγ), fatty acid oxidation system in mitochondria (PPARα), lipid homeostasis, adipogenesis (PPARγ), and cell proliferation, differentiation and migration in wound healing and inflammatory processes [11, 168]. *Bmal1* expression is stimulated by PPARγ in the blood vessels [169]. Rhythmic regulation of lipid metabolism is achieved through direct or indirect interaction of PPARα and PPARγ with PER2 [103, 170]. PER3 also interacts with PPARγ, repressing the activity of this nuclear receptor [171]. Another clock modulator, peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) regulates the expression of *Bmal1* and *Rev-erbα* through coactivation of RORs. PGC-1α is activated by SIRT1 which inhibits

PPARγ activity [172-174]. Lastly, DBP is involved in the regulation of gluconeogenesis and lipogenesis [175].

Feeding homeostasis is under the control of circulating hormones such as ghrelin and leptin which send information about peripheral energy levels to the brain. Oxyntic gland cells in the stomach release ghrelin upon anticipating a scheduled mealtime. Subsequently, ghrelin receptors are activated, leading to promotion of feeding behavior as well as an advance in locomotor activity, that is, food anticipatory activity (FAA) [176]. The clock proteins PER1 and PER2 are coexpressed with ghrelin by oxyntic cells in a circadian manner, indicating a connection of the molecular clock machinery with rhythmic ghrelin release [177]. Leptin, on the other hand, is produced and released by white adipose tissue. Secretion of leptin causes suppression of food intake as well as stimulation of metabolic processes to waste excess energy storage by modulating the transcription of target genes such as transcription factor STAT3. Leptin has been shown to oscillate in a circadian fashion in the plasma of rats, therefore leptin receptors might be activated in a time-dependent manner [178]. However, details of the mechanism through which leptin exerts its effect is yet to be discovered. Taken together, these data illustrates the existence of a complex crosstalk between nutrient sensors and the molecular clock machinery at the level of peripheral tissues [179].

2.7. Circadian Regulation of Metabolism

Oscillating levels of hormones including insulin and glucagon from pancreas, adiponectin and leptin from adipose, and ghrelin from stomach [180] as well as periodic operation of metabolic processes reveal that metabolism goes through circadian alterations. Rhythmic activities of metabolic pathways are consistently reflected by levels and activities of the rate-limiting enzymes. These metabolic processes include glucose metabolism, lipid metabolism, bile acid metabolism, amino acid metabolism, fat and muscle mass regulation, oxidative metabolism, and xenobiotic metabolism [147, 181].

2.7.1. Glucose Homeostasis

Glucose, lipid and amino acid metabolisms are centrally modulated by the liver, function of which is under the control of the circadian clock. The rate-limiting enzymes of glycolysis and gluconeogenesis exhibit diurnal oscillations, and the former peaks in the early evening while the latter peaks in the early evening. One example is the rate-limiting gluconeogenic enzyme, phosphoenolpyruvate carboxylase (PEPCK) that is transcriptionally regulated by the liver clock. Two major transcription factors CREB and forkhead box protein O1 (FOXO1) drive the expression of PEPCK [182, 183]. Rhythmically released glucagon activates CREB, thereby PEPCK [184]. CRYs, on the other hand, repress PEPCK transcription through inhibition of glucagon and glucocorticoid signaling. Since CRYs repress the expression of gluconeogenic genes, loss of CRYs in the liver results in constitutively high levels of circulating glucose or glucose intolerance [185]. Unlike CRYs, hepatic depletion of BMAL1 results in inhibition of diurnal expression of glucose metabolic genes, hence hypoglycemia in the rest phase, while insulin release is impaired in pancreatic ß cells in the absence of

BMAL1. In contrast, hyperglycemia and hypoinsulinemia are developed when *Clock* is mutated [186, 187]. REV-ERBs and RORs also directly regulate the expression of PEPCK. Loss of REV-ERBs leads to high levels of circulating glucose in the fasting phase, while RORα stimulates Glucose 6-phosphatase (G6Pase) [108, 188].

2.7.2. Lipid Homeostasis

Circadian clocks directly or indirectly regulate the modulators of lipid metabolism that exhibit hepatic oscillation. REV-ERBα inhibits the transcription of lipid metabolic genes by recruiting the nuclear receptor corepressor (NCoR) and histone deacetylase 3 (HDAC3) [189]. Impairment of the REV-ERB/NCoR/HDAC3 complex as well as *Clock* mutation and BMAL1 depletion have been shown to cause hepatic steatosis and hyperlipidemia [108, 190, 191]. The hepatic and plasma levels of triglyceride, cholesterol, and lipoproteins are regulated by REV-ERBα through modulation of the diurnal oscillation of sterol element binding protein 1c (SREBP1c) which coordinates the fatty acid and triglyceride biosynthesis [192, 193]. Fatty acid oxidation is stimulated by PPARα that peaks in the early evening. Consumption of fatty acids is induced at the beginning of the night-time feeding phase through the synchronized oscillation of the PPARα, the PPARγ-coactivator (PGC-1α), and the amplifier of the PPARα/PGC-1α module (lipin 1). By this means, the hepatocyte is prepared to meet the elevated energy expenditure from physical activities [147].

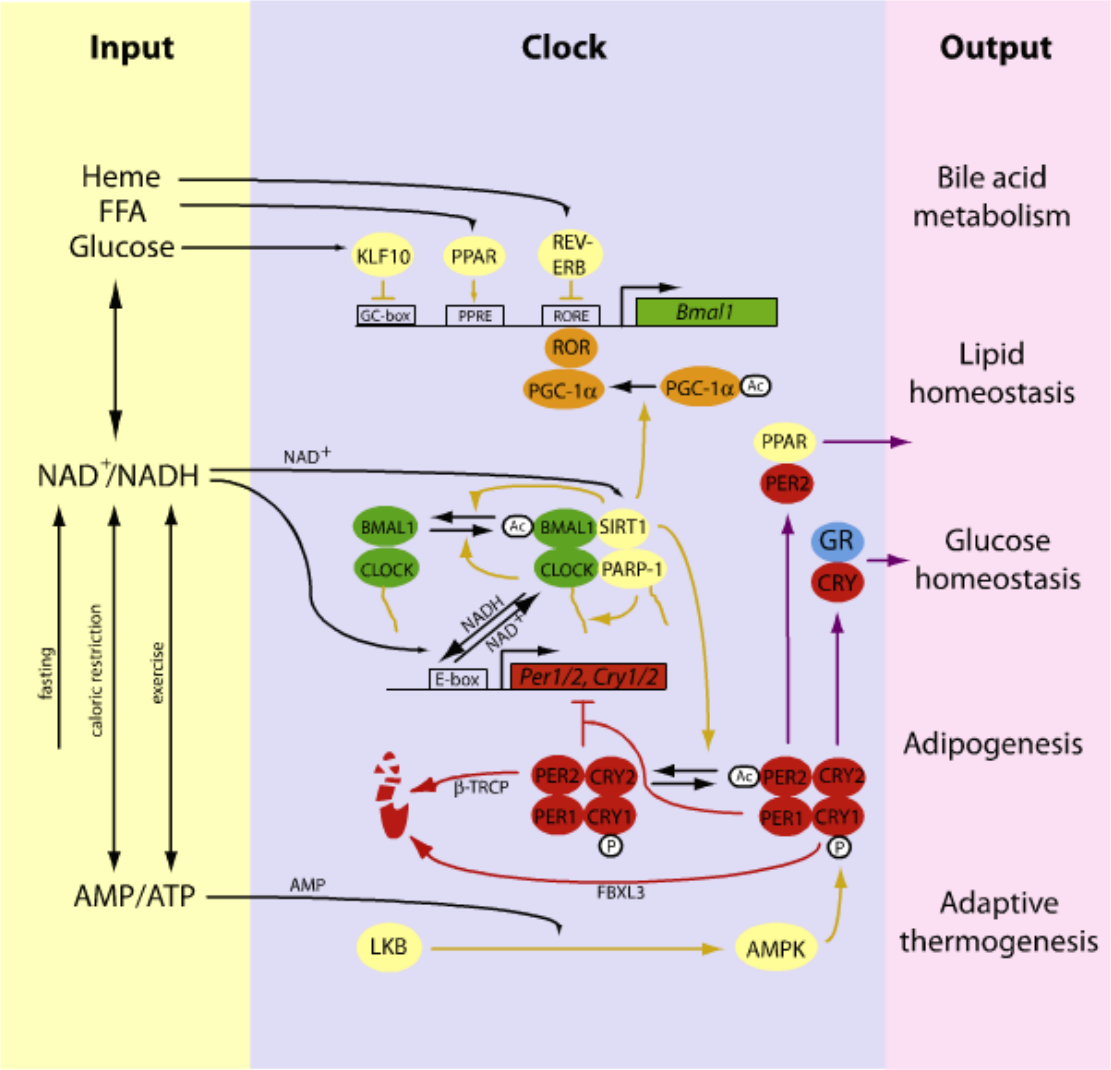


Figure 2. 8. Schematic illustration of the interactions between metabolic input signals (yellow box) and metabolic regulators (yellow, orange) with clock components of the positive limb (green) and the negative limb (red) of the circadian clock mechanism in the liver. Adapted from *[11]*

2.8. Discovery of Novel Clock Components

A great number of the core clock components were identified through various methods including phenotype-driven or forward mutagenesis and reverse genetics, behavioral phenotyping, and molecular cloning [33, 194]. In the forward mutagenesis approach, random mutagenesis is applied to the germ line, and the offspring of the mutagenized animals are subsequently screened for abnormal circadian phenotype. Subsequently, genes affected by these mutations are identified using candidate gene and positional cloning methods. Circadian clock genes *period* and *Clock* have been discovered through this method in both fruit flies and mice. The first gene discovered to be a component of the circadian clock is *period*. It was identified by Konopka and Benzer in 1971 using *Drosophila melanogaster* as a model system. As a result of screening 2000 descendant fruitflies, they identified three mutant clock phenotypes, namely long-period, short-period, and arrhythmic [33]. About 10 years later, another group showed that all three of these mutations occurred on the same gene, *period*. Similarly, the *Clock* mutants discovered through the screen exhibited an abnormally long period of circadian activity in mice. This phenotype was heritable, and circadian rhythmicity eventually disappeared in mice homozygous for the mutation after several days in constant darkness. This showed that *Clock* is required for normal mammalian circadian function [38]. *Bmal1*, on the other hand, was discovered using a different approach in which iterative search of human expressed sequence tags was employed using bHLH-PAS domains of the huAHR, huARNT, drSIM, and the PAS domain of drPER as query sequences [195]. The involvement of *Bmal1* in the circadian clock machinery was proved when phenotypic alterations of the circadian rhythms in *Bmal1*-deficient mice were investigated. The loss of BMAL1 resulted in immediate and complete loss of circadian rhythmicity in constant darkness [196]. On the other hand, cryptochromes were first related to the circadian clock when mouse CRY1 was found to be highly expressed in the suprachiasmatic nucleus (SCN) with a rhythmic expression pattern.

Function of CRYs in the clockwork was proved after observing long-period and phase-shift phenotypes in CRY2 knockout mice [194].

In addition to these elegant experimental methods, the process of clock gene discovery is greatly accelerated using a naive Bayesian integration strategy by which publicly available, and experimentally derived genome-wide data sets are integrated, and a list of high probability core clock components are identified. There are five main attributes used in the naive Bayesian integration strategy to define a core clock component:

1. core clock transcripts oscillate with a frequency of ~24 hour
2. core clock proteins interact with other clock components
3. cellular and behavioral phenotypes are generated when core clock components are inhibited or knocked out
4. core clock components are ubiquitously expressed
5. core clock components are conserved through phylogeny

The naive Bayesian integration method assumes that all features are independent from each other given the value of the class variable, which is called conditional independence [197]. Since attributes that better differentiate the known core clock genes from non-clock genes have higher values in the ultimate classification schemes, the relative contribution of each attribute arises naturally [198, 199]. For each attribute of a core clock component, data sets are identified to evaluate the behavior of core clock components and all other genes in the genome. Following, the degree of enrichment for a gene at a particular point in the distribution, named Bayes factor, is calculated. These Bayes factors are derived from independent data sets; hence all genes in the genome are ranked from the highest probability to the lowest probability clock components.

2.8.1. Leptin receptor overlapping transcript-like 1 (LEPROTL1)

Leptin receptor overlapping transcript-like 1 (LEPROTL1) is one of the high probability core clock components identified by the naive Bayesian integration strategy. The role of LEPROTL1 in the core clock function was validated in this study among hundreds of other candidate clock genes. Leptin and its receptor are involved in the regulation of energy balance and body weight [200]. The leptin receptor has a single transmembrane domain, and several isoforms of this receptor are encoded by the leptin receptor gene including OB-Ra, OB-Rc, OB-Rd (short isoforms), and OB-Rb (long isoform) [201]. A negative regulator of the leptin receptor expression on the cell surface is the leptin receptor overlapping transcript-like 1 (LEPROTL1) which has four membrane-spanning domains of 20 or 21 residues with N- and C- termini facing the cytoplasmic side. This integral membrane protein is ubiquitously expressed in various tissues, and composed of 131 amino acid residues, thus it has very short connecting loops at the luminal side of the membranes as as a not much longer hydrophilic loop. At the steady state, LEPROTL1 is mainly found in the trans-Golgi network (TGN), and less extensively in endosomes. Therefore, LEPROTL1 is a membrane protein of the TGN-endosomal system. Once LEPROTL1 traffics to the cell surface, it is transferred to endosomes after endocytosis together with leptin receptors instead of recycling back to the TGN. Final destination of LEPROTL1 and leptin receptors that are internalized to endosomes are lysosomes where they likely undergo lysosomal degradation. One possible function of LEPROTL1 is to regulate postinternalization steps of OB-R trafficking in the endocytic pathway, which is supported by diminution of lysosomal degradation of OB-R upon si-RNA depletion of LEPROTL1. As LEPROTL1 levels decrease, OB-R expression on the plasma membrane is induced likely through increasing their recycling to the cell surface [202]. LEPROTL1 has been also shown to regulate growth hormone receptor (GHR) expression on the plasma membrane in a similar way of OB-R regulation [203].

Chapter 3

MATERIALS AND METHODS

3.1. Preparation of Chemically Competent DH5α and Transformation

E.coli DH5α strain was streaked to an LB agar plate, and incubated overnight at 37^0C. Following day a single colony was inoculated to 5 ml LB medium, and grown overnight. Grown bacteria culture was subsequently inoculated to 500 ml LB medium, and incubated at 37^0C until the OD_{600} of the culture reaches to ~ 0.8 – 0.9. The culture was cooled afterwards in the cold room, and centrifuged in pre-chilled centrifuge tubes at 4053 rcf, 4^0C for 10 minutes. The pellet was resuspended in 250 ml of ice cold $CaCl_2$ solution, centrifuged at 4053 rcf for another 10 minutes. Supernatant was discarded. Washing step was repeated three times with 100 ml, 50 ml, and 10 ml solution. Lastly, pellet was resuspended in 6 ml ice cold $CaCl_2$ solution, and aliquoted to pre-chilled sterile microfuge tubes. Aliquots were frozen in liquid nitrogen and stored at -80^0C.

3.2. Polymerase Chain Reaction (PCR)

Fifty genes were selected from the list created by the naive Bayesian method. Primers for each gene were designed as forward and reverse primers include different restriction sites. All the primer sequences are listed in Appendix A. Genes were amplified by polymerase chain reaction. The reaction mixture contained 1X *Pfx* Amplification Buffer, 1X PCR_x Enhancer Solution, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM

dTTP, 1 mM $MgSO_4$, 0.3 μM of each primer, 25 ng template DNA, and 1 unit of Platinum® *Pfx* DNA Polymerase (Thermo Scientific). The reaction conditions are as follows: 94^{0}C for 15 seconds, 55^{0}C for 30 seconds, and 72^{0}C for 1 minute per kb, 30 cycles. PCR products were visualized in 1% agarose gel, and the ones that gave the correct-sized band were purified by peqGOLD Cycle-Pure Kit (Peqlab).

3.3. Gene cloning and transformation

pACT vector in which genes were to be inserted was obtained by Plasmid Maxiprep Kit (SIGMA-ALDRICH GenElute™ HP). Subsequently, amplified genes and pACT vector were double digested by EcoRV-HF (NEB) and NotI-HF (NEB), or BamHI (NEB) and NotI-HF (NEB), or SalI (Fermentas) and NotI (Fermentas) at 37^{0}C for 4 hours. After digested pACT vector was extracted from 0.8% agarose gel by Gel Extraction Kit (QIAquick, Qiagen), ligation of genes and vector that have the same flanking sequences were performed at room temperature for 5 minutes using Quick Ligation™ Kit (NEB). Ligation products were transformed to chemically competent *E.coli* DH5α cells by heat shock transformation method. Ligation products were added into competent cells, and incubated on ice for 10 minutes. Cells were heat-shocked at 42^{0}C for 90 seconds, and immediately put on ice. They were incubated at 37^{0}C shaker incubator for 30 minutes after addition of 1 ml LB medium, and spread on LB agar plates supplemented with 100μg/ml ampicillin. Following overnight incubation at 37^{0}C, transformants were inoculated into LB medium containing 100μg/ml ampicillin and grown overnight at 37^{0}C in the incubator shaker. Plasmid isolation was then performed from grown cells using Plasmid Miniprep Kit (peqGOLD, Peqlab).

3.4. Cell culture

HEK293T cells were grown in high-glucose Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% FBS (Sigma-Aldrich), 100 μg/ml streptomycin and 100 μg/ml penicillin (HyClone), and 2 mM L-Glutamine (Sigma-Aldrich). Cells were grown at 37^0C in a humidified cell culture incubator supplied with constant 5% CO_2. To passage the confluent cells, medium was first aspirated, then cells were resuspended in 5 ml of culture medium. 1 ml of the resuspended cells was homogenously seeded to another plate containing fresh medium.

3.5. Mammalian two-hybrid assay

Confluent HEK293T cells were splitted to a 10 cm plate 24 hours prior to transfection, as 70-80% confluent fresh cells are used for transfection. DNA mixture to be transfected was prepared as follows: 50 ng PG5-*luc*, 50 ng clock gene in pBIND (*Per1, Per2, Per3, Cry1, Cry2, Clock, Bmal1, Npas2*), and 50 ng candidate gene in pACT (selected fifty genes). 300 ng/wel, the total DNA amount was equalized among different plates by adding 150 ng empty pCMV-Sport6 plasmid. As a positive control, a mixture of 50 ng PG5-*luc*, 50 ng pBIND-*Bmal1*, 125 ng pACT-*Clock* was used, while only 50 ng PG5-*luc* and 250 ng Sport6 were included in the negative control. DNA mixtures were diluted in 50 μl serum-free DMEM, to which 0.9 μl TurboFect transfection reagent (Thermo Scientific) was added. After mixing thoroughly, the reaction mixture was incubated for 20 minutes at room temperature. In the mean time, medium was aspirated from the 10 cm plate, and cells were resuspended in 5 ml of 2X DMEM supplemented with 20% fetal bovine serum, 4 mM L-Glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin, then counted in the hemocytometer, and cells (4×10^4 cells/ well) were seeded to 96-well plate in 50 μl. After 20-minute incubation, 50 μl reaction mixtures were dispensed to each well containing cells. Assays were carried out in

duplicates. The plate was rocked gently, and put into the incubator. At 36 h post-transfection, 100 µl britelite™ plus was added to each well, shaked gently, finally luciferase activity was measured in the Fluoroskan Ascent FL (Thermo Scientific, USA) plate reader after a few minutes of incubation. Luciferase values are background subtracted and normalized to *Renilla* luciferase activity for *LEPROTL1*.

3.6. Protein overexpression and Western blot

HEK293T cells (3×10^4cells/well) were plated in 2 ml of growth medium in 6-well plates and grown 24 h- prior to transfection (~ 70 – 80% confluent cells) with plasmids carrying the gene of interest. For each well, 2 µg of DNA was mixed with 3 µl of TurboFect transfection reagent (Thermo Scientific) in 200 µl of serum-free DMEM. The reaction mixture was incubated for 20 minutes at room temperature, and then added dropwise to each well. Plate was rocked gently and incubated at 37^0C. At 48 h post-transfection, medium was aspirated, cells were washed once with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO4, pH 7.4), and scraped using a micropipette. Collected cells were then centrifuged at 1800 x g for 5 minutes. Subsequently, pellet was dissolved in 150 µl of distilled water, and mixed with 50 µl of 4X protein loading dye (200 mM Tris-Cl, pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol). After vortexing, cells were boiled at 95^0C for 5 minutes, and then put on ice. Finally, they were centrifuged at 16,000 x g for 3 minutes, and soluble phase was loaded to 10% SDS-PAGE gel. Following, proteins were transferred to polyvinylidene difluoride membrane (Biotrace PVDF, Pall Corporation, FL, and USA), blocked with 4% milk powder for 45 min, and incubated in primary antibody for 1 h at room temperature. Proteins were visualized by ECL system.

Chapter 4

RESULTS

4.1. Gene Cloning

55 high probability core clock components identified by the naive Bayesian integration strategy were amplified from their template plasmid DNA, pCMV-Sport6 vector. Amplified PCR products were then run in 1% agarose gel. Gel photos of all the screened genes are in Appendix A. Subsequently, PCR products were purified by PCR purification kit and double digested with restriction enzymes whose cut sites were included in the primers of each gene. After digesting the pACT (one of the mammalian two-hybrid vector) with the same enzymes, PCR products were cloned into the vector.

Since the interaction was detected between core clock components and LEPROTL1 and SOAT1 proteins (the results will be shown in below), cloning results of these genes will be presented here. All the other gene cloning data are given in Appendix A, B, and C. The size of the open reading frame of *Leprotl1* and *SOAT1* are 396 bp, and 1653 bp in length, respectively. Amplification of the both genes were achieved by PCR and products can be seen in Figure 4.1, single band below 500 bp (Figure 4.1A), and a band above 1500 bp (Figure 4.1B). To clone PCR products into pACT vectors, both PCR products and vector are subjected to the EcoRV and NotI digestion. Subsequently, PCR products of the genes and vectors were ligated using T4 DNA ligase. Then ligated products were transformed to *E.coli* DH5α cells. Plasmids were purified from colonies grown in LB agar in the presence of the ampicilin. Plasmids were then digested with the

EcoRV and NotI to select the plasmids consist of the PCR products Analysis of digested plasmids indicated that *Leprotl1* and *SOAT1* were successfully cloned to pACT vector (Figure 4.1 - A, B).

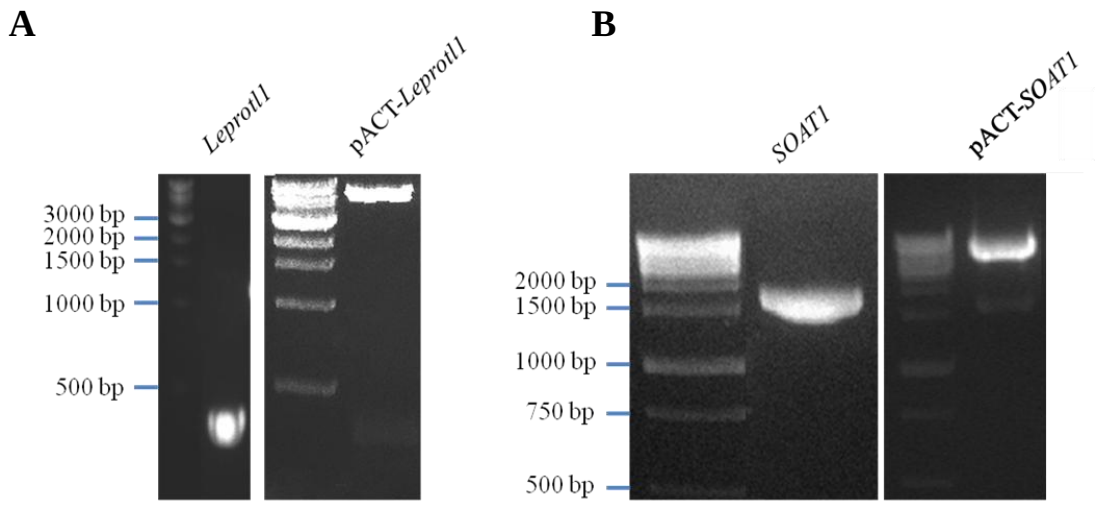


Figure 4. 1. Cloning of *Leprotl1* and *SOAT1*. (A) *Leprotl1* was amplified by PCR and gave a single band with a size of approx 400 bp. To confirm the existence of the insert within the vector, plasmid was cut with restriction enzymes that were initially used to insert *Leprotl1* into the vector (B) *SOAT1* was amplified by PCR and gave a single band with a size of approx 1600 bp. To confirm the existence of the insert within the vector, plasmid was cut with restriction enzymes that were initially used to insert *SOAT1* into the vector.

4.2. Mammalian Two-Hybrid Assay

Mammalian two-hybrid assay is an extremely powerful method for detection of protein-protein interactions in mammalian cell culture. The basic principle behind the mammalian two-hybrid assay is the transcriptional activation of the reporter gene by two different modular domains of certain transcription factors fused to two potentially interactive proteins of interest. One of these domains is a DNA-binding domain, which binds to a specific DNA sequence, and the other one is a transcriptional activation domain, which interacts with the basal transcriptional machinery. The DNA-binding domain and the transcriptional activation domain are brought in close proximity when one protein fused to a DNA-binding domain interacts with a second protein fused to a transcriptional activation domain, leading to recruitment of RNA polymerase II complexes at the TATA box and an increase in transcription of firefly luciferase reporter gene. The yeast GAL4 DNA-binding domain is transcribed from the pBIND vector, while the herpes simplex virus VP16 activation domain is contained in the pACT vector, and the PG5*luc* vector contains the firefly luciferase reporter gene downstream of five GAL4 binding sites as well as a minimal TATA box. In this study, core clock component genes *mPer1*, *mPer2*, *mPer3*, *mCry1*, *mCry2*, *mClock*, *mBmal1*, and *mNpas2* were cloned into the pBIND vector, whereas candidate clock genes were cloned into the pACT vector (Figure 4.2). Among 55 high probability core clock components, LEPROTL1 was shown to influentially interact with both mCRY1 and mCRY2 proteins (Figure 4.3 - A), while SOAT1 appears to interact with only mCRY1 (Figure 4.4-A). After the primary screening of both genes with core clock components, detailed analysis of this interactions were carried out to eliminate the possibility of the false interaction as results of the self activation on the reporter plasmids. For this purpose several controls were used in the assay. As being the negative control, only reporter plasmid (PG5*luc*) was used to determine the background signal. In addition, *mCry1* and PG5*luc*, *mCry2* and PG5*luc*, *Leprotl1* and PG5*luc*, and *SOAT1* and PG5*luc* were used to control whether reporter plasmid is activated by the presence of each gene

itself. As can be seen in Figure 4.3-B, C and 4.4-B, C, luciferase activity was detected between genes and Crys, but not in the controls, suggesting that luciferase activity was not engendered by false interactions. In all experiments Firefly luciferase activity were normalized to *Renilla* luciferase to eliminate differences in transfection efficiency. For interaction of LEPROTL1 and mCRY1/2, almost 10-fold increase was observed (Figure 4.3-B), while interaction of SOAT1 with mCRY1 resulted in about 4-fold increase compared to the background firefly luciferase activity (Figure 4.3 - B).

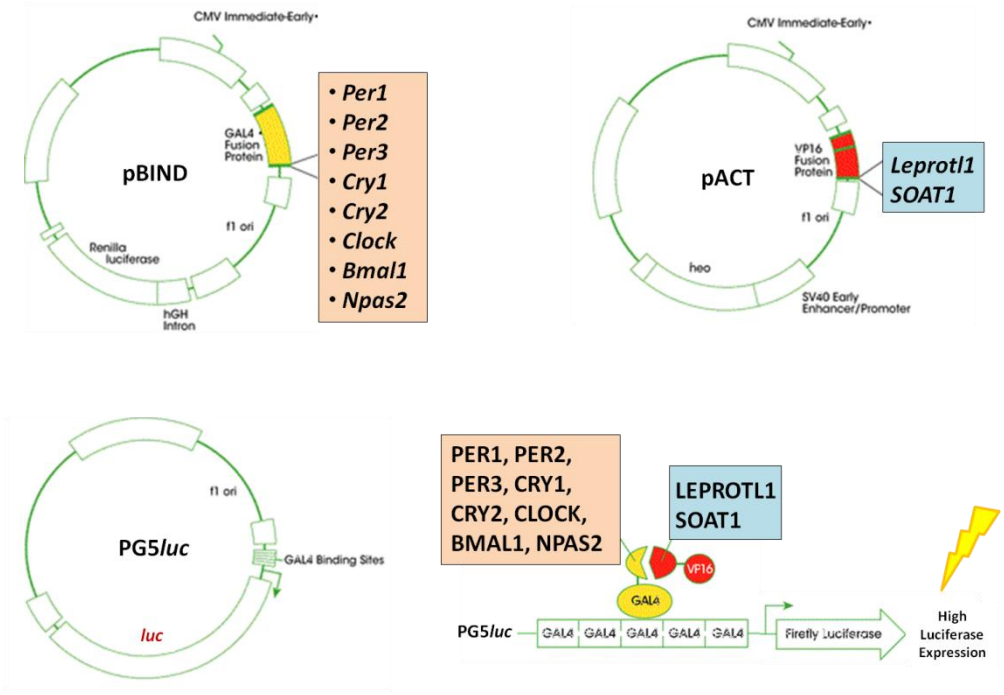


Figure 4. 2. Schematic representation of the mammalian two-hybrid assay system.

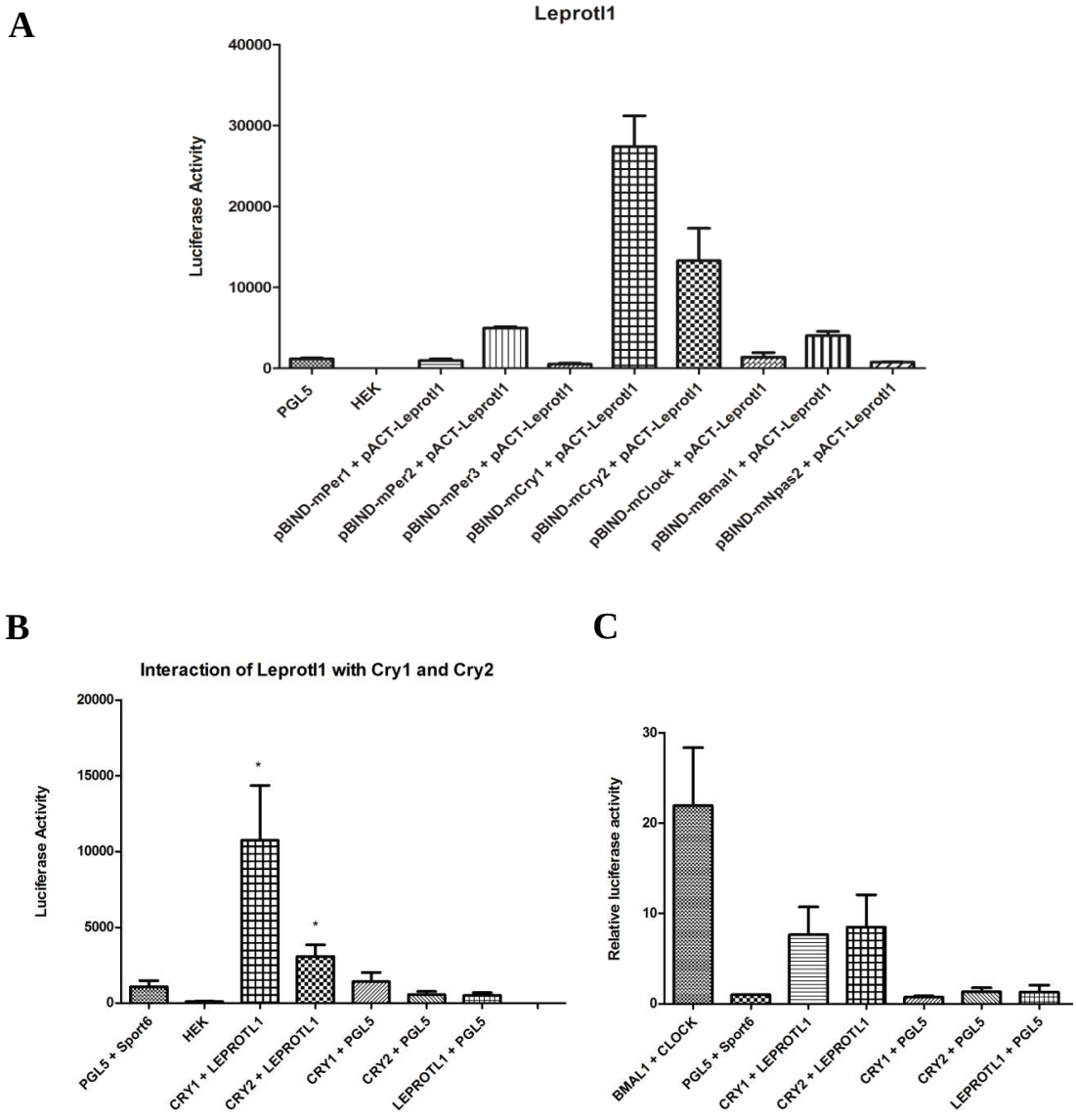


Figure 4. 3. (A) Mammalian two-hybrid assay to test the possible interactions between LEPROTL1 and the core clock components. (B) The experiment was repeated three times and mean values are plotted, as seen LEPROTL1 seems to interact with both CRY1 and CRY2. *(C)* Firefly luciferase values were normalized to *Renilla* luciferase to eliminate the effect of differing transfection efficiency among wells. All experiments were carried out in duplicate and repeated at least three times. Statistical analysis were carried by t-test ($p<0.05$).

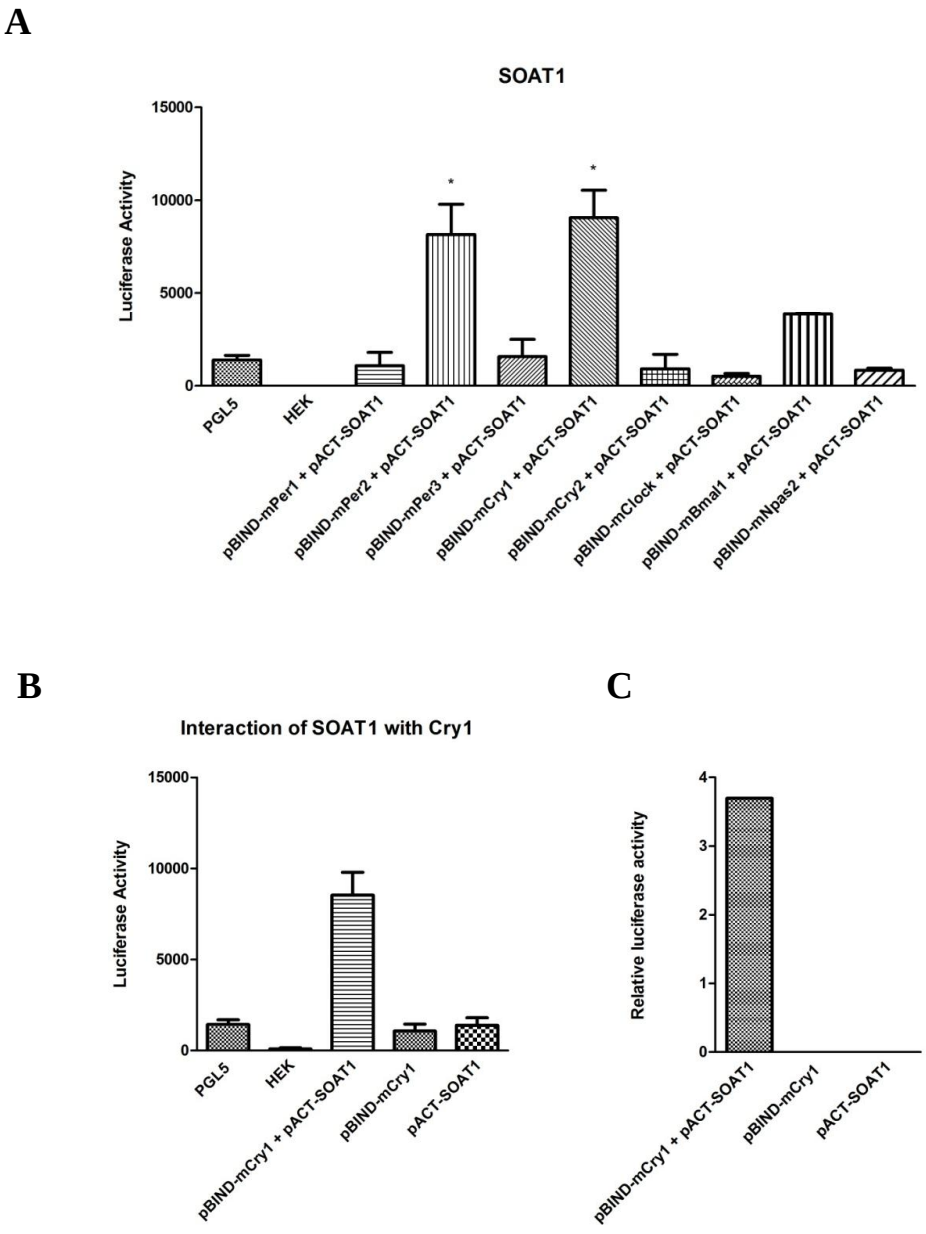
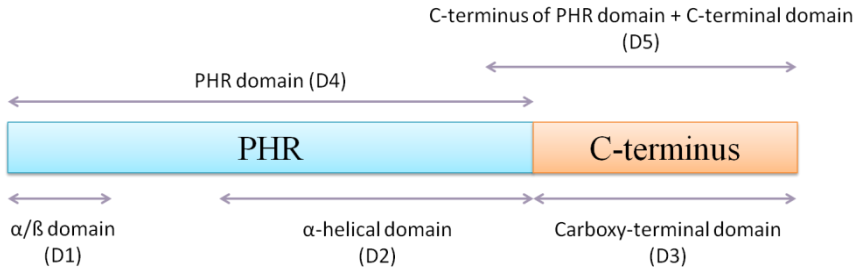


Figure 4. 4. (A) Mammalian two-hybrid assay to test the possible interactions between SOAT1 and the core clock components. (B) The experiment was repeated two times and mean values are plotted, as seen SOAT1 seems to interact with CRY1. *(C)* Firefly luciferase values were normalized to *Renilla* luciferase to eliminate the effect of differing transfection efficiency among wells. All experiments were carried out in duplicate and repeated at least three times. Statistical analysis were carried by t-test ($p<0.05$).

To further confirm the interaction and to investigate which part of mCRY1 interacts with LEPROTL1, mammalian two-hybrid assay was performed using different mCRY1 domains (Figure 4.5-A). These domains include α/ß domain (D1) and α-helical domain (D2) within the photolyase homology region (PHR), carboxy-terminal domain (D3), PHR domain (D4), and C-terminus of PHR with C-terminal domain (D5). PHR is a highly conserved core domain at the N-terminus, whereas the C-terminal tail domain (CTD) has diverged during evolution. The PHR appears to confer a significant role to CRY1 in clock function because a critical region within the highly conserved α-helical domain of CRY1 PHR (from amino acid 313 to 426) has been demonstrated to be essential to restore circadian rhythms. In contrast, the CTD is dispensable for Cry1 function. However, it plays important roles in modulating rhythm amplitude and period length [204]. Biochemical and biophysical tests show that CTD is highly unstructured when expressed alone, but it bears a rigid structure by interacting with the PHR domain [205]. Since PER2 and FBXL3 have been previously shown to interact with CTD of CRY1 [206], we generated D5 domain to observe a possible interaction with LEPROTL1. Based on known structure and domain function of photolyase/cryptochrome proteins, we divided CRY1 protein into five regions. Although LEPROTL1 interacted with full-length mCRY1, this interaction was not seen with mCRY1 domains, indicating a need for intact mCRY1 for interaction (Figure 4.5-B).

A



B

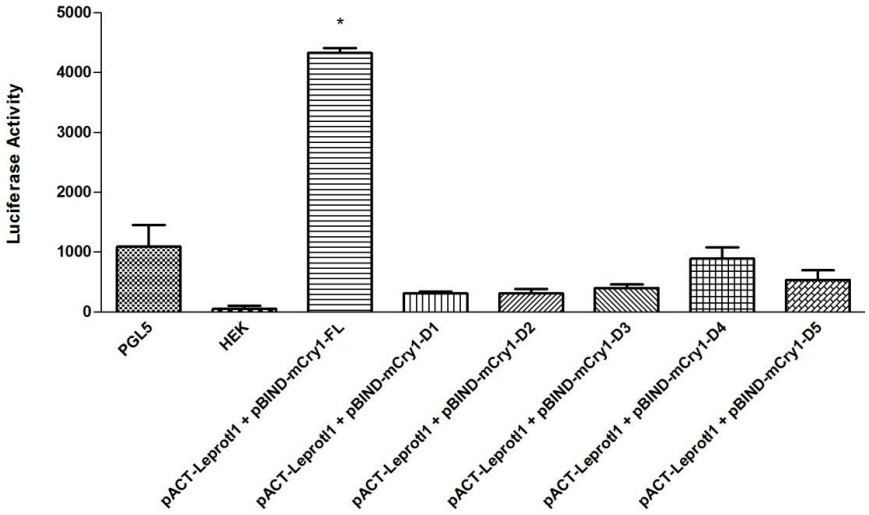


Figure 4. 5. Interaction of LEPROTL1 with mCRY1 domains. (A) Schematic illustration of mCRY1 domains, from D1 to D5. (B) Mammalian two-hybrid assay of LEPROTL1 and mCRY1 domains.

4.3. The Effect of *Leprotl1* on Circadian Rhythm

To investigate the role of *Leprotl1* in the rhythmic oscillations, phenotypic alterations in the absence of *Leprotl1* were observed. NIH 3T3 and U2OS cells were transfected with siRNA designed against *Leprotl1*. Bioluminescence rhythms were recorded from luciferase-based circadian reporter plasmid, Bmal1-luc reporter that was transfected to NIH 3T3 and U2OS cell lines together with siRNA-*Leprotl1*. As it is seen in Figure 4.6-A, knockdown of *Leprotl1* resulted in amplitude damping, period-lengthening and phase advance in U2OS cells, while only amplitude damping was observed in NIH 3T3 cells. Quantification of both mRNA and protein levels at the end of the fifth day of recording ensured endurance of the siRNA effect (data not shown). Amplitude damping is indicative of decrease in the expression of circadian reporter gene *Bmal1*, while period-lengthening means that one cycle of the oscillation takes more than 24 h. Phase advance, on the other hand, is the delay in the expression of the circadian reporter gene. Cellular clock phenotypes of siRNA knockdown of known clock genes have been previously investigated, and it was shown that knockdown of BMAL1 or PER1 results in low-amplitude oscillations and loss of rhythmicity, knockdown of CLOCK leads to amplitude damping and phase delay, knockdown of CRY1 leads to shorter period length and rapid damping, and knockdown of PER2 or CRY2 results in period lengthening (Figure 4.6-B) [207]. In other words, loss of core clock components results in phase shifts, and alterations in amplitude magnitude and period length. Since siRNA knockdown of Leprotl1 brought about a similar cellular clock phenotype, it stands to reason that Leprotl1 might be indeed a core clock component.

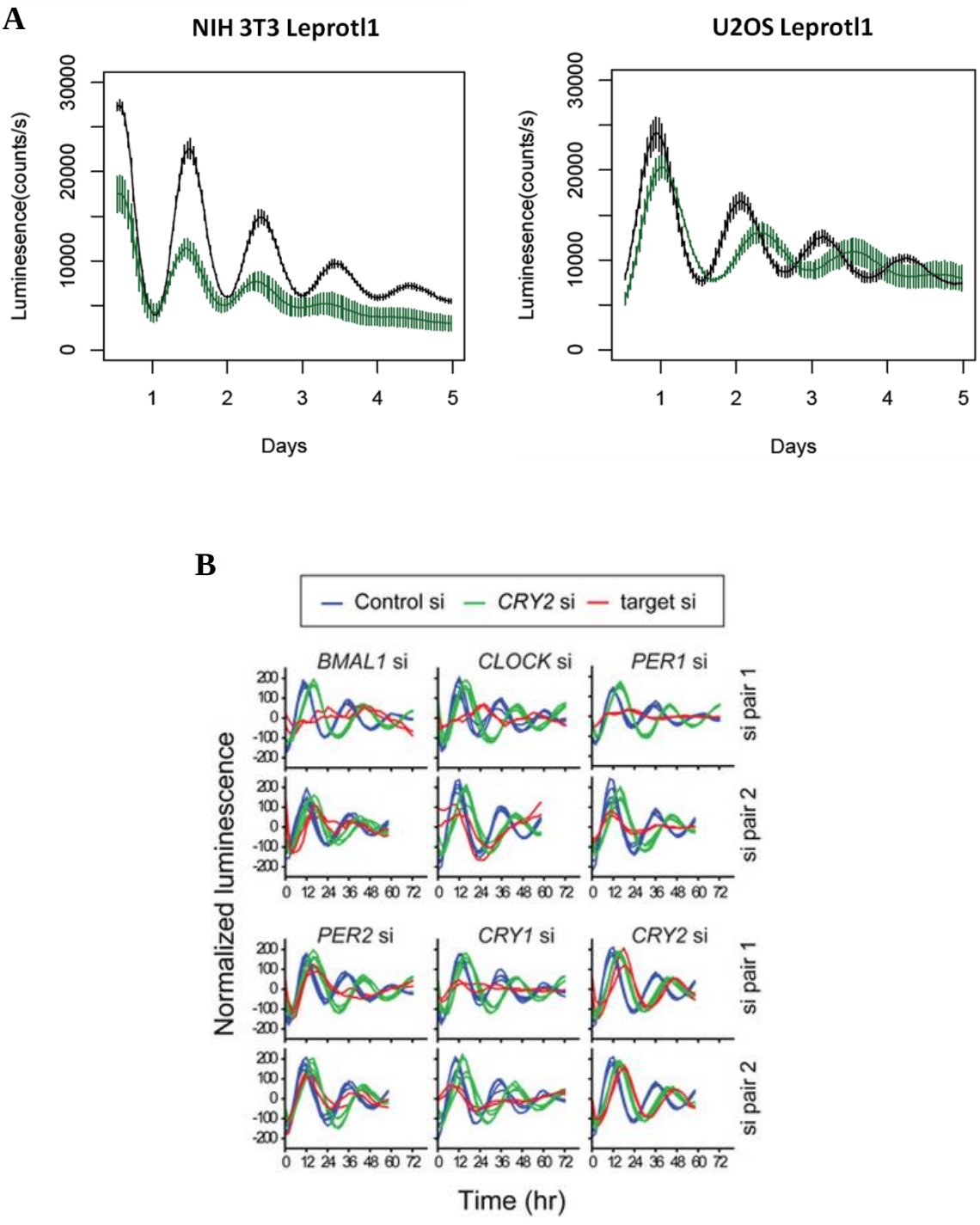


Figure 4. 6. (A) Effect of Leprotl1 depletion on generation of rhytmic oscillations, conducted at University of Pennsylvania by Ron Anafi, MD, PhD. (B) Cellular clock phenotypes of siRNA knockdown of known clock genes. Adapted from *[207]*

4.4. Expression of LEPROTL1 and CRYs

Next, expression of LEPROTL1 CRYs was examined. To achieve this, first the genes were cloned to expression vectors pCMV-Sport6 and pcDNA4-myc-his-A, respectively. Primers of *Leprotl1* contained FLAG-tag for future co-immunoprecipitation (Figure 4.7). *Leprotl1* was sent to Macrogen for sequencing, the sequence was analyzed by BLAST and no mutation was detected (Figure 4.8).

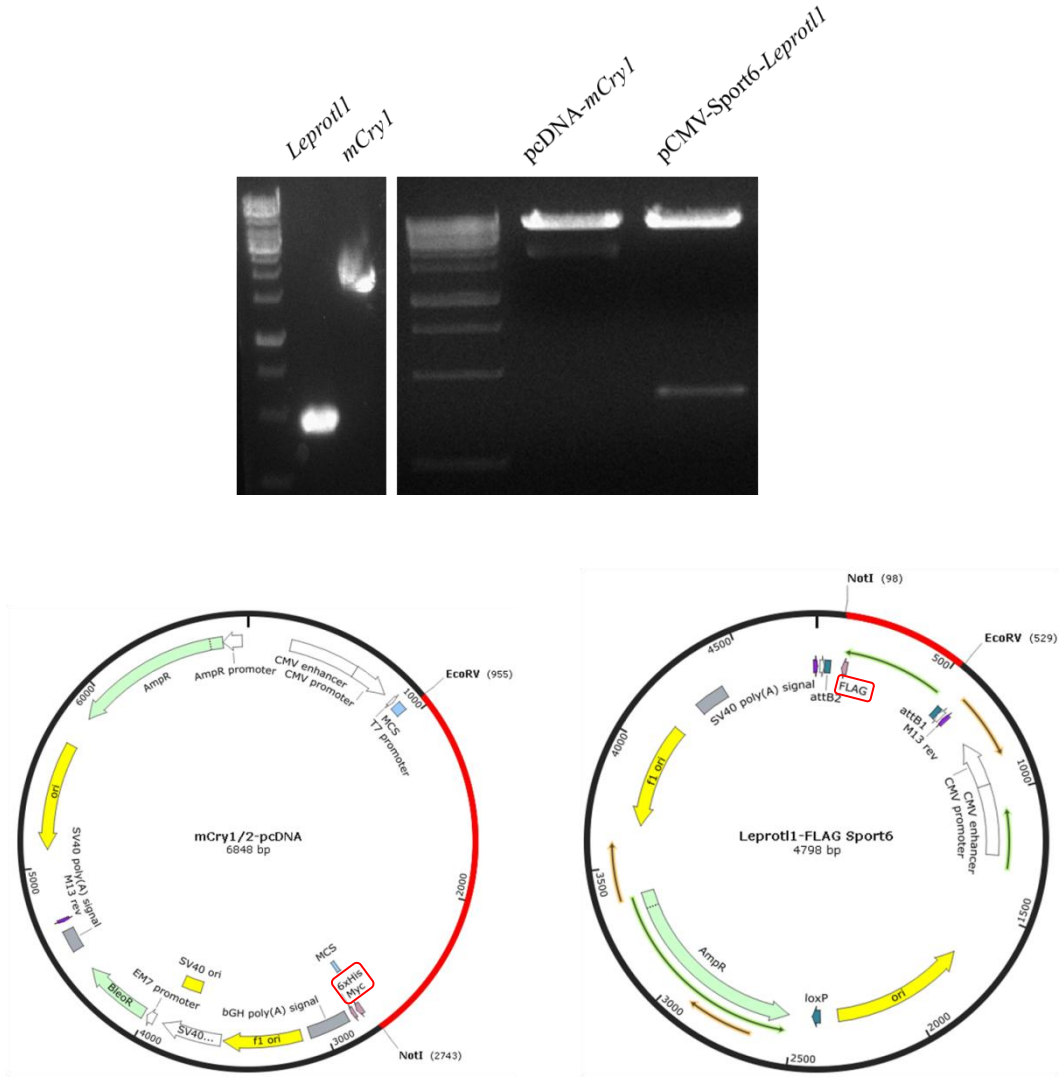


Figure 4. 7. Cloning of FLAG-tagged *Leprotl1* (420 bp) into pCMV-Sport6, and *mCry1/2* (1821 bp) into pcDNA4-myc-his-A expression vector.

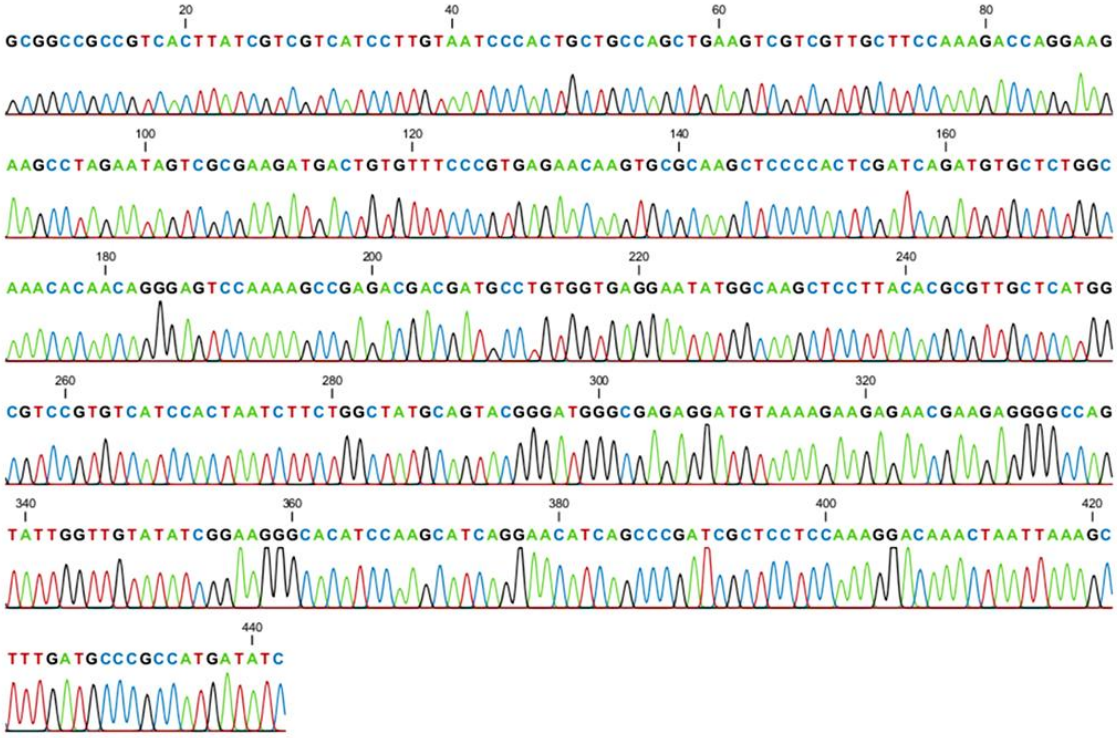


Figure 4. 8. Sequence analysis of *Leprotl1*. No mutations were detected.

CRY1 and CRY2 transfected cells were collected after 48 h of transfection, pelleted, and soluble part was run in 10% SDS-PAGE after adding 4X SDS loading dye and boiling at 95°C for 5 minutes. Proteins were then transferred to PVDF membrane, and blocked with 4% milk powder for 45 minutes. Subsequently, membrane was incubated with HRP-conjugated anti-His or anti-FLAG antibody at room temperature for 1 h. As can be seen in Figure 4.6, CRY1 and CRY2 were greatly expressed. Both gave single band around 70 kDa as expected size of CRYs (~ 67 kDa) together with c-myc (1.20 kDa) and Poly-His (0.84 kDa) tags is ~69 kDa, while LEPROTL1 (~15 kDa) together with FLAG (1.01 kDa) tag gave an intense band above 15 kDa.

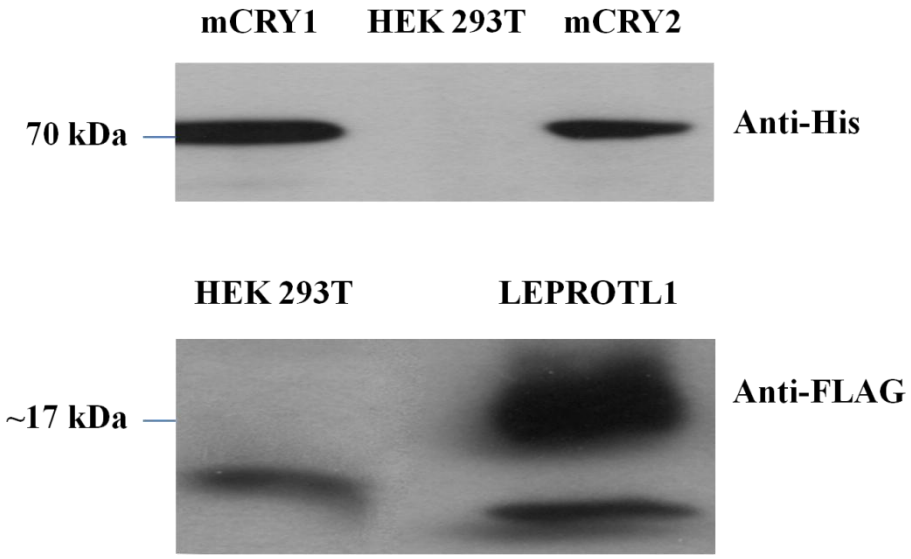


Figure 4. 9. Expression of Poly-His-tagged mCRY1 and mCRY2 from pcDNA4-myc-His-A vector, and FLAG-tagged LEPROTL1 from pCMV-Sport6 vector.

Chapter 5

DISCUSSION

Circadian clocks have been extensively studied during the last decade. As a result of these studies, plenty of proteins have been found to be either directly or indirectly involved in the molecular machinery of the circadian clock. In addition to the core clock components that make the clockwork operate, many more regulators of this daily timing system have been identified so far. Since the circadian clock regulates most of the physiological and behavioral processes, it stands to reason that a wide array of proteins with diverse functions takes part in the proper functioning of the clock. For discovery of new clock components or modifiers, certain experimental methods including forward mutagenesis and reverse genetics, behavioral phenotyping, and molecular cloning have been used. However, computational methods become more popular nowadays because of their time-saving features [194, 195].

The naive Bayesian strategy, being one of these advantageous computational methods, has provided the basis of this study. Here, we aimed to experimentally test the likely interactions of high probability candidate clock genes identified by this strategy with already established core clock components. 55 genes were cloned and screened one by one with mammalian two-hybrid assay, a powerful method to detect protein-protein interactions. The entire screen yielded two hits at the end, *Leprotl1* and *SOAT1*. In all assays, BMAL1/CLOCK was used as a positive control, and PG5*luc* was used as a negative control to give a background signal, while *mCry1*, *mCry2*, *Leprotl1*, or *SOAT1* was tested with PG5*luc* to see whether reporter plasmid is self-activated by each gene

itself, which would cause false interaction signals. However, luciferase activity resulted from the interaction of genes and mCRYs was remarkably higher compared to these controls.

SOAT1 is a Sterol O-acyltransferase and catalyzes the formation of fatty acid-cholesterol esters. It also plays a role in lipoprotein assembly and dietary cholesterol absorption. Since *Leprotl1* was tested prior to *SOAT1*, the project proceeded with the identification of the role of *Leprotl1* in the circadian timing system. For this purpose, first the effect of *Leprotl1* on circadian rhythm was investigated. siRNA experiments conducted by our collaborator at University of Pennsylvania revealed that LEPROTL1 is indeed required for proper circadian oscillations as *Leprotl1*-depleted cell lines showed altered oscillations in terms of either amplitude or phase. Amplitude damping is associated with the robustness of the oscillators. For instance, ClockΔ19 mutant mice are known to exhibit damped amplitude of circadian rhythms, and this effect is accompanied by various physiological and behavioral deficiencies in mice. Together with observed phase-shift and period-lengthening phenotypes, deterioration in the circadian system has been shown to appear as disruptions in the sleep-wake cycle and system-wide physiology [208].

LEPROTL1 has been previously demonstrated to negatively regulate the cell surface expression of leptin receptors as targeting *Leprotl1* resulted in increased leptin receptor levels on the cell surface. Leptin receptors are thought to be activated in a time-dependent manner because leptin release was found to show rhythmic pattern in the plasma of rats [200]. Considering the close relationship of the circadian clock with metabolism, it is very likely that *Leprotl1* has a modulatory function on the circadian clock machinery. More importantly, LEPROTL1 might be a pharmacological target to increase leptin receptor levels, thereby enhance leptin sensitivity in obese patients who have dramatically increased circulating leptin levels. LEPROTL1 is a small

transmembrane protein mainly localized in trans-Golgi network, and to a lesser extent in endosomes. There are in fact only a few studies on LEPROTL1, and one of them claims LEPROTL1 to traffic to the plasma membrane, and then get internalized in the same endocytic compartments as the leptin receptor. It was shown that both N- and C-terminus of the protein face the cytoplasmic side of the membrane, therefore mCRY1 and mCRY2 might be interacting with these accessible termini when LEPROTL1-carrying endosome is fused to the plasma membrane. Otherwise, cytoplasmic N- and C-terminus would face the interior endosome, rendering them inaccessible for soluble proteins like CRYs. To investigate which domains of mCRY1 interacts with LEPROTL1, full-length mCRY1 was divided into five different domains, and interaction of each domain with LEPROTL1 was tested. However, we failed to detect any interaction between mCRY1 domains and LEPROTL1, despite of high luciferase activity resulted from interaction of full-length mCRY1 with LEPROTL1. The most likely reason is that mCRY1 requires its intact conformation to interact with other proteins. Another explanation is that we might have chopped the mCRY1 protein from the interaction site. Further studies will investigate the interaction sites by using random mutagenesis approach on both LEPROTL1 and mCRYs.

The exact mechanism by which feeding entrains the peripheral clocks is unknown. LEPROTL1 might be involved in this mechanism such that increased levels of leptin during the feeding state act on leptin receptors to inhibit appetite binding, and LEPROTL1 might somehow receive signals from leptin or its receptor, and exert its effect on circadian clock mechanism through interacting with mCRYs (Figure 5.1).

To our knowledge, the relationship of LEPROTL1 with the circadian clock was shown for the first time in this thesis work. As acting on the heart of metabolism, LEPROTL1 generates an additional link between the circadian clock and metabolism, and it might be a promising target for treatment of clock-related metabolic diseases.

Future perspectives involve biochemical validation of the interaction of LEPROTL1 and SOAT1 with mCRYs by co-immunoprecipitation assay. Following, the effect of *SOAT1* on the circadian rhythm should be investigated as conducted on *Leprotl1*. Last but not least, locomotor activity of knock-out mice should be examined to observe the effect of these genes on the circadian clock in organismal level.

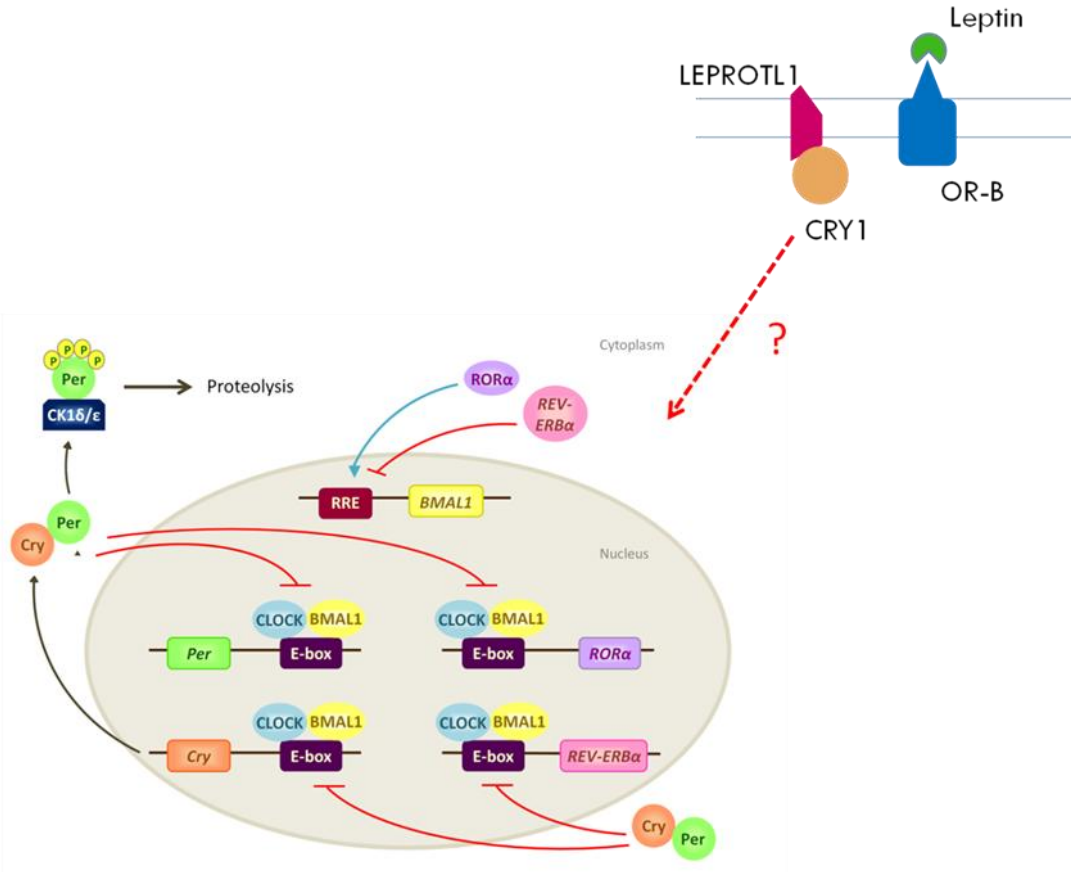


Figure 5. 1. One possible mechanism for food entrainment by leptin signaling.

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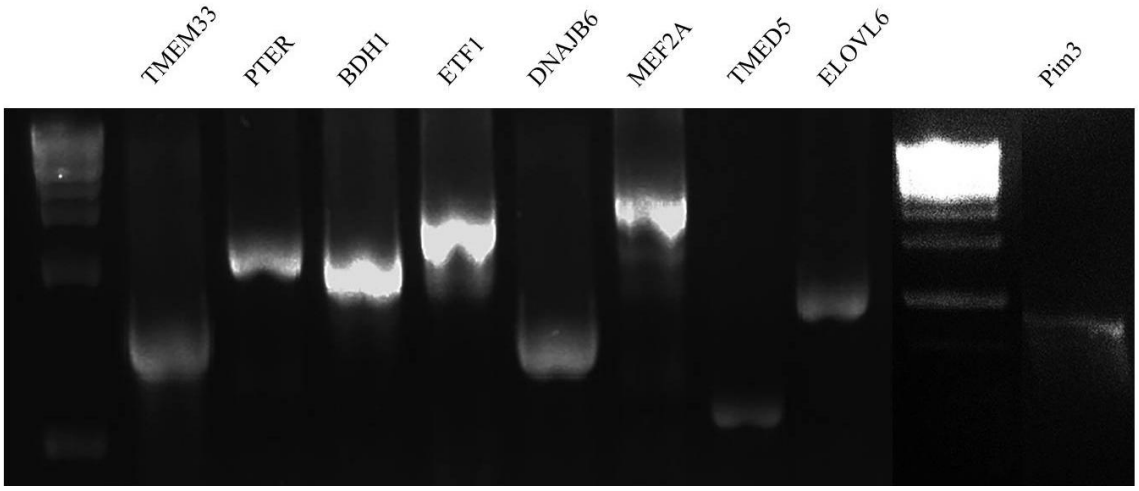
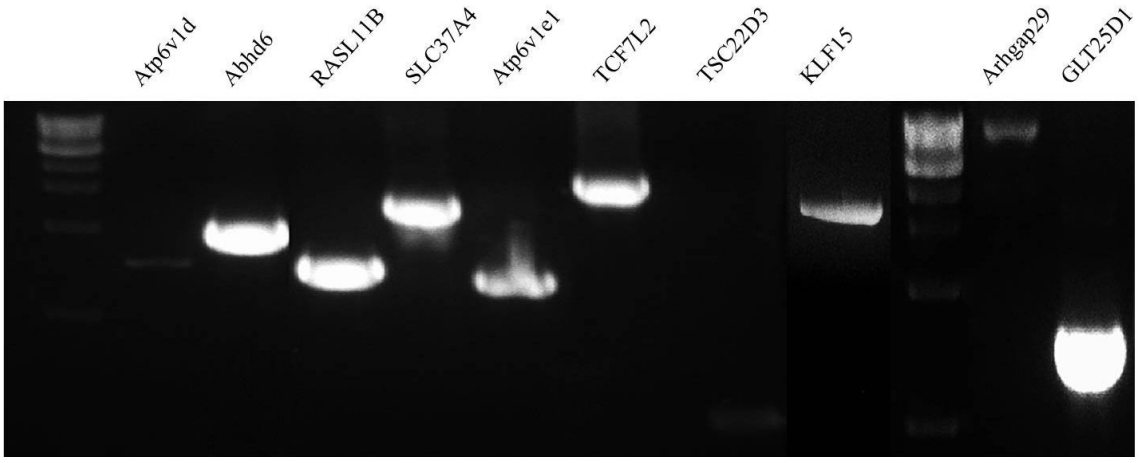
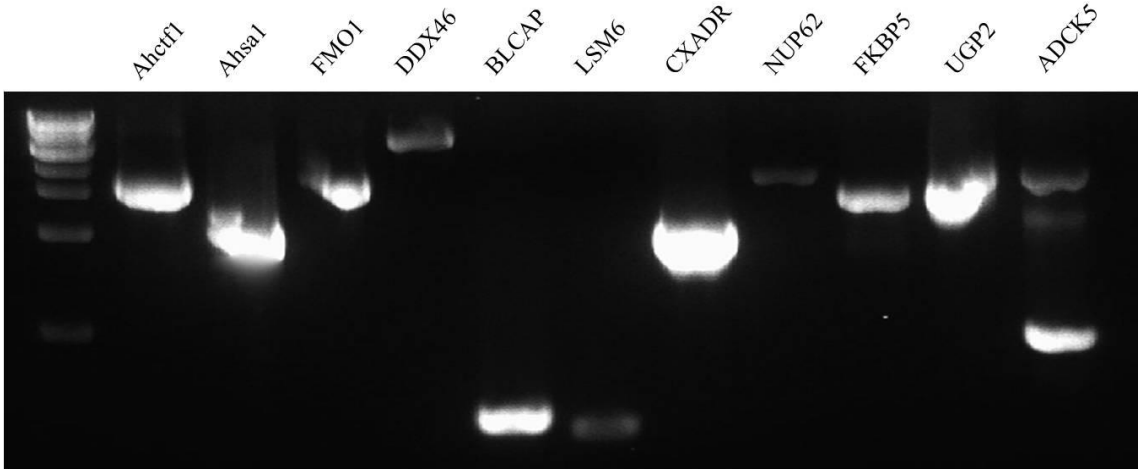
VITA

Gülsüm Elif Muku was born in Eskişehir, Turkey, on September 14, 1989. She received her B.Sc. Degree in Molecular Biology and Genetics from Middle East Technical University, Ankara, in 2011. From September 2011 to August 2013 she worked as teaching and research assistant at Koç University, Istanbul, Turkey as a member of Biochemistry and Molecular Biology Laboratory. She has worked on "Identification of Novel Components of the Mammalian Circadian Clock" during her M.Sc. study. She will continue her education with Ph.D. at Pennsylvania State University, USA.

APPENDIX A: PCR Data of the Screened Genes



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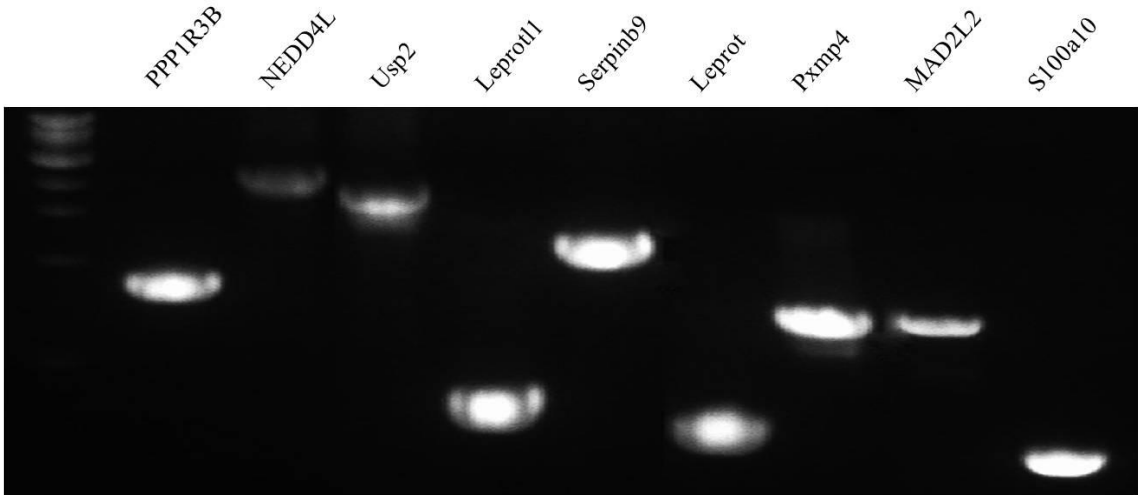
Ahsa1
FMO1
DDX46
BLCAP
LSM6
CXADR
NUP62
FKBP5
UGP2
ADCK5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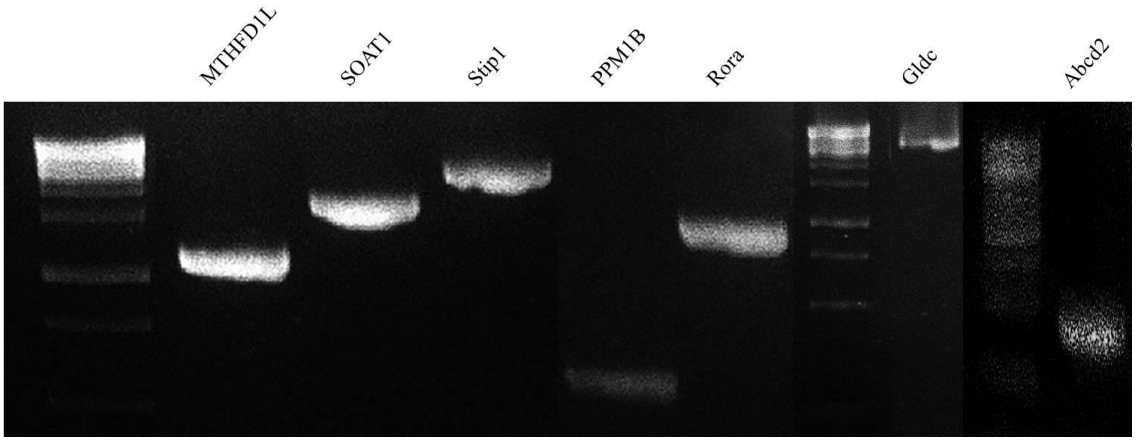
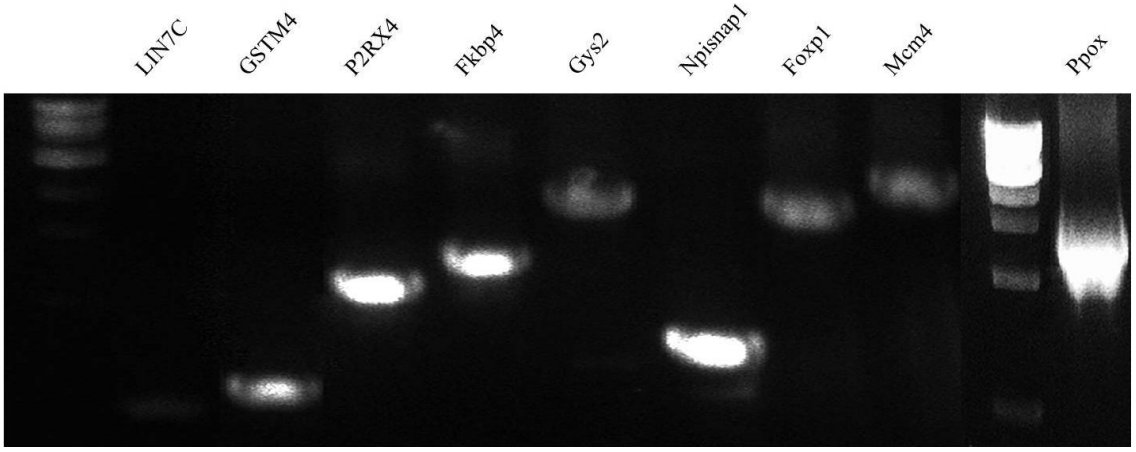
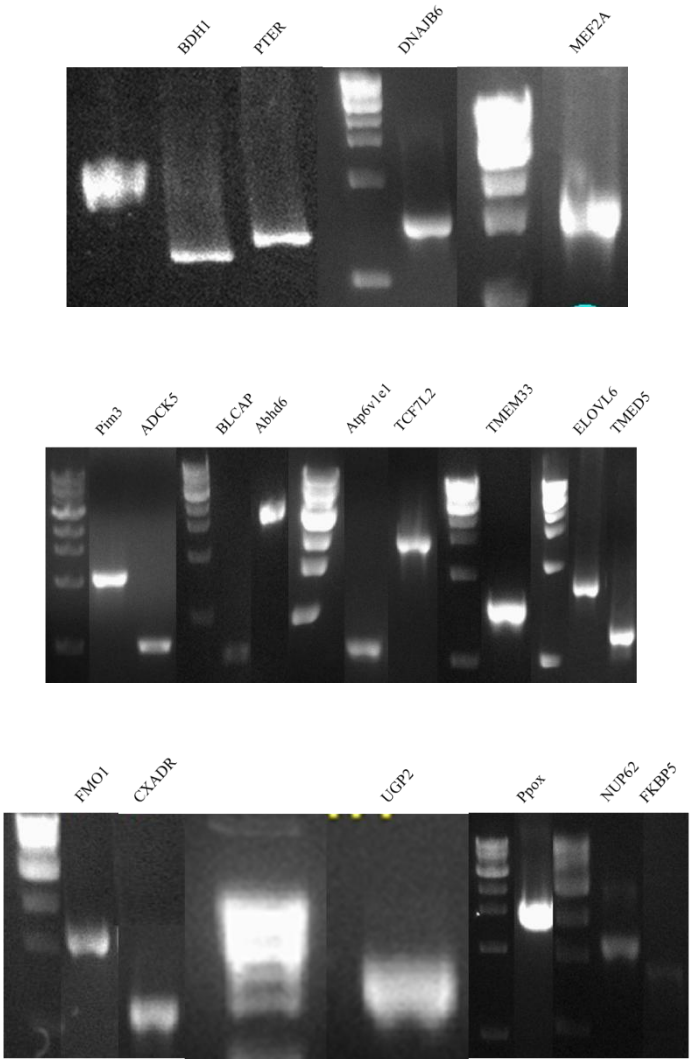
NEDD4L
Usp2
Leprotl1
Serpinb9
Leprot
Pxmp4
MAD2L2
S100a10


MTHFD1L
SOAT1
Stip1
PPM1B
Rora
Gldc
Abcd2

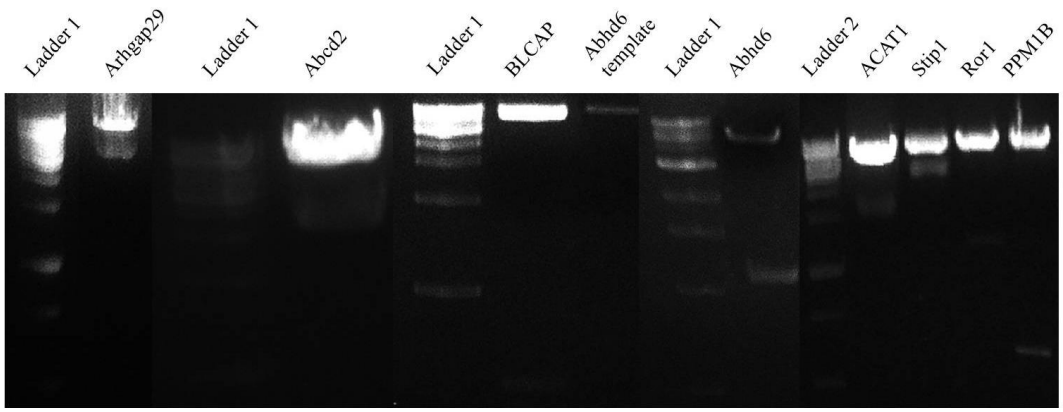

LIN7C
GSTM4
P2RX4
Fkbp4
Gys2
Npisnap1
Foxp1
Mcm4
Ppox

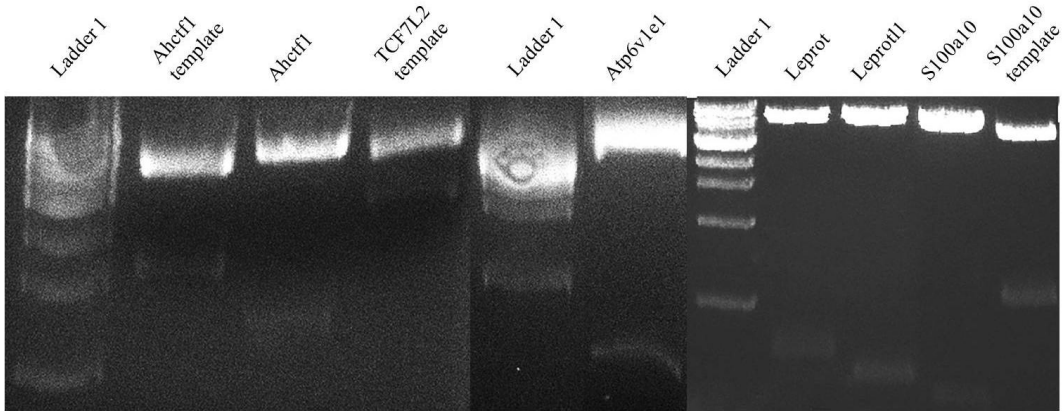
APPENDIX B: Colony PCR Results of the Constructs

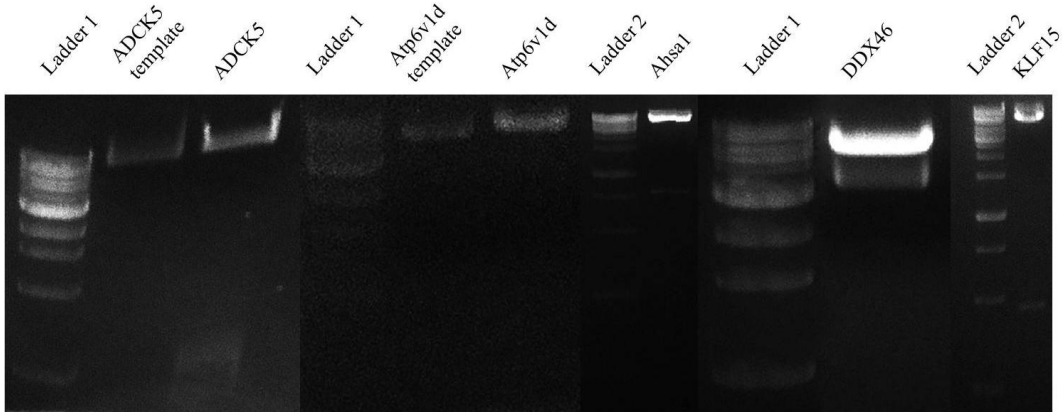
For confirmation of the constructs, first colony PCR was performed. After checking 19 constructs, colony PCR procedure was skipped as it gave false positives.

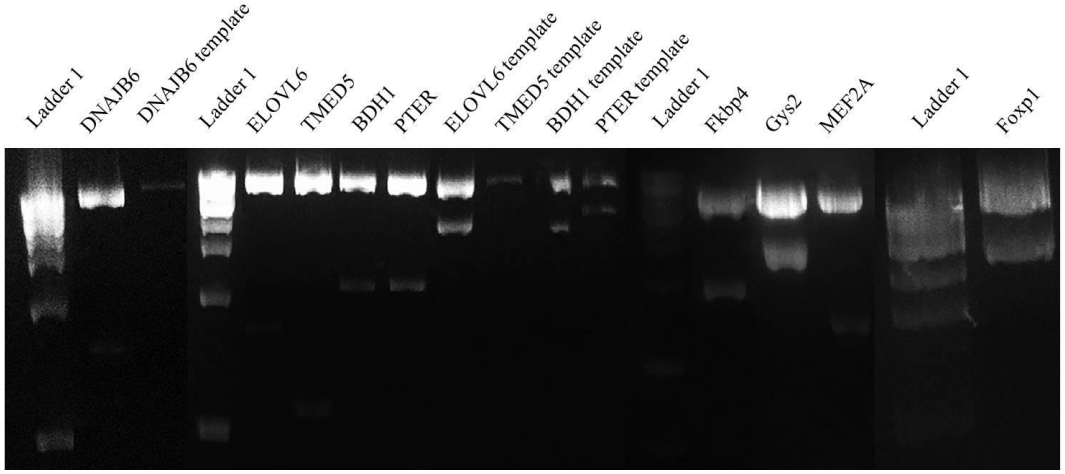


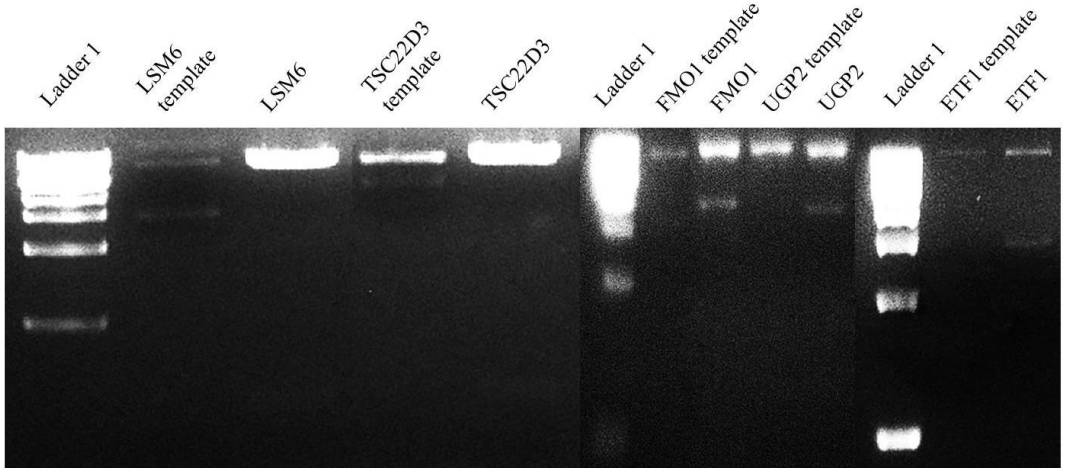
APPENDIX C: Restriction Enzyme Digestion to Confirm the Inserts

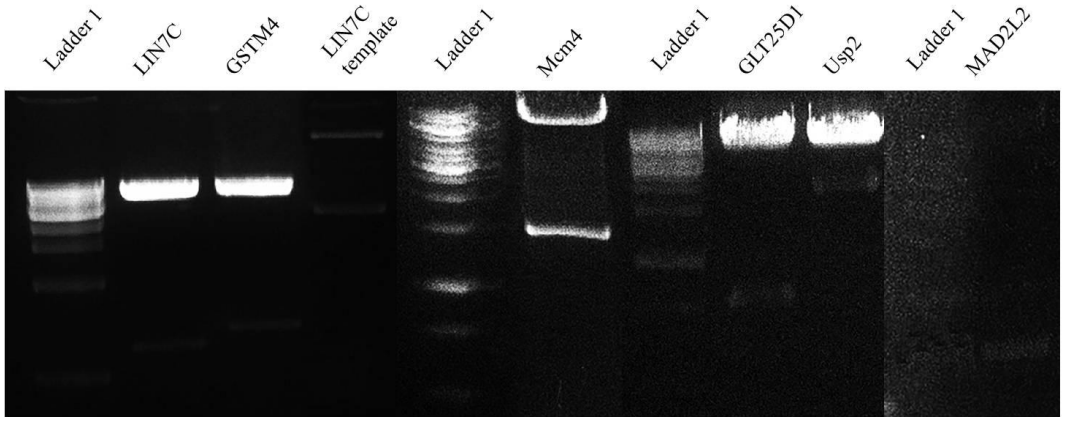


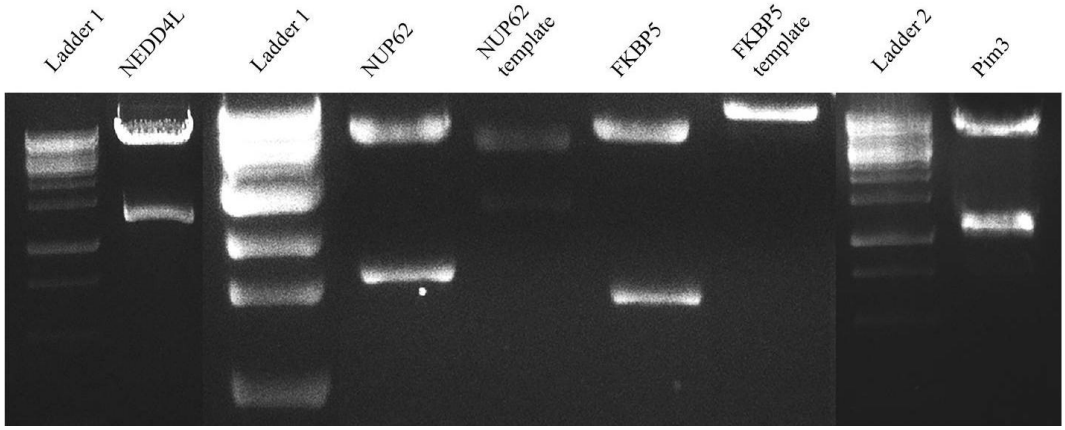


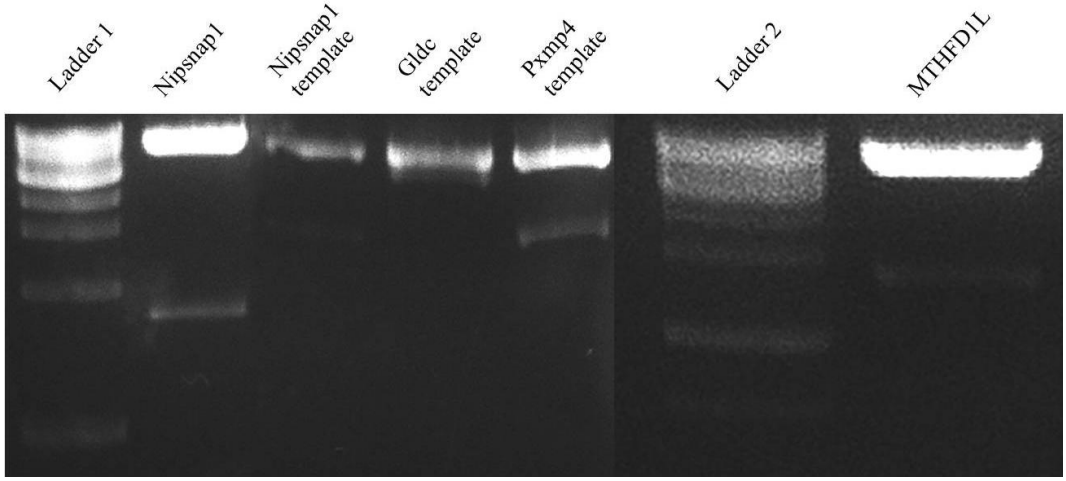

Ladder 1
ADCK5 template
ADCK5
Ladder 1
Atp6v1d template
Atp6v1d
Ladder 2
Ahsa1
Ladder 1
DDX46
Ladder 2
KLF15

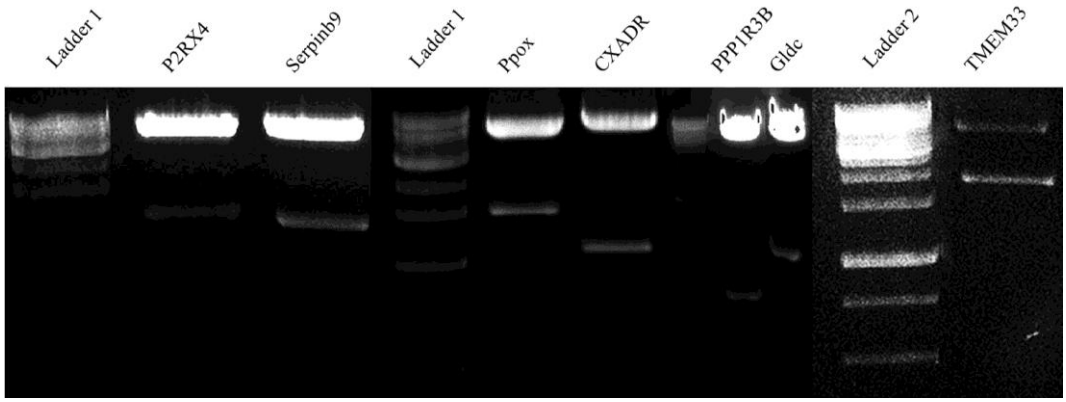

Ladder 1
DNAJB6
DNAJB6 template
Ladder 1
ELOVL6
TMED5
BDH1
PTER
ELOVL6 template
TMED5 template
BDH1 template
PTER template
Ladder 1
Fkbp4
Gys2
MEF2A
Ladder 1
Foxp1

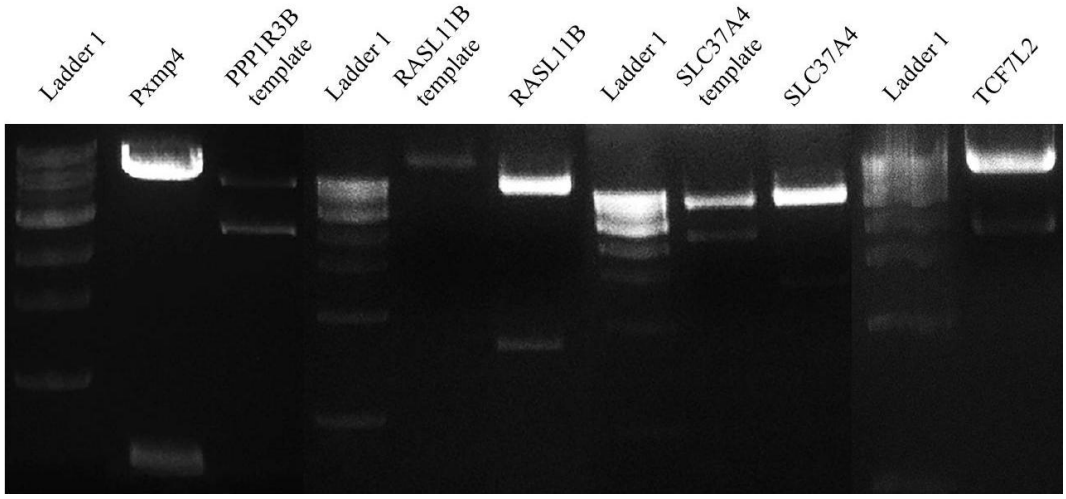

Ladder 1
LSM6 template
LSM6
TSC22D3 template
TSC22D3
Ladder 1
FMO1 template
FMO1
UGP2 template
UGP2
Ladder 1
ETF1 template
ETF1

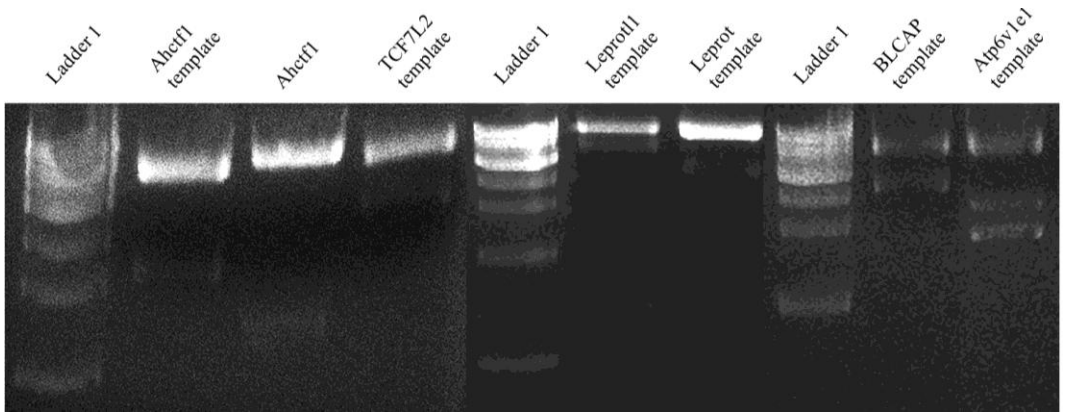

Ladder 1
LIN7C
GSTM4
LIN7C template
Ladder 1
Mcm4
Ladder 1
GLT25D1
Usp2
Ladder 1
MAD2L2

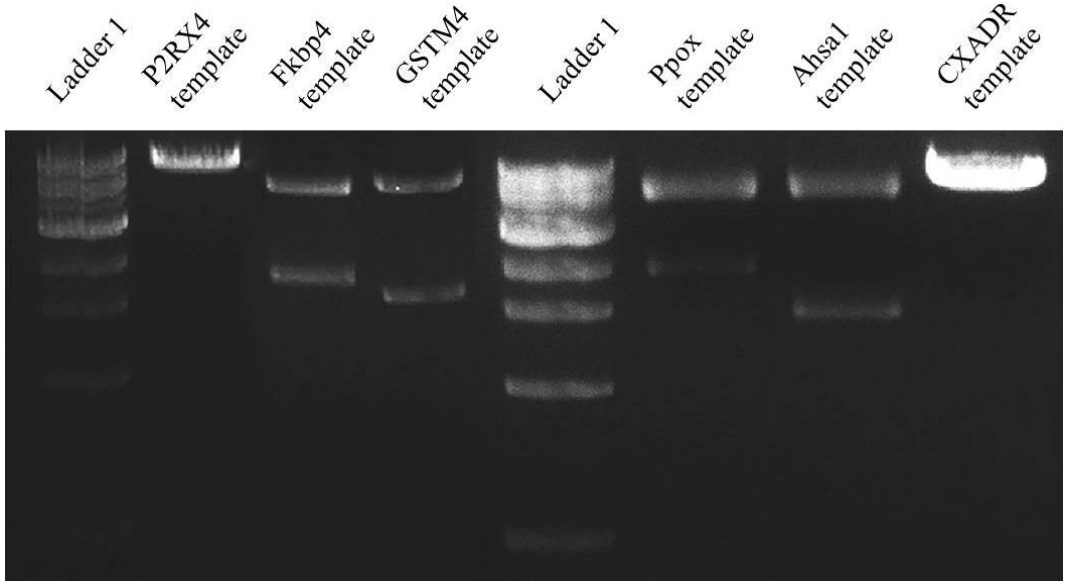

Ladder 1
NEDD4L
Ladder 1
NUP62
NUP62 template
FKBP5
FKBP5 template
Ladder 2
Pim3


Ladder 1
Nipsnap1
Nipsnap1 template
Glde template
Pxmp4 template
Ladder 2
MTHFD1L

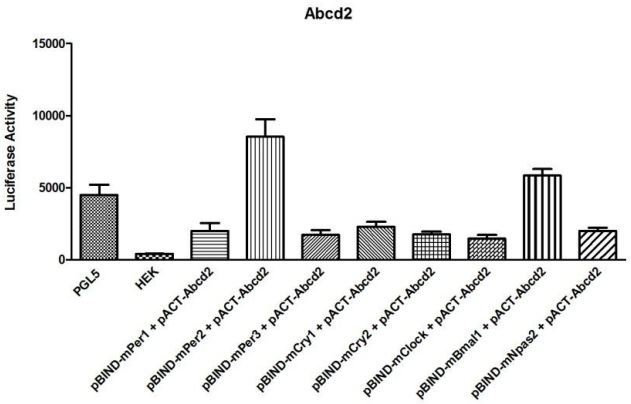

Ladder 1
P2RX4
Serpinb9
Ladder 1
Ppox
CXADR
PPP1R3B
Gldc
Ladder 2
TMEM33

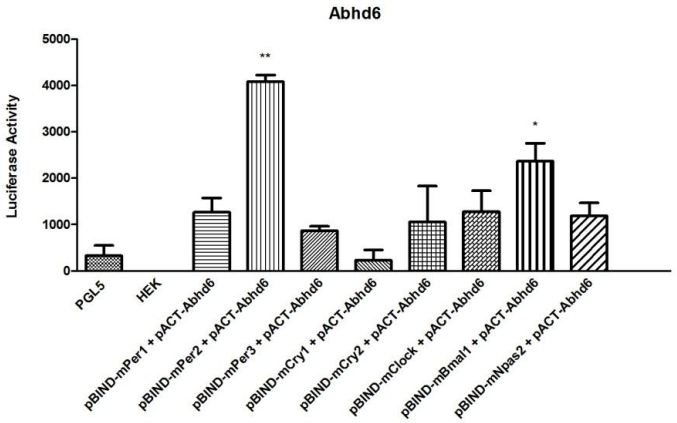

Ladder 1
Pxmp4
PPP1R3B template
Ladder 1
RASL11B template
RASL11B
Ladder 1
SLC37A4 template
SLC37A4
Ladder 1
TCF7L2

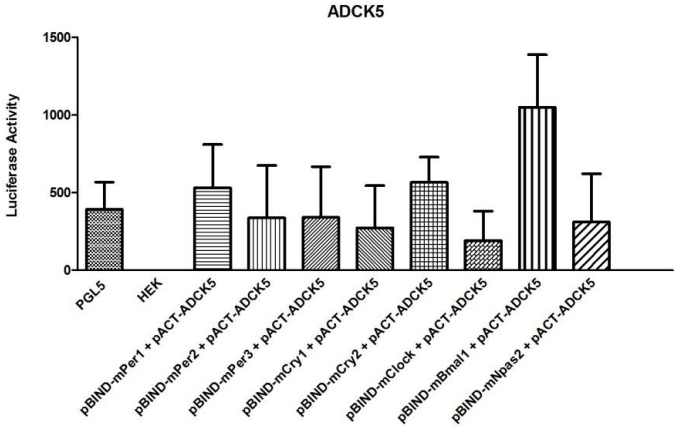

Ladder 1
Ahctf1 template
Ahctf1
TCF7L2 template
Ladder 1
Leprotl1 template
Leprot template
Ladder 1
BLCAP template
Atp6v1e1 template

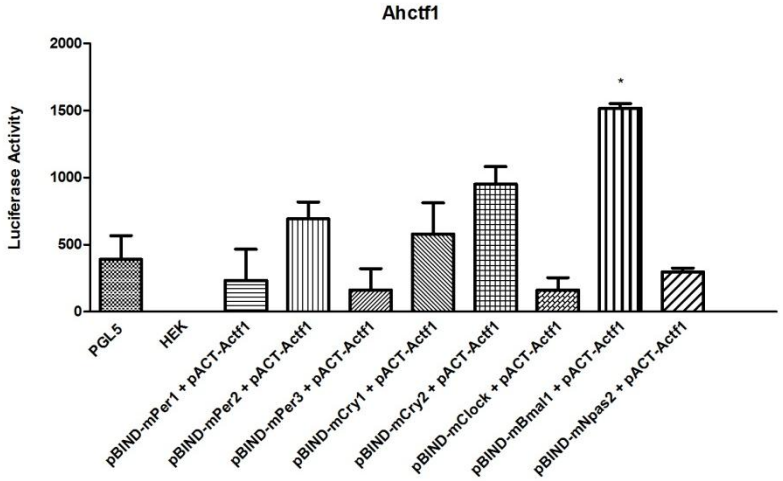

Ladder 1
P2RX4 template
Fkbp4 template
GSTM4 template
Ladder 1
Ppox template
Ahsa1 template
CXADR template

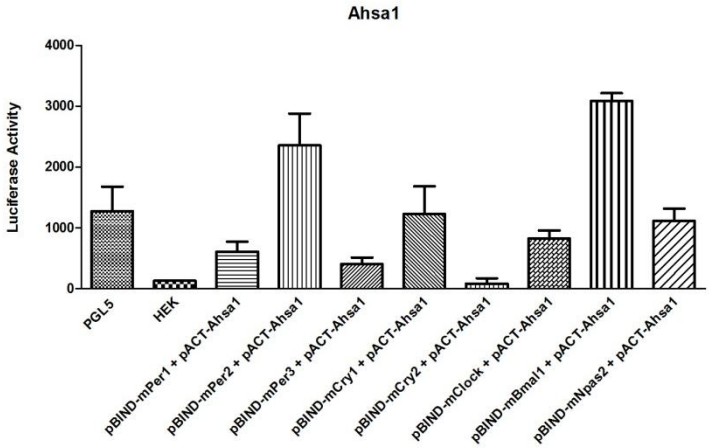
APPENDIX D: Mammalian Two Hybrid Screen Data

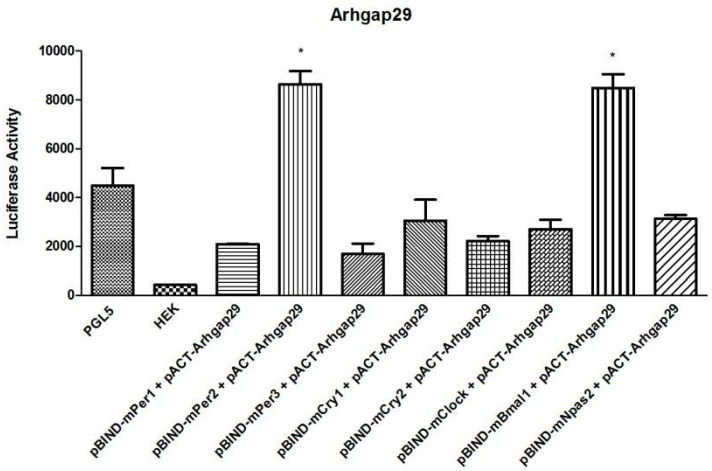


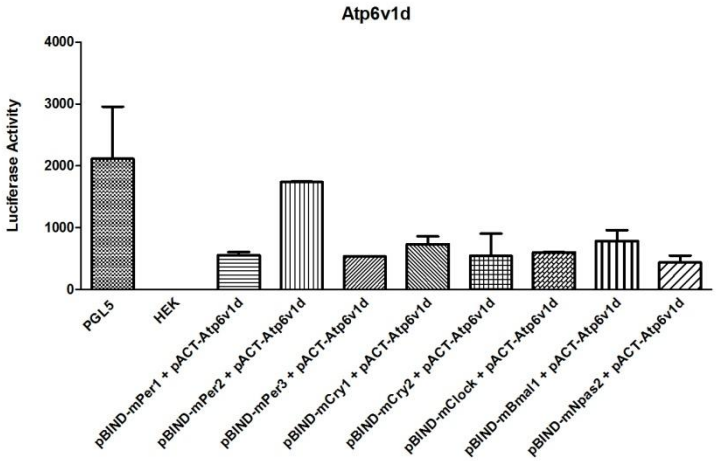





Ahctf1
Luciferase Activity
2000
1500
1000
500
0
*
PGL5
HEK
pBIND-mPer1 + pACT-Actf1
pBIND-mPer2 + pACT-Actf1
pBIND-mPer3 + pACT-Actf1
pBIND-mCry1 + pACT-Actf1
pBIND-mCry2 + pACT-Actf1
pBIND-mClock + pACT-Actf1
pBIND-mBmal1 + pACT-Actf1
pBIND-mNpas2 + pACT-Actf1


Ahsa1
Luciferase Activity
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-Ahsa1
pBIND-mPer2 + pACT-Ahsa1
pBIND-mPer3 + pACT-Ahsa1
pBIND-mCry1 + pACT-Ahsa1
pBIND-mCry2 + pACT-Ahsa1
pBIND-mClock + pACT-Ahsa1
pBIND-mBmal1 + pACT-Ahsa1
pBIND-mNpas2 + pACT-Ahsa1


Arhgap29
Luciferase Activity
10000
8000
6000
4000
2000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-Arhgap29
pBIND-mPer2 + pACT-Arhgap29
pBIND-mPer3 + pACT-Arhgap29
pBIND-mCry1 + pACT-Arhgap29
pBIND-mCry2 + pACT-Arhgap29
pBIND-mClock + pACT-Arhgap29
pBIND-mBmal1 + pACT-Arhgap29
pBIND-mNpas2 + pACT-Arhgap29


Atp6v1d
Luciferase Activity
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-Atp6v1d
pBIND-mPer2 + pACT-Atp6v1d
pBIND-mPer3 + pACT-Atp6v1d
pBIND-mCry1 + pACT-Atp6v1d
pBIND-mCry2 + pACT-Atp6v1d
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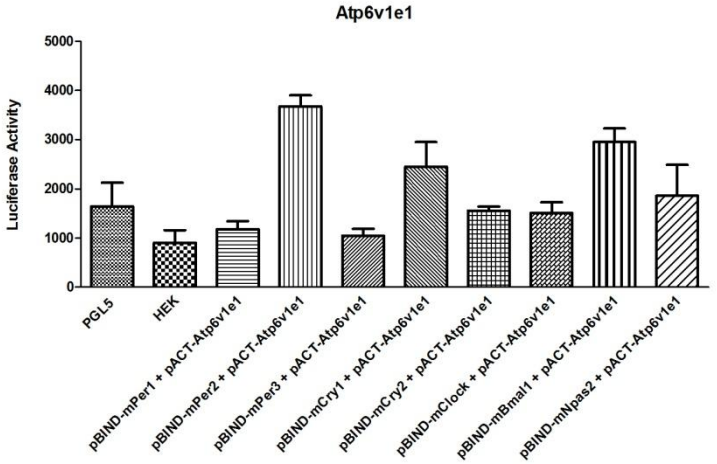
pBIND-mNpas2 + pACT-Atp6v1d


Atp6v1e1
Luciferase Activity
5000
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-Atp6v1e1
pBIND-mPer2 + pACT-Atp6v1e1
pBIND-mPer3 + pACT-Atp6v1e1
pBIND-mCry1 + pACT-Atp6v1e1
pBIND-mCry2 + pACT-Atp6v1e1
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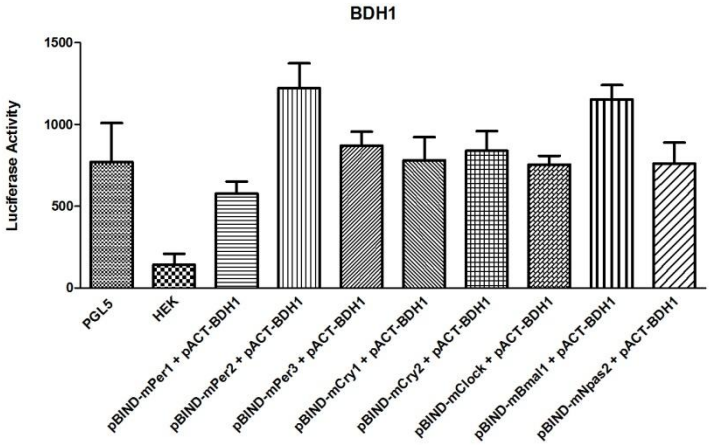
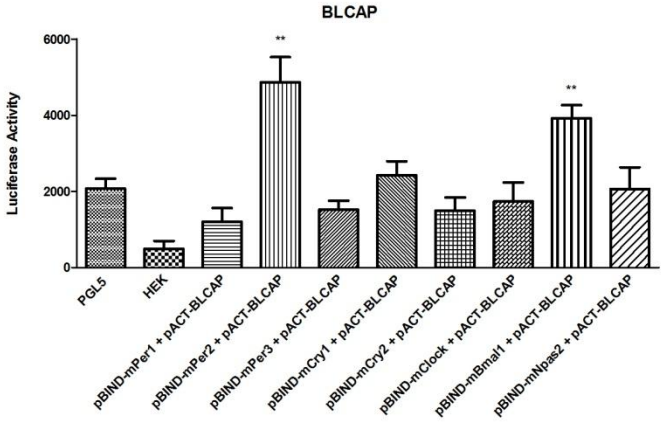

BDH1
Luciferase Activity
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-BDH1
pBIND-mPer2 + pACT-BDH1
pBIND-mPer3 + pACT-BDH1
pBIND-mCry1 + pACT-BDH1
pBIND-mCry2 + pACT-BDH1
pBIND-mClock + pACT-BDH1
pBIND-mBmal1 + pACT-BDH1
pBIND-mNpas2 + pACT-BDH1


BLCAP
Luciferase Activity
6000
4000
2000
0
**
**
PGL5
HEK
pBIND-mPer1 + pACT-BLCAP
pBIND-mPer2 + pACT-BLCAP
pBIND-mPer3 + pACT-BLCAP
pBIND-mCry1 + pACT-BLCAP
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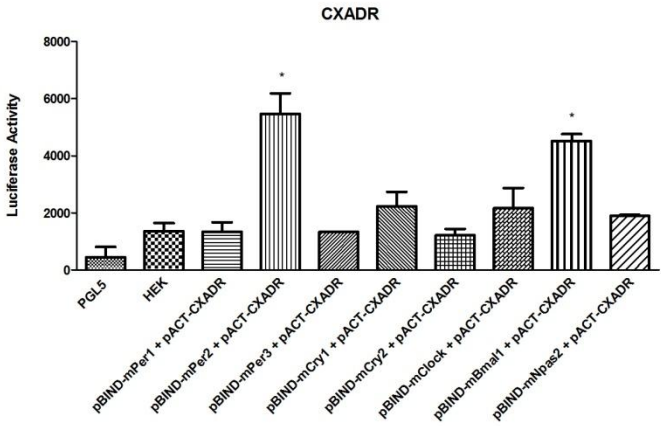
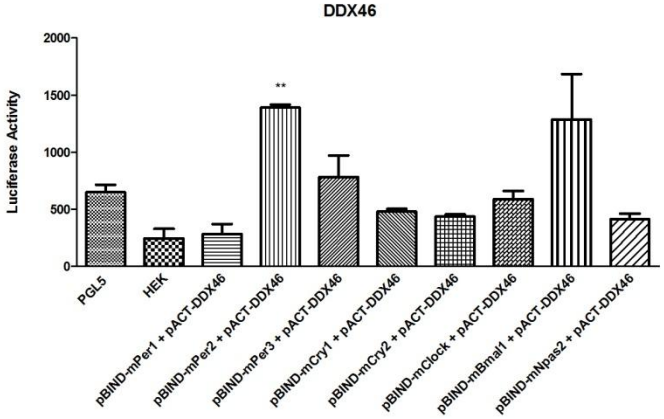
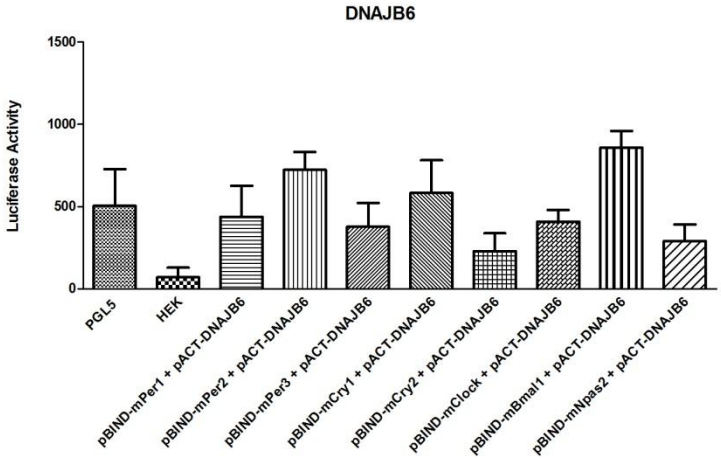
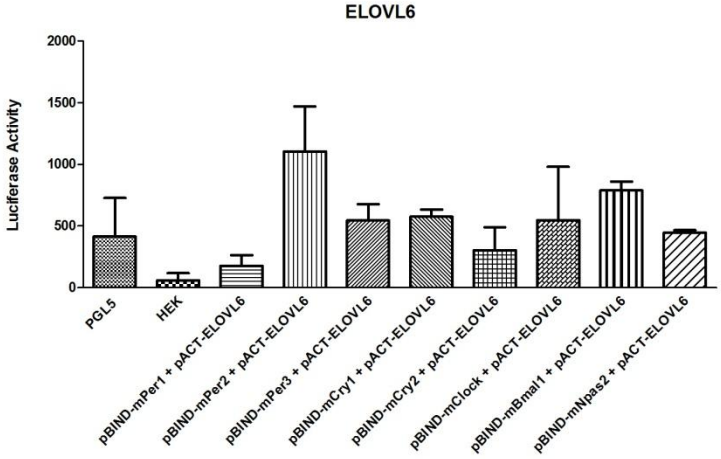
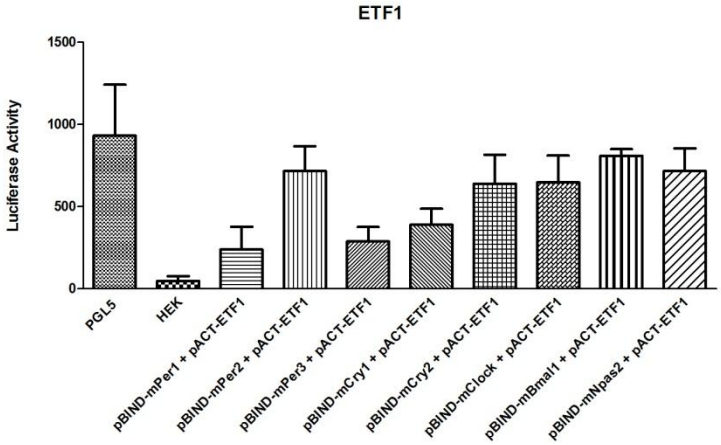
pBIND-mNpas2 + pACT-BLCAP

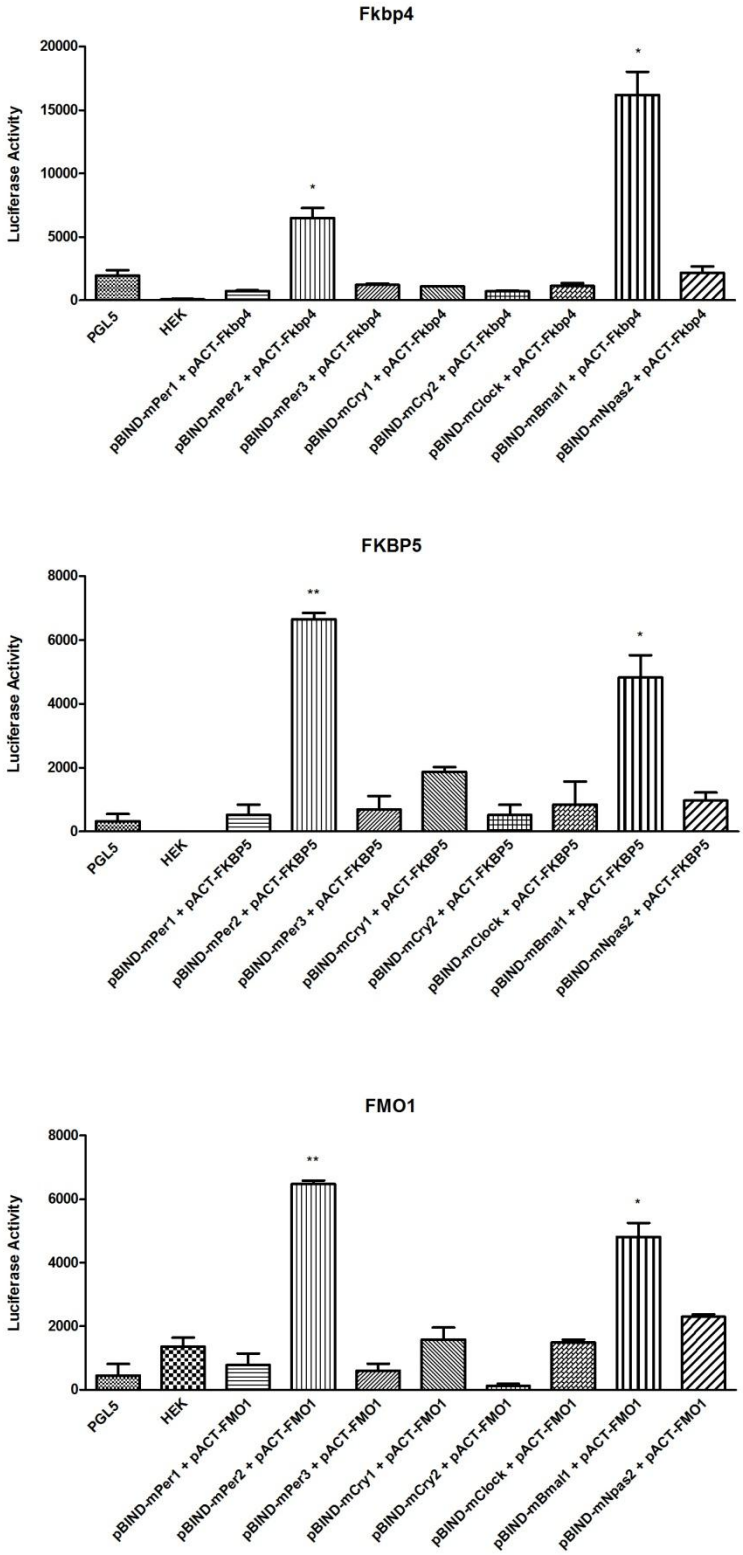

CXADR
Luciferase Activity
8000
6000
4000
2000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-CXADR
pBIND-mPer2 + pACT-CXADR
pBIND-mPer3 + pACT-CXADR
pBIND-mCry1 + pACT-CXADR
pBIND-mCry2 + pACT-CXADR
pBIND-mClock + pACT-CXADR
pBIND-mBmal1 + pACT-CXADR
pBIND-mNpas2 + pACT-CXADR

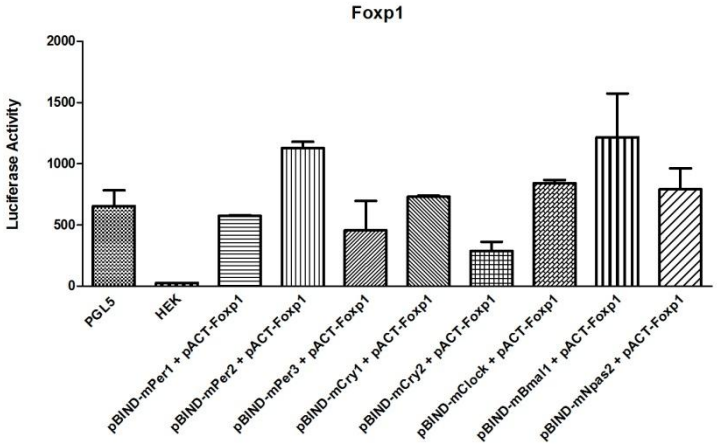

DDX46
Luciferase Activity
2000
1500
1000
500
0
**
PGL5
HEK
pBIND-mPer1 + pACT-DDX46
pBIND-mPer2 + pACT-DDX46
pBIND-mPer3 + pACT-DDX46
pBIND-mCry1 + pACT-DDX46
pBIND-mCry2 + pACT-DDX46
pBIND-mClock + pACT-DDX46
pBIND-mBmal1 + pACT-DDX46
pBIND-mNpas2 + pACT-DDX46

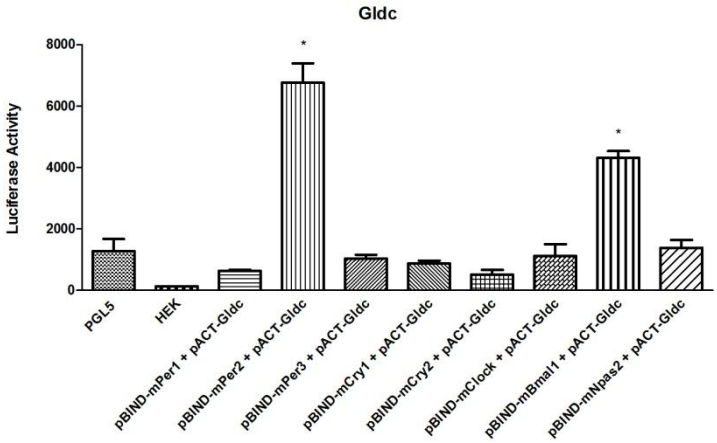

DNAJB6
Luciferase Activity
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-DNAJB6
pBIND-mPer2 + pACT-DNAJB6
pBIND-mPer3 + pACT-DNAJB6
pBIND-mCry1 + pACT-DNAJB6
pBIND-mCry2 + pACT-DNAJB6
pBIND-mClock + pACT-DNAJB6
pBIND-mBmal1 + pACT-DNAJB6
pBIND-mNpas2 + pACT-DNAJB6

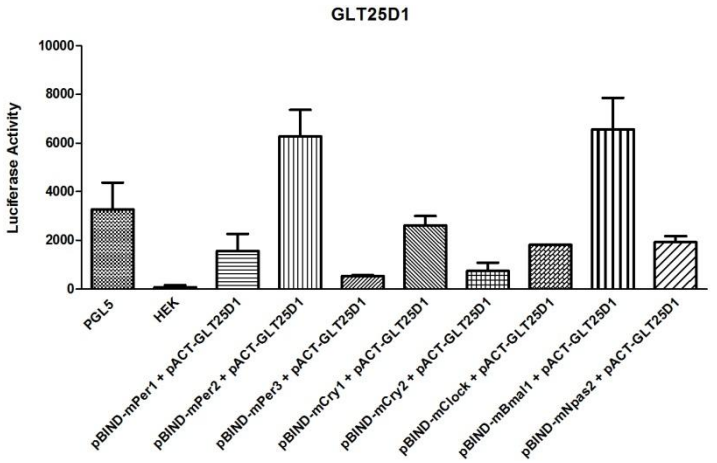

ELOVL6
Luciferase Activity
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-ELOVL6
pBIND-mPer2 + pACT-ELOVL6
pBIND-mPer3 + pACT-ELOVL6
pBIND-mCry1 + pACT-ELOVL6
pBIND-mCry2 + pACT-ELOVL6
pBIND-mClock + pACT-ELOVL6
pBIND-mBmal1 + pACT-ELOVL6
pBIND-mNpas2 + pACT-ELOVL6

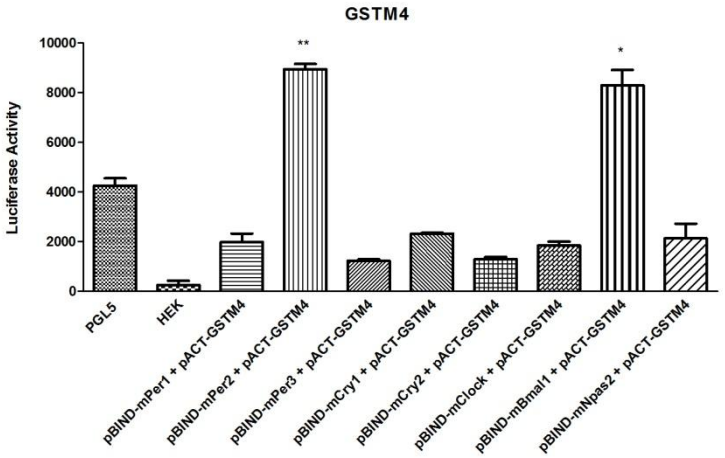

ETF1
Luciferase Activity
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-ETF1
pBIND-mPer2 + pACT-ETF1
pBIND-mPer3 + pACT-ETF1
pBIND-mCry1 + pACT-ETF1
pBIND-mCry2 + pACT-ETF1
pBIND-mClock + pACT-ETF1
pBIND-mBmal1 + pACT-ETF1
pBIND-mNpas2 + pACT-ETF1

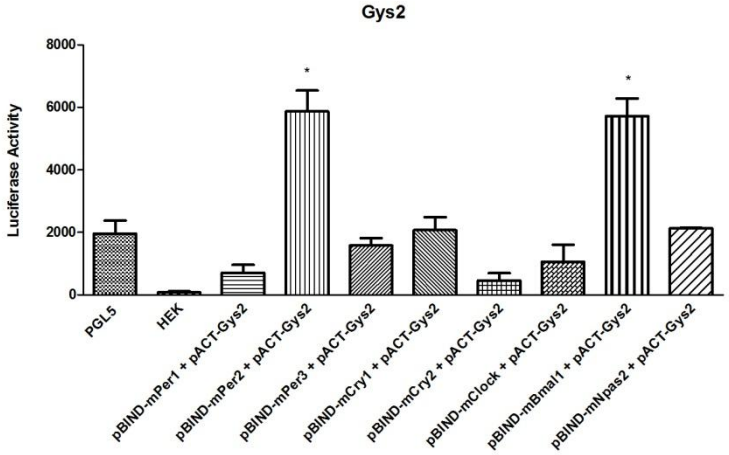

Fkbp4
Luciferase Activity
20000
15000
10000
5000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-Fkbp4
pBIND-mPer2 + pACT-Fkbp4
pBIND-mPer3 + pACT-Fkbp4
pBIND-mCry1 + pACT-Fkbp4
pBIND-mCry2 + pACT-Fkbp4
pBIND-mClock + pACT-Fkbp4
pBIND-mBmal1 + pACT-Fkbp4
pBIND-mNpas2 + pACT-Fkbp4
FKBP5
Luciferase Activity
8000
6000
4000
2000
0
**
*
PGL5
HEK
pBIND-mPer1 + pACT-FKBP5
pBIND-mPer2 + pACT-FKBP5
pBIND-mPer3 + pACT-FKBP5
pBIND-mCry1 + pACT-FKBP5
pBIND-mCry2 + pACT-FKBP5
pBIND-mClock + pACT-FKBP5
pBIND-mBmal1 + pACT-FKBP5
pBIND-mNpas2 + pACT-FKBP5
FMO1
Luciferase Activity
8000
6000
4000
2000
0
**
*
PGL5
HEK
pBIND-mPer1 + pACT-FMO1
pBIND-mPer2 + pACT-FMO1
pBIND-mPer3 + pACT-FMO1
pBIND-mCry1 + pACT-FMO1
pBIND-mCry2 + pACT-FMO1
pBIND-mClock + pACT-FMO1
pBIND-mBmal1 + pACT-FMO1
pBIND-mNpas2 + pACT-FMO1

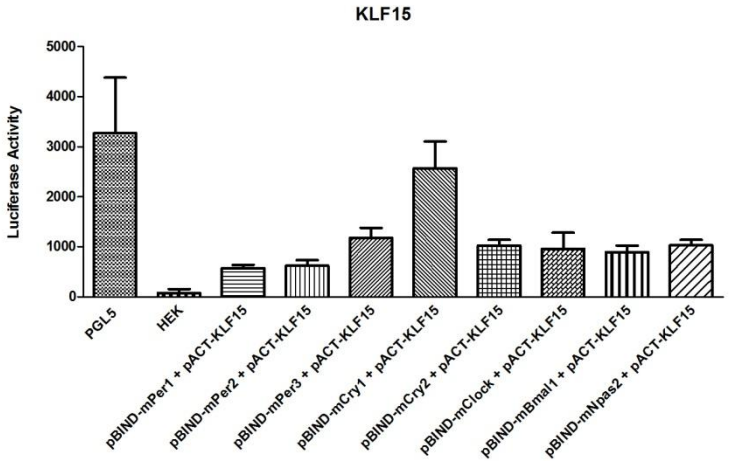

Foxp1
Luciferase Activity
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-Foxp1
pBIND-mPer2 + pACT-Foxp1
pBIND-mPer3 + pACT-Foxp1
pBIND-mCry1 + pACT-Foxp1
pBIND-mCry2 + pACT-Foxp1
pBIND-mClock + pACT-Foxp1
pBIND-mBmal1 + pACT-Foxp1
pBIND-mNpas2 + pACT-Foxp1

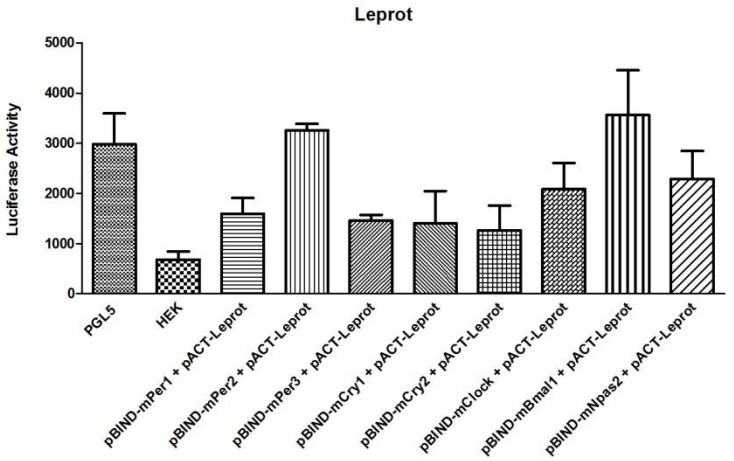

Gldc
Luciferase Activity
8000
6000
4000
2000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-Gldc
pBIND-mPer2 + pACT-Gldc
pBIND-mPer3 + pACT-Gldc
pBIND-mCry1 + pACT-Gldc
pBIND-mCry2 + pACT-Gldc
pBIND-mClock + pACT-Gldc
pBIND-mBmal1 + pACT-Gldc
pBIND-mNpas2 + pACT-Gldc

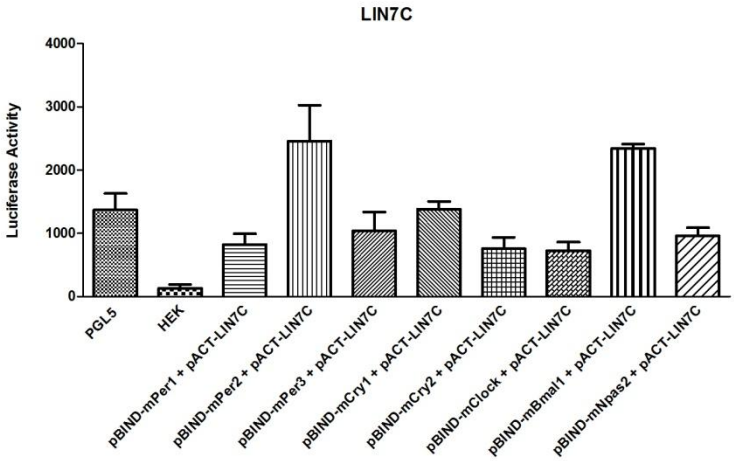

GLT25D1
Luciferase Activity
10000
8000
6000
4000
2000
0
PGL5
HEK
pBIND-mPer1 + pACT-GLT25D1
pBIND-mPer2 + pACT-GLT25D1
pBIND-mPer3 + pACT-GLT25D1
pBIND-mCry1 + pACT-GLT25D1
pBIND-mCry2 + pACT-GLT25D1
pBIND-mClock + pACT-GLT25D1
pBIND-mBmal1 + pACT-GLT25D1
pBIND-mNpas2 + pACT-GLT25D1

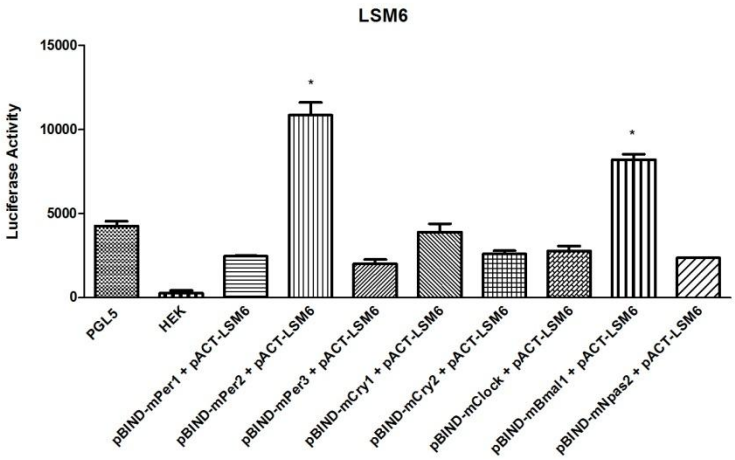

GSTM4
Luciferase Activity
10000
8000
6000
4000
2000
0
**
*
PGL5
HEK
pBIND-mPer1 + pACT-GSTM4
pBIND-mPer2 + pACT-GSTM4
pBIND-mPer3 + pACT-GSTM4
pBIND-mCry1 + pACT-GSTM4
pBIND-mCry2 + pACT-GSTM4
pBIND-mClock + pACT-GSTM4
pBIND-mBmal1 + pACT-GSTM4
pBIND-mNpas2 + pACT-GSTM4

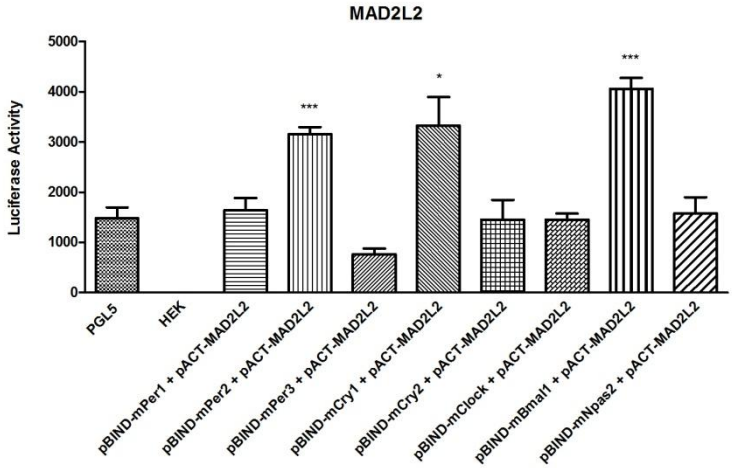

Gys2
Luciferase Activity
8000
6000
4000
2000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-Gys2
pBIND-mPer2 + pACT-Gys2
pBIND-mPer3 + pACT-Gys2
pBIND-mCry1 + pACT-Gys2
pBIND-mCry2 + pACT-Gys2
pBIND-mClock + pACT-Gys2
pBIND-mBmal1 + pACT-Gys2
pBIND-mNpas2 + pACT-Gys2

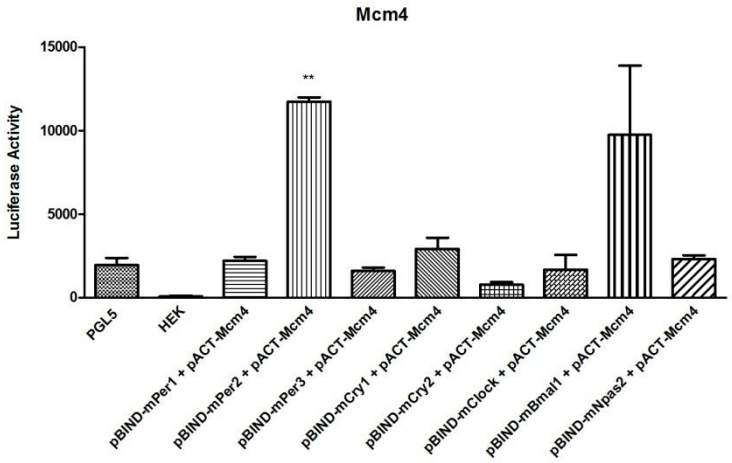

KLF15
Luciferase Activity
5000
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-KLF15
pBIND-mPer2 + pACT-KLF15
pBIND-mPer3 + pACT-KLF15
pBIND-mCry1 + pACT-KLF15
pBIND-mCry2 + pACT-KLF15
pBIND-mClock + pACT-KLF15
pBIND-mBmal1 + pACT-KLF15
pBIND-mNpas2 + pACT-KLF15

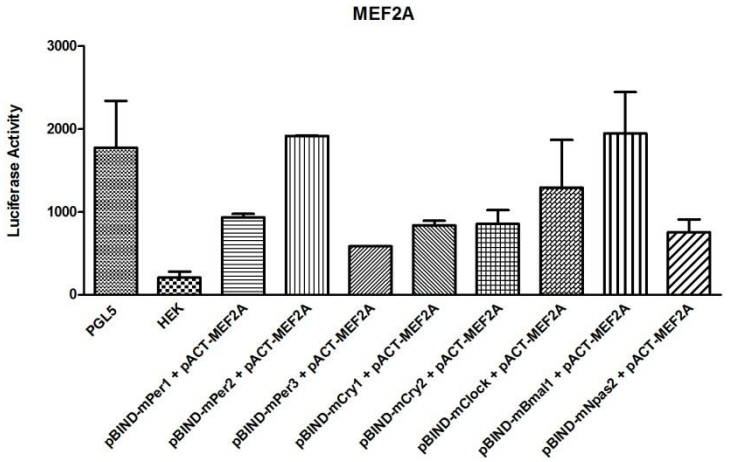

Leprot
Luciferase Activity
5000
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-Leprot
pBIND-mPer2 + pACT-Leprot
pBIND-mPer3 + pACT-Leprot
pBIND-mCry1 + pACT-Leprot
pBIND-mCry2 + pACT-Leprot
pBIND-mClock + pACT-Leprot
pBIND-mBmal1 + pACT-Leprot
pBIND-mNpas2 + pACT-Leprot

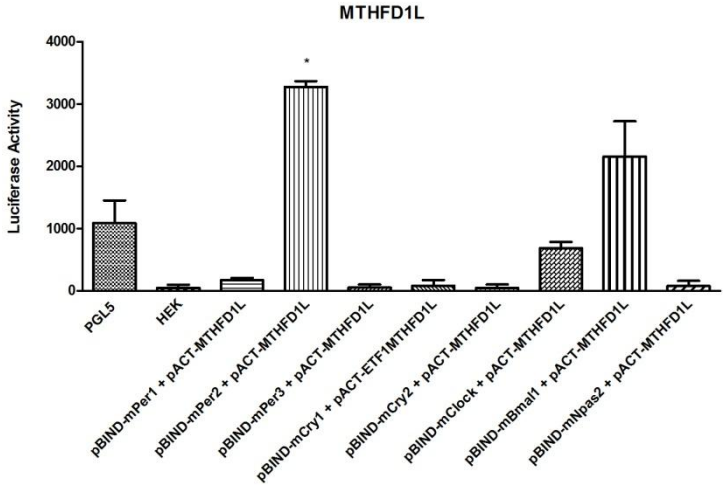

LIN7C
Luciferase Activity
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-LIN7C
pBIND-mPer2 + pACT-LIN7C
pBIND-mPer3 + pACT-LIN7C
pBIND-mCry1 + pACT-LIN7C
pBIND-mCry2 + pACT-LIN7C
pBIND-mClock + pACT-LIN7C
pBIND-mBmal1 + pACT-LIN7C
pBIND-mNpas2 + pACT-LIN7C

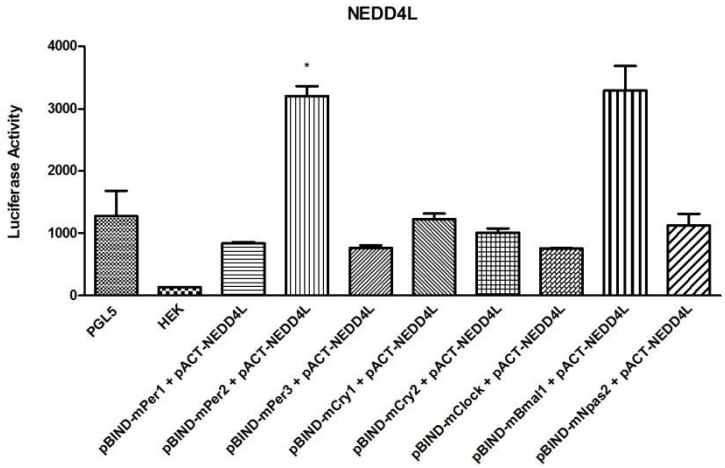

LSM6
Luciferase Activity
15000
10000
5000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-LSM6
pBIND-mPer2 + pACT-LSM6
pBIND-mPer3 + pACT-LSM6
pBIND-mCry1 + pACT-LSM6
pBIND-mCry2 + pACT-LSM6
pBIND-mClock + pACT-LSM6
pBIND-mBmal1 + pACT-LSM6
pBIND-mNpas2 + pACT-LSM6

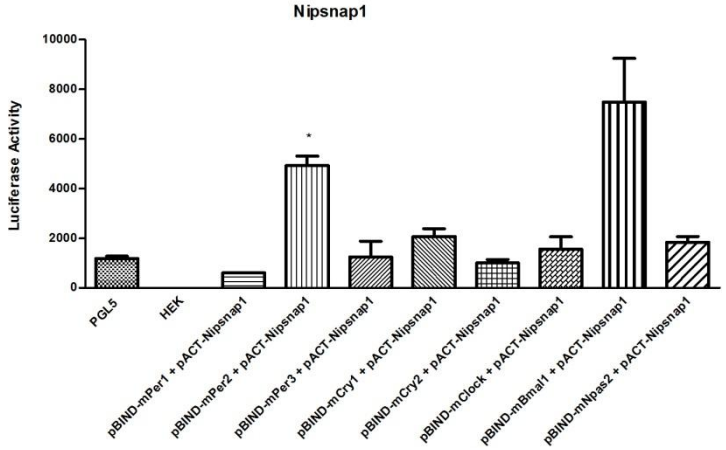

MAD2L2
Luciferase Activity
5000
4000
3000
2000
1000
0
***
*
***
PGL5
HEK
pBIND-mPer1 + pACT-MAD2L2
pBIND-mPer2 + pACT-MAD2L2
pBIND-mPer3 + pACT-MAD2L2
pBIND-mCry1 + pACT-MAD2L2
pBIND-mCry2 + pACT-MAD2L2
pBIND-mClock + pACT-MAD2L2
pBIND-mBmal1 + pACT-MAD2L2
pBIND-mNpas2 + pACT-MAD2L2

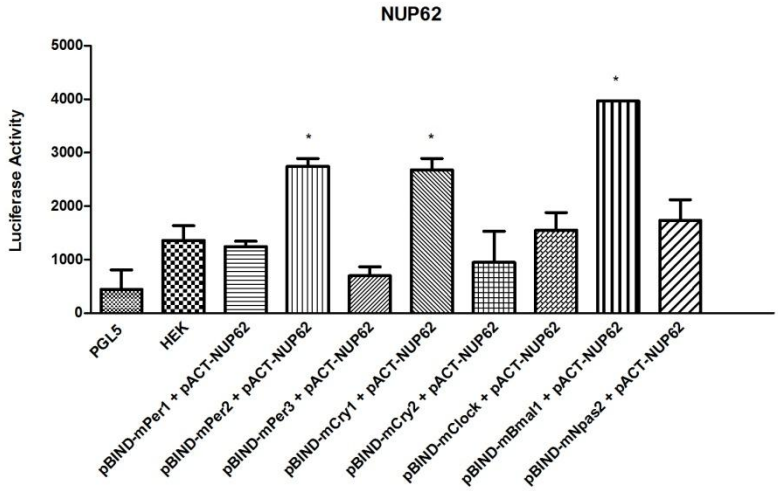

Mcm4
Luciferase Activity
15000
10000
5000
0
**
PGL5
HEK
pBIND-mPer1 + pACT-Mcm4
pBIND-mPer2 + pACT-Mcm4
pBIND-mPer3 + pACT-Mcm4
pBIND-mCry1 + pACT-Mcm4
pBIND-mCry2 + pACT-Mcm4
pBIND-mClock + pACT-Mcm4
pBIND-mBmal1 + pACT-Mcm4
pBIND-mNpas2 + pACT-Mcm4

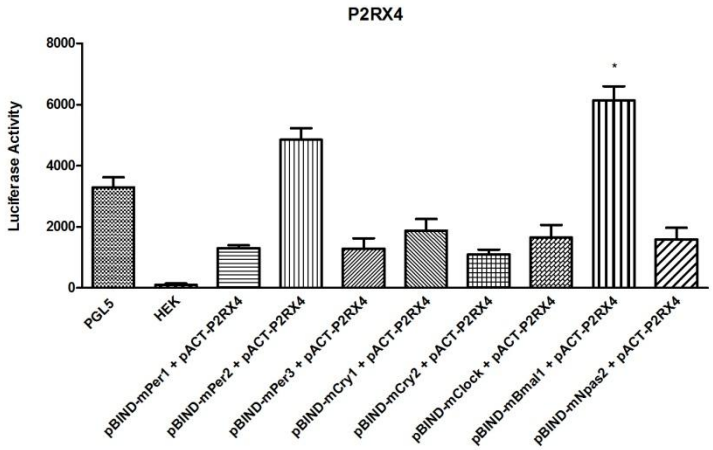

MEF2A
Luciferase Activity
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-MEF2A
pBIND-mPer2 + pACT-MEF2A
pBIND-mPer3 + pACT-MEF2A
pBIND-mCry1 + pACT-MEF2A
pBIND-mCry2 + pACT-MEF2A
pBIND-mClock + pACT-MEF2A
pBIND-mBmal1 + pACT-MEF2A
pBIND-mNpas2 + pACT-MEF2A

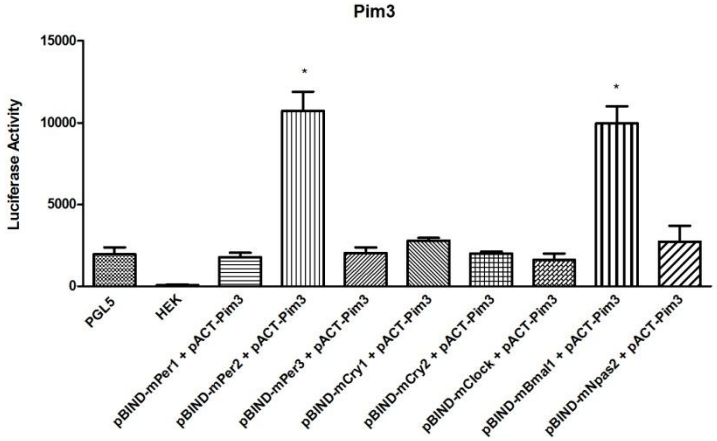

MTHFD1L
Luciferase Activity
4000
3000
2000
1000
0
*
PGL5
HEK
pBIND-mPer1 + pACT-MTHFD1L
pBIND-mPer2 + pACT-MTHFD1L
pBIND-mPer3 + pACT-MTHFD1L
pBIND-mCry1 + pACT-ETF1MTHFD1L
pBIND-mCry2 + pACT-MTHFD1L
pBIND-mClock + pACT-MTHFD1L
pBIND-mBmal1 + pACT-MTHFD1L
pBIND-mNpas2 + pACT-MTHFD1L

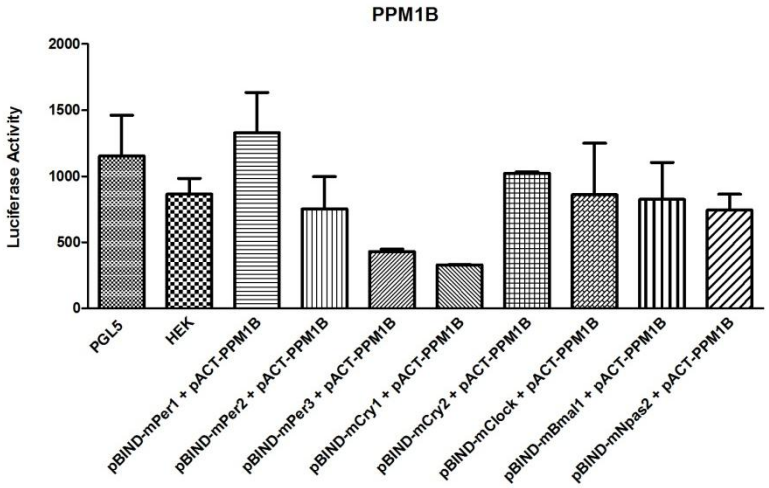

NEDD4L
Luciferase Activity
4000
3000
2000
1000
0
*
PGL5
HEK
pBIND-mPer1 + pACT-NEDD4L
pBIND-mPer2 + pACT-NEDD4L
pBIND-mPer3 + pACT-NEDD4L
pBIND-mCry1 + pACT-NEDD4L
pBIND-mCry2 + pACT-NEDD4L
pBIND-mClock + pACT-NEDD4L
pBIND-mBmal1 + pACT-NEDD4L
pBIND-mNpas2 + pACT-NEDD4L

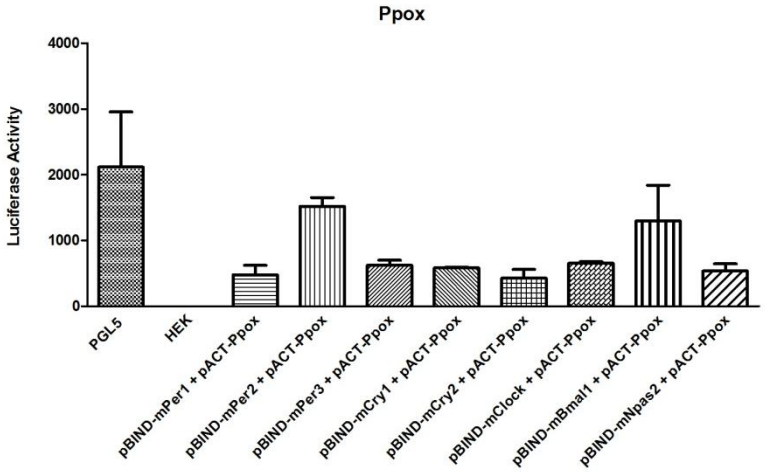

Nipsnap1
Luciferase Activity
10000
8000
6000
4000
2000
0
*
PGL5
HEK
pBIND-mPer1 + pACT-Nipsnap1
pBIND-mPer2 + pACT-Nipsnap1
pBIND-mPer3 + pACT-Nipsnap1
pBIND-mCry1 + pACT-Nipsnap1
pBIND-mCry2 + pACT-Nipsnap1
pBIND-mClock + pACT-Nipsnap1
pBIND-mBmal1 + pACT-Nipsnap1
pBIND-mNpas2 + pACT-Nipsnap1

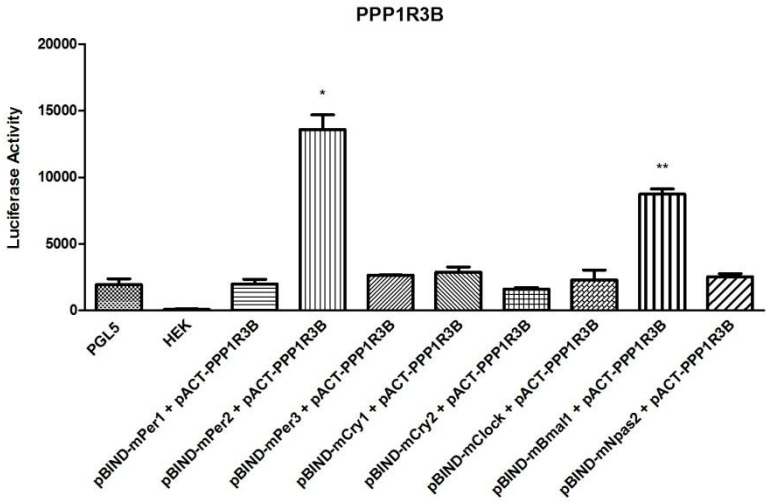

NUP62
Luciferase Activity
5000
4000
3000
2000
1000
0
*
*
*
PGL5
HEK
pBIND-mPer1 + pACT-NUP62
pBIND-mPer2 + pACT-NUP62
pBIND-mPer3 + pACT-NUP62
pBIND-mCry1 + pACT-NUP62
pBIND-mCry2 + pACT-NUP62
pBIND-mClock + pACT-NUP62
pBIND-mBmal1 + pACT-NUP62
pBIND-mNpas2 + pACT-NUP62

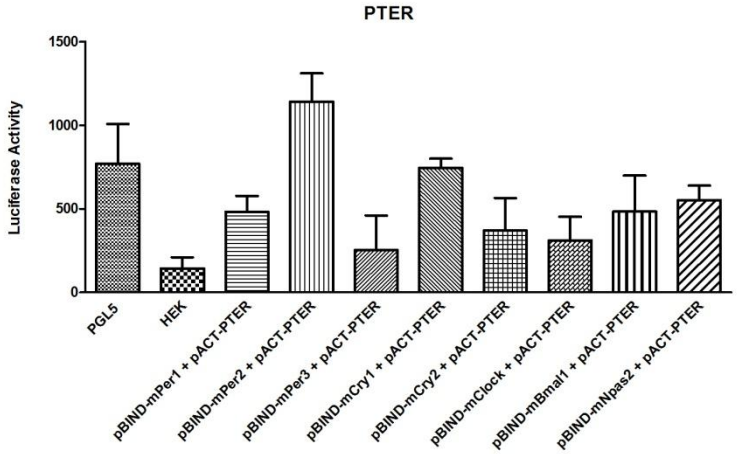

P2RX4
Luciferase Activity
8000
6000
4000
2000
0
*
PGL5
HEK
pBIND-mPer1 + pACT-P2RX4
pBIND-mPer2 + pACT-P2RX4
pBIND-mPer3 + pACT-P2RX4
pBIND-mCry1 + pACT-P2RX4
pBIND-mCry2 + pACT-P2RX4
pBIND-mClock + pACT-P2RX4
pBIND-mBmal1 + pACT-P2RX4
pBIND-mNpas2 + pACT-P2RX4

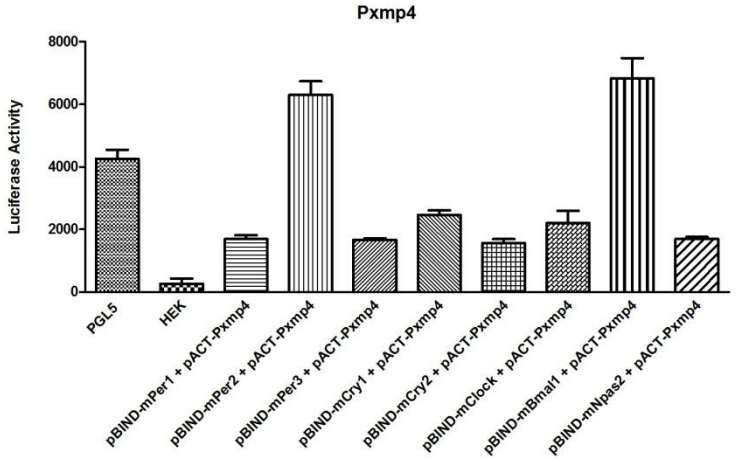

Pim3
Luciferase Activity
15000
10000
5000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-Pim3
pBIND-mPer2 + pACT-Pim3
pBIND-mPer3 + pACT-Pim3
pBIND-mCry1 + pACT-Pim3
pBIND-mCry2 + pACT-Pim3
pBIND-mClock + pACT-Pim3
pBIND-mBmal1 + pACT-Pim3
pBIND-mNpas2 + pACT-Pim3

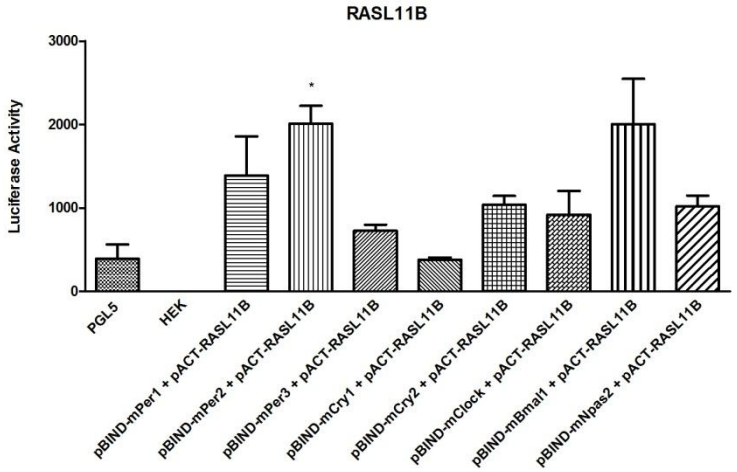

PPM1B
Luciferase Activity
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-PPM1B
pBIND-mPer2 + pACT-PPM1B
pBIND-mPer3 + pACT-PPM1B
pBIND-mCry1 + pACT-PPM1B
pBIND-mCry2 + pACT-PPM1B
pBIND-mClock + pACT-PPM1B
pBIND-mBmal1 + pACT-PPM1B
pBIND-mNpas2 + pACT-PPM1B

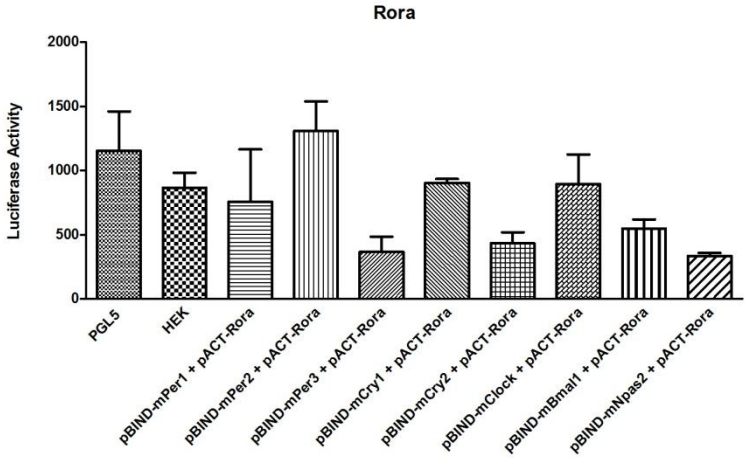

Ppox
Luciferase Activity
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-Ppox
pBIND-mPer2 + pACT-Ppox
pBIND-mPer3 + pACT-Ppox
pBIND-mCry1 + pACT-Ppox
pBIND-mCry2 + pACT-Ppox
pBIND-mClock + pACT-Ppox
pBIND-mBmal1 + pACT-Ppox
pBIND-mNpas2 + pACT-Ppox

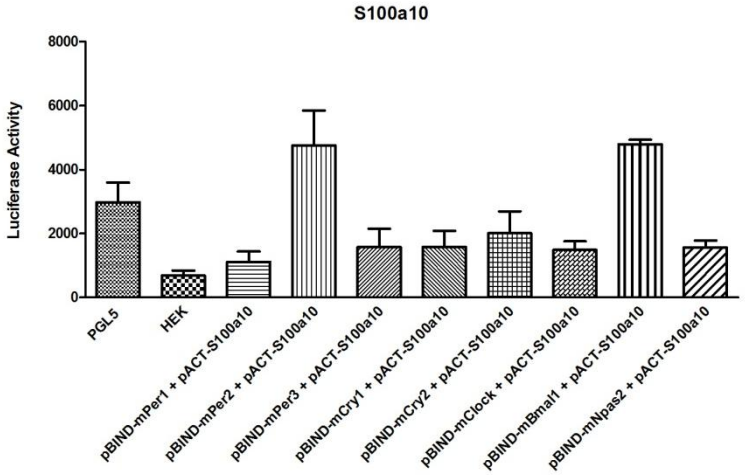

PPP1R3B
Luciferase Activity
20000
15000
10000
5000
0
*
**
PGL5
HEK
pBIND-mPer1 + pACT-PPP1R3B
pBIND-mPer2 + pACT-PPP1R3B
pBIND-mPer3 + pACT-PPP1R3B
pBIND-mCry1 + pACT-PPP1R3B
pBIND-mCry2 + pACT-PPP1R3B
pBIND-mClock + pACT-PPP1R3B
pBIND-mBmal1 + pACT-PPP1R3B
pBIND-mNpas2 + pACT-PPP1R3B

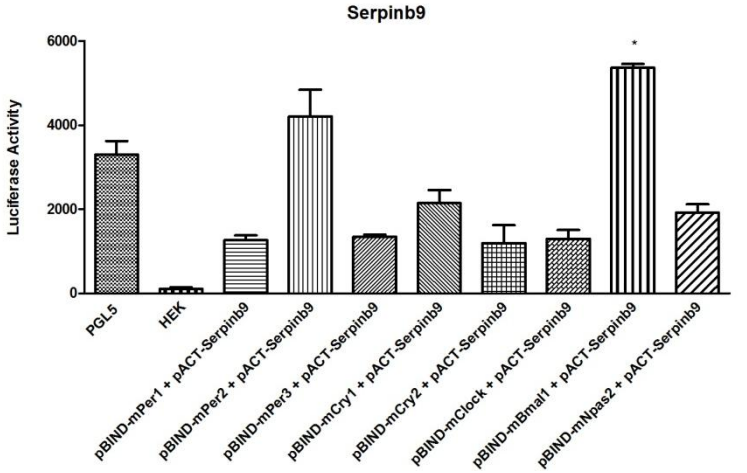

PTER
Luciferase Activity
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-PTER
pBIND-mPer2 + pACT-PTER
pBIND-mPer3 + pACT-PTER
pBIND-mCry1 + pACT-PTER
pBIND-mCry2 + pACT-PTER
pBIND-mClock + pACT-PTER
pBIND-mBmal1 + pACT-PTER
pBIND-mNpas2 + pACT-PTER

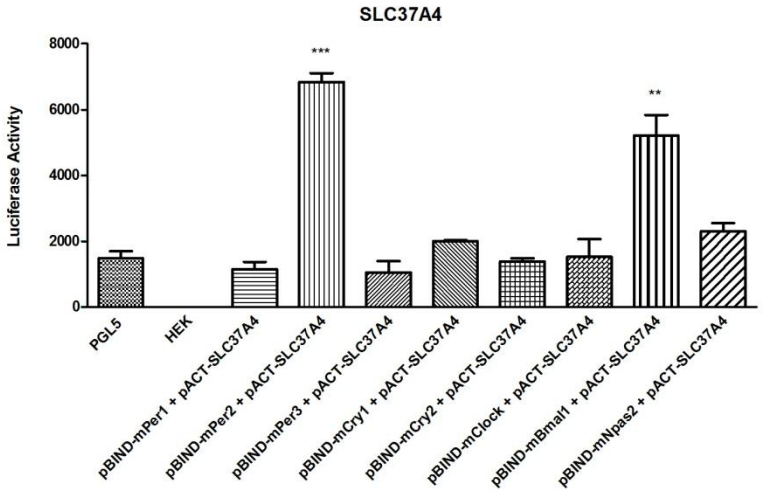

Pxmp4
Luciferase Activity
8000
6000
4000
2000
0
PGL5
HEK
pBIND-mPer1 + pACT-Pxmp4
pBIND-mPer2 + pACT-Pxmp4
pBIND-mPer3 + pACT-Pxmp4
pBIND-mCry1 + pACT-Pxmp4
pBIND-mCry2 + pACT-Pxmp4
pBIND-mClock + pACT-Pxmp4
pBIND-mBmal1 + pACT-Pxmp4
pBIND-mNpas2 + pACT-Pxmp4

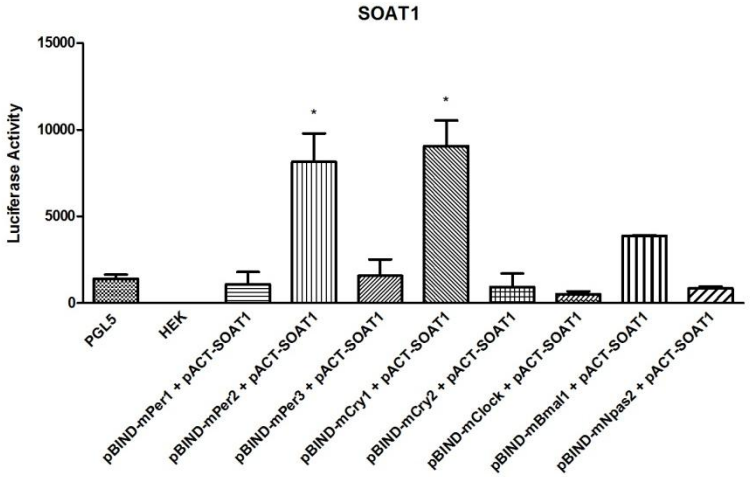

RASL11B
Luciferase Activity
3000
2000
1000
0
*
PGL5
HEK
pBIND-mPer1 + pACT-RASL11B
pBIND-mPer2 + pACT-RASL11B
pBIND-mPer3 + pACT-RASL11B
pBIND-mCry1 + pACT-RASL11B
pBIND-mCry2 + pACT-RASL11B
pBIND-mClock + pACT-RASL11B
pBIND-mBmal1 + pACT-RASL11B
pBIND-mNpas2 + pACT-RASL11B

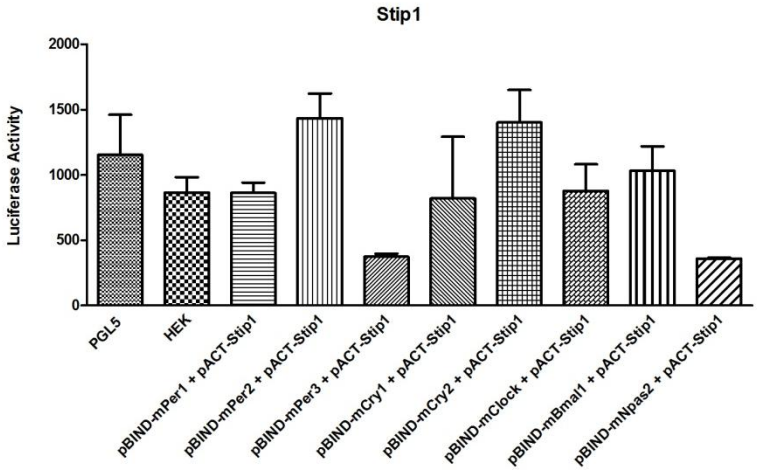

Rora
Luciferase Activity
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-Rora
pBIND-mPer2 + pACT-Rora
pBIND-mPer3 + pACT-Rora
pBIND-mCry1 + pACT-Rora
pBIND-mCry2 + pACT-Rora
pBIND-mClock + pACT-Rora
pBIND-mBmal1 + pACT-Rora
pBIND-mNpas2 + pACT-Rora

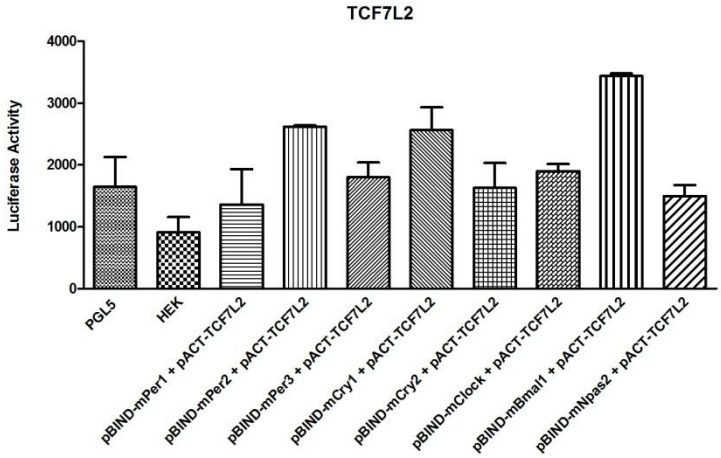

S100a10
Luciferase Activity
8000
6000
4000
2000
0
PGL5
HEK
pBIND-mPer1 + pACT-S100a10
pBIND-mPer2 + pACT-S100a10
pBIND-mPer3 + pACT-S100a10
pBIND-mCry1 + pACT-S100a10
pBIND-mCry2 + pACT-S100a10
pBIND-mClock + pACT-S100a10
pBIND-mBmal1 + pACT-S100a10
pBIND-mNpas2 + pACT-S100a10

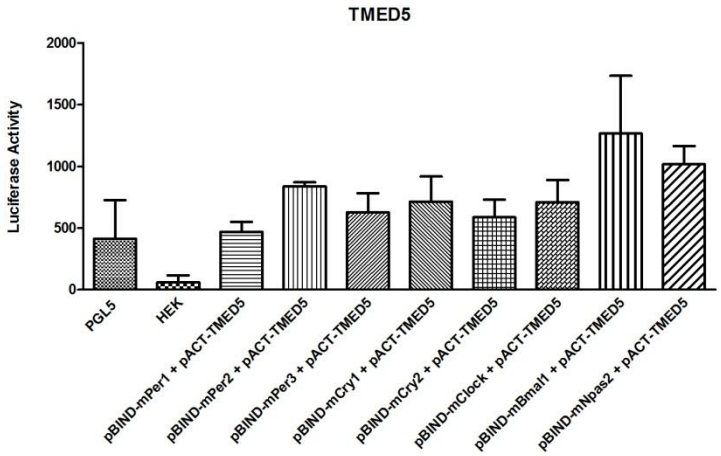

Serpinb9
Luciferase Activity
6000
4000
2000
0
*
PGL5
HEK
pBIND-mPer1 + pACT-Serpinb9
pBIND-mPer2 + pACT-Serpinb9
pBIND-mPer3 + pACT-Serpinb9
pBIND-mCry1 + pACT-Serpinb9
pBIND-mCry2 + pACT-Serpinb9
pBIND-mClock + pACT-Serpinb9
pBIND-mBmal1 + pACT-Serpinb9
pBIND-mNpas2 + pACT-Serpinb9

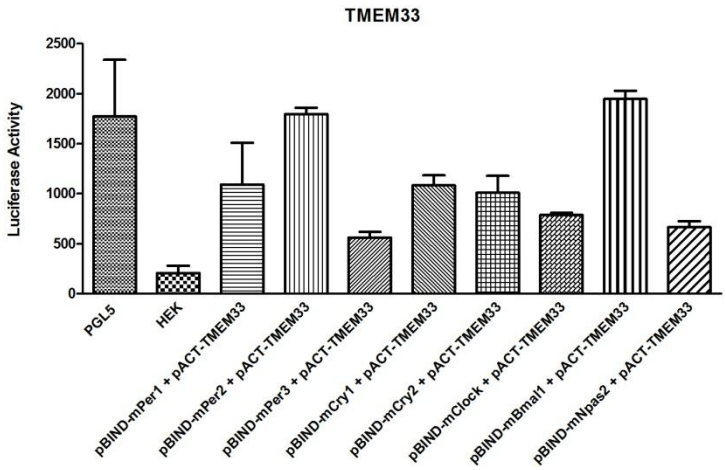

SLC37A4
Luciferase Activity
8000
6000
4000
2000
0
***
**
PGL5
HEK
pBIND-mPer1 + pACT-SLC37A4
pBIND-mPer2 + pACT-SLC37A4
pBIND-mPer3 + pACT-SLC37A4
pBIND-mCry1 + pACT-SLC37A4
pBIND-mCry2 + pACT-SLC37A4
pBIND-mClock + pACT-SLC37A4
pBIND-mBmal1 + pACT-SLC37A4
pBIND-mNpas2 + pACT-SLC37A4

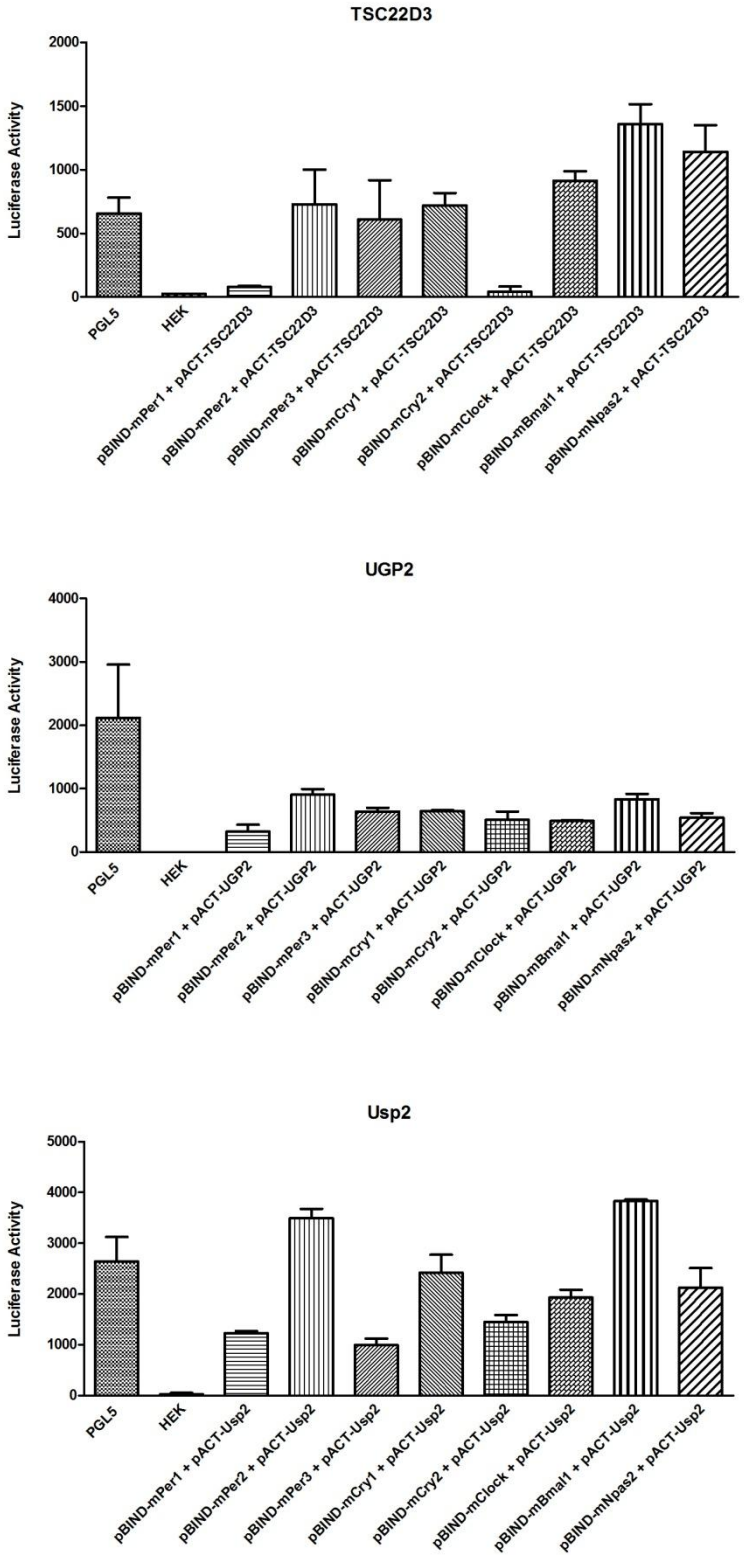

SOAT1
Luciferase Activity
15000
10000
5000
0
*
*
PGL5
HEK
pBIND-mPer1 + pACT-SOAT1
pBIND-mPer2 + pACT-SOAT1
pBIND-mPer3 + pACT-SOAT1
pBIND-mCry1 + pACT-SOAT1
pBIND-mCry2 + pACT-SOAT1
pBIND-mClock + pACT-SOAT1
pBIND-mBmal1 + pACT-SOAT1
pBIND-mNpas2 + pACT-SOAT1


Stip1
Luciferase Activity
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-Stip1
pBIND-mPer2 + pACT-Stip1
pBIND-mPer3 + pACT-Stip1
pBIND-mCry1 + pACT-Stip1
pBIND-mCry2 + pACT-Stip1
pBIND-mClock + pACT-Stip1
pBIND-mBmal1 + pACT-Stip1
pBIND-mNpas2 + pACT-Stip1

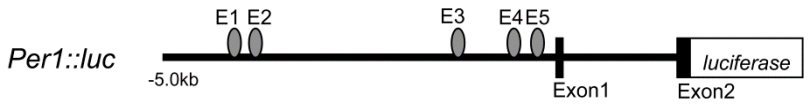

TCF7L2
Luciferase Activity
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-TCF7L2
pBIND-mPer2 + pACT-TCF7L2
pBIND-mPer3 + pACT-TCF7L2
pBIND-mCry1 + pACT-TCF7L2
pBIND-mCry2 + pACT-TCF7L2
pBIND-mClock + pACT-TCF7L2
pBIND-mBmal1 + pACT-TCF7L2
pBIND-mNpas2 + pACT-TCF7L2

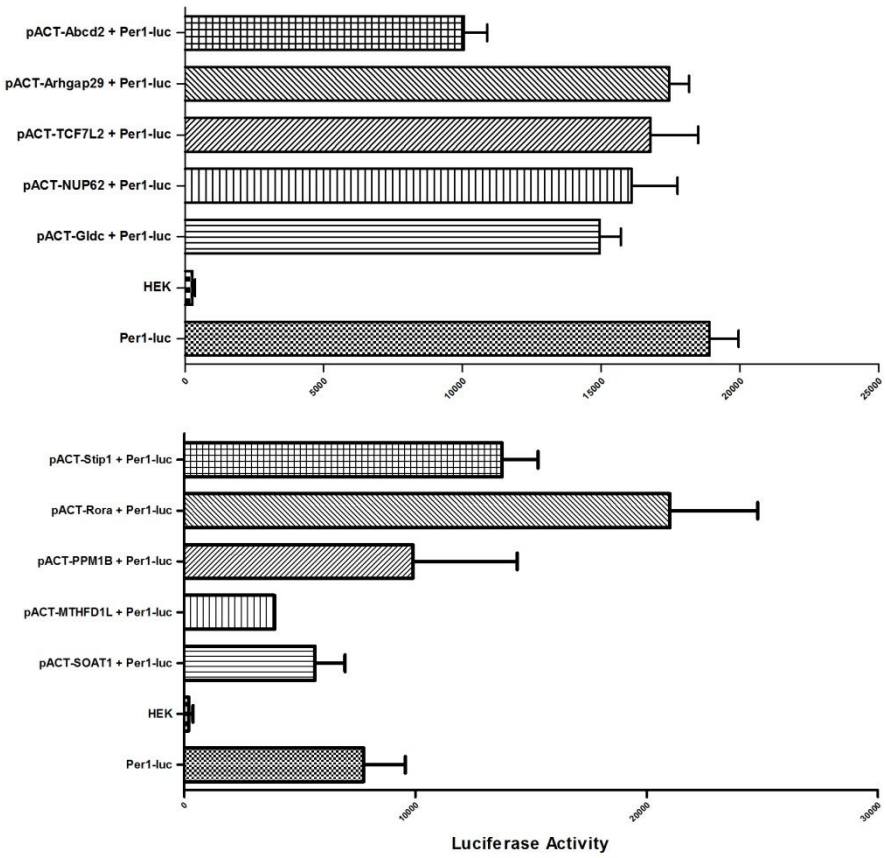

TMED5
Luciferase Activity
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-TMED5
pBIND-mPer2 + pACT-TMED5
pBIND-mPer3 + pACT-TMED5
pBIND-mCry1 + pACT-TMED5
pBIND-mCry2 + pACT-TMED5
pBIND-mClock + pACT-TMED5
pBIND-mBmal1 + pACT-TMED5
pBIND-mNpas2 + pACT-TMED5

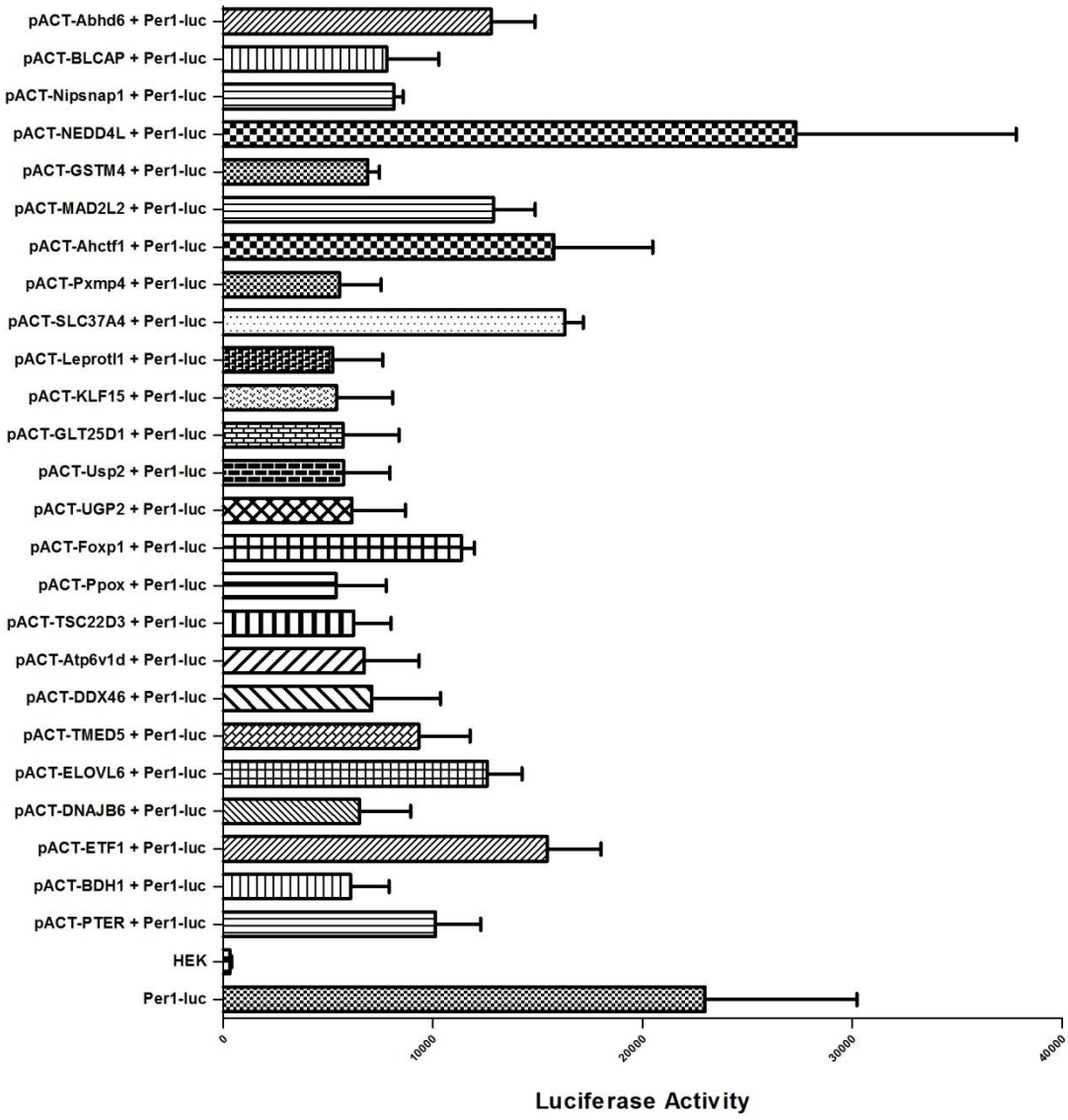

TMEM33
Luciferase Activity
2500
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-TMEM33
pBIND-mPer2 + pACT-TMEM33
pBIND-mPer3 + pACT-TMEM33
pBIND-mCry1 + pACT-TMEM33
pBIND-mCry2 + pACT-TMEM33
pBIND-mClock + pACT-TMEM33
pBIND-mBmal1 + pACT-TMEM33
pBIND-mNpas2 + pACT-TMEM33

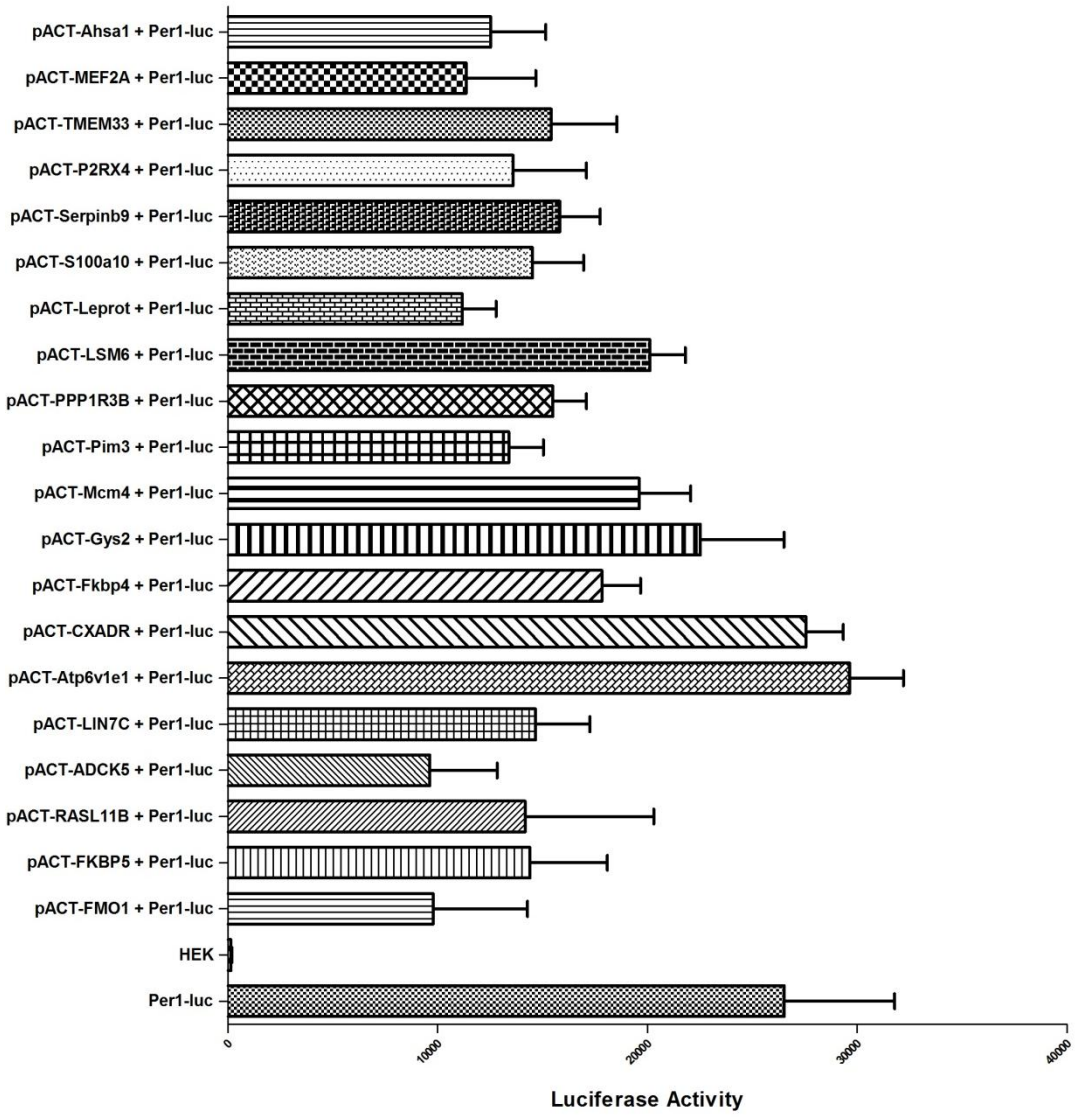

TSC22D3
Luciferase Activity
2000
1500
1000
500
0
PGL5
HEK
pBIND-mPer1 + pACT-TSC22D3
pBIND-mPer2 + pACT-TSC22D3
pBIND-mPer3 + pACT-TSC22D3
pBIND-mCry1 + pACT-TSC22D3
pBIND-mCry2 + pACT-TSC22D3
pBIND-mClock + pACT-TSC22D3
pBIND-mBmal1 + pACT-TSC22D3
pBIND-mNpas2 + pACT-TSC22D3
UGP2
Luciferase Activity
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-UGP2
pBIND-mPer2 + pACT-UGP2
pBIND-mPer3 + pACT-UGP2
pBIND-mCry1 + pACT-UGP2
pBIND-mCry2 + pACT-UGP2
pBIND-mClock + pACT-UGP2
pBIND-mBmal1 + pACT-UGP2
pBIND-mNpas2 + pACT-UGP2
Usp2
Luciferase Activity
5000
4000
3000
2000
1000
0
PGL5
HEK
pBIND-mPer1 + pACT-Usp2
pBIND-mPer2 + pACT-Usp2
pBIND-mPer3 + pACT-Usp2
pBIND-mCry1 + pACT-Usp2
pBIND-mCry2 + pACT-Usp2
pBIND-mClock + pACT-Usp2
pBIND-mBmal1 + pACT-Usp2
pBIND-mNpas2 + pACT-Usp2

Interaction of candidate genes with E-box regions of *Per1::luc* promoter was also investigated as this promoter consists of five E-boxes. Since core clock components BMAL1/CLOCK acts on E-boxes of clock-controlled genes, it stands to reason that these candidate genes might be involved indirectly in the regulation of the clock through E-boxes instead of directly interacting with core clock components.

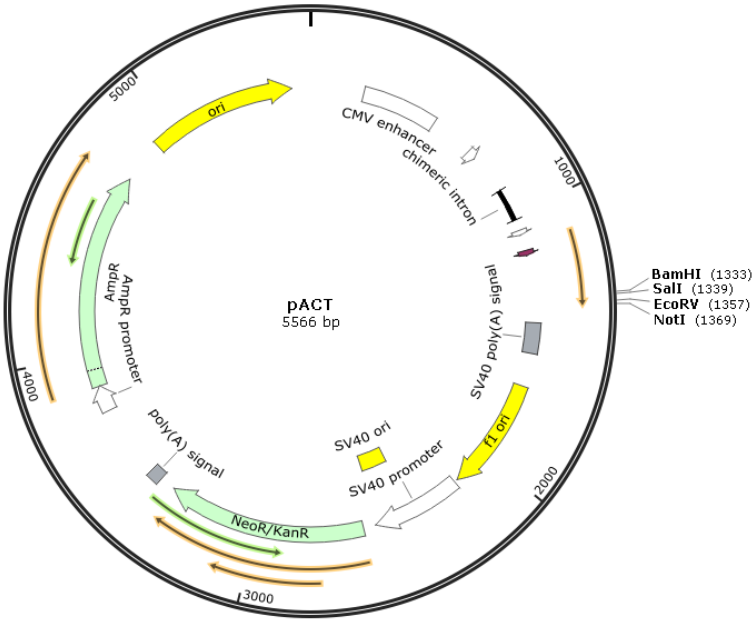


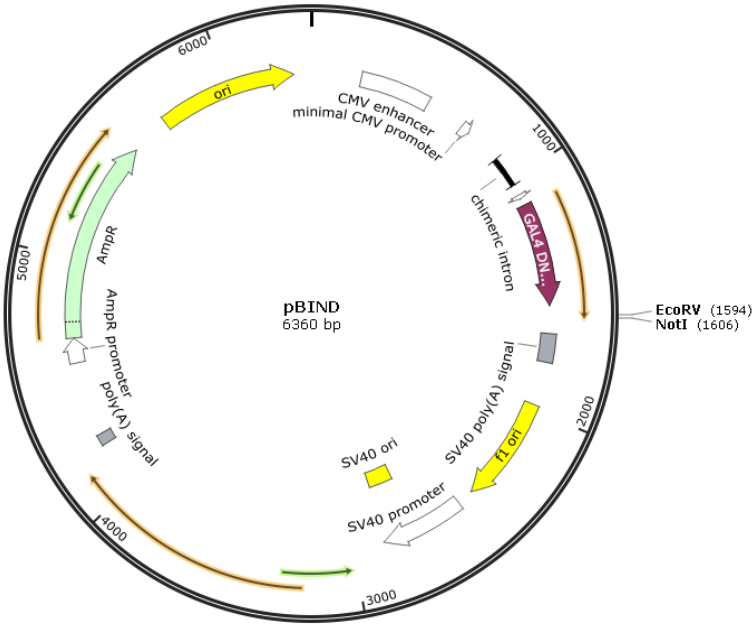


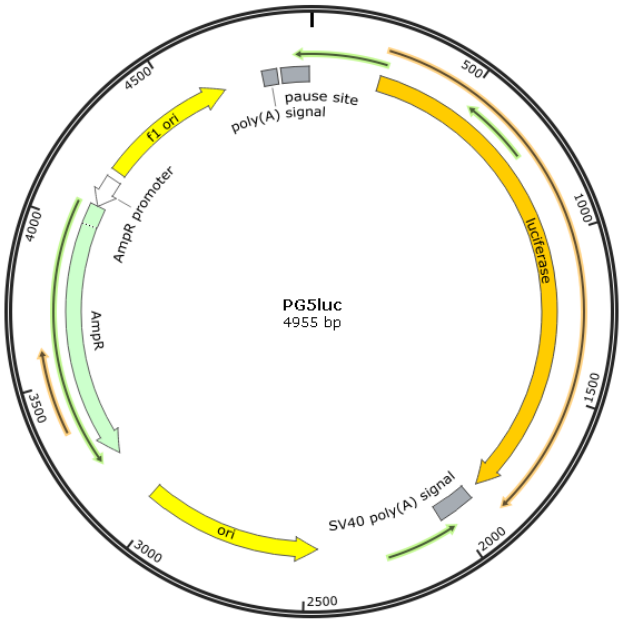

pACT-Abhd6 + Per1-luc
pACT-BLCAP + Per1-luc
pACT-Nipsnap1 + Per1-luc
pACT-NEDD4L + Per1-luc
pACT-GSTM4 + Per1-luc
pACT-MAD2L2 + Per1-luc
pACT-Ahctf1 + Per1-luc
pACT-Pxmp4 + Per1-luc
pACT-SLC37A4 + Per1-luc
pACT-Leprotl1 + Per1-luc
pACT-KLF15 + Per1-luc
pACT-GLT25D1 + Per1-luc
pACT-Usp2 + Per1-luc
pACT-UGP2 + Per1-luc
pACT-Foxp1 + Per1-luc
pACT-Ppox + Per1-luc
pACT-TSC22D3 + Per1-luc
pACT-Atp6v1d + Per1-luc
pACT-DDX46 + Per1-luc
pACT-TMED5 + Per1-luc
pACT-ELOVL6 + Per1-luc
pACT-DNAJB6 + Per1-luc
pACT-ETF1 + Per1-luc
pACT-BDH1 + Per1-luc
pACT-PTER + Per1-luc
HEK
Per1-luc
0
10000
20000
30000
40000
Luciferase Activity

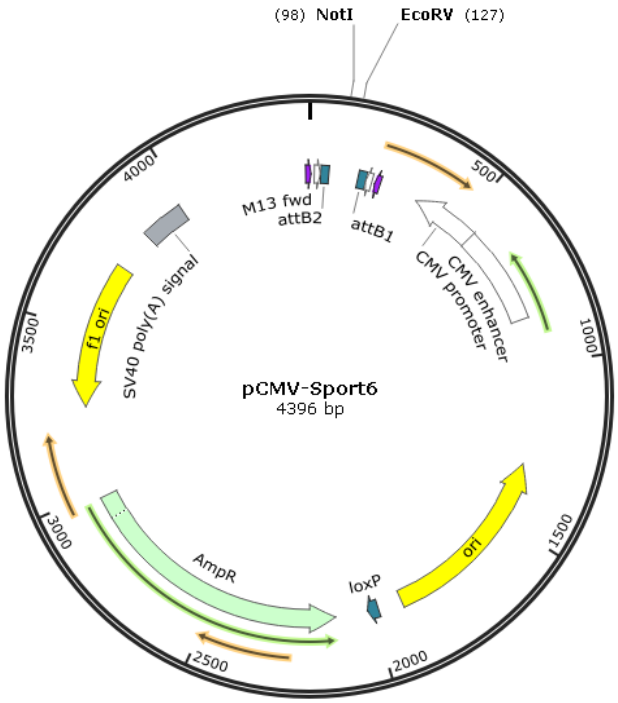

pACT-Ahsa1 + Per1-luc
pACT-MEF2A + Per1-luc
pACT-TMEM33 + Per1-luc
pACT-P2RX4 + Per1-luc
pACT-Serpinb9 + Per1-luc
pACT-S100a10 + Per1-luc
pACT-Leprot + Per1-luc
pACT-LSM6 + Per1-luc
pACT-PPP1R3B + Per1-luc
pACT-Pim3 + Per1-luc
pACT-Mcm4 + Per1-luc
pACT-Gys2 + Per1-luc
pACT-Fkbp4 + Per1-luc
pACT-CXADR + Per1-luc
pACT-Atp6v1e1 + Per1-luc
pACT-LIN7C + Per1-luc
pACT-ADCK5 + Per1-luc
pACT-RASL11B + Per1-luc
pACT-FKBP5 + Per1-luc
pACT-FMO1 + Per1-luc
HEK
Per1-luc
0
10000
20000
30000
40000
Luciferase Activity

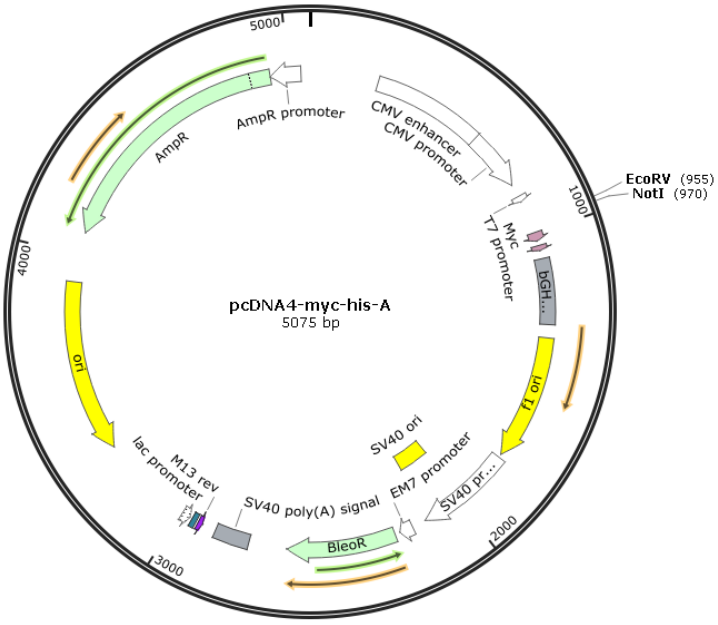
APPENDIX E: Vector Maps






pause site
poly(A) signal
f1 ori
AmpR promoter
AmpR
luciferase
PG5luc
4955 bp
SV40 poly(A) signal
ori
500
1000
1500
2000
2500
3000
3500
4000
4500


(98) NotI
EcoRV (127)
M13 fwd
attB2
attB1
CMV enhancer
CMV promoter
SV40 poly(A) signal
f1 ori
pCMV-Sport6
4396 bp
AmpR
loxP
ori
500
1000
1500
2000
2500
3000
3500
4000


5000
AmpR promoter
CMV enhancer
CMV promoter
EcoRV (955)
NotI (970)
1000
AmpR
T7 promoter
Myc
bGH...
4000
pcDNA4-myc-his-A
5075 bp
ori
f1 ori
SV40 ori
M13 rev
lac promoter
EM7 promoter
SV40 pr...
SV40 poly(A) signal
BleoR
2000
3000

APPENDIX F: Primer Sequences

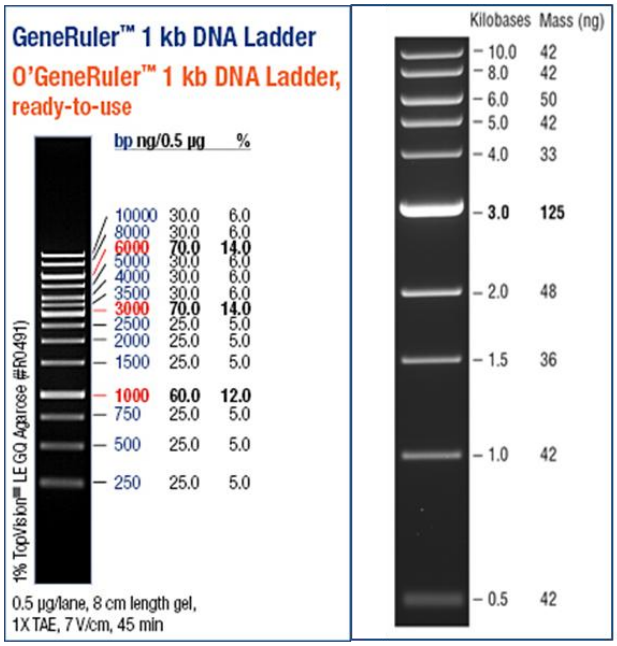
Gene *(M. musculus)*	Forward primer	Reverse primer
Abcd2	5’ CGTTGATATCAATGATACACATGCTAAATG 3’	5’ CCTGCGGCCGCTTAGGATGTCTTTTCTGCA 3’
Abhd6	5’ CGTTGATATCAATGGATCTCGATGTGGTTA 3’	5’ CCTGCGGCCGCTCAGTTCAGCTTCTTGTTG 3’
Ahctf1	5’ CGTTGATATCAATGCAAGACTTGACAGCTC 3’	5’ CCTGCGGCCGCTTACAGCATTTTCCTGCGT 3’
Ahsa1	5’ CGTTGATATCAATGGCCAAGTGGGGTGAGG 3’	5’ CCTGCGGCCGCCTAAAACAAGCGTGCACCA 3’
Arhgap29	5’ CAGGGATCCGTATGATTGCTCACAAACAGA 3’	5’ CCTGCGGCCGCCTACACAAACTGTGGGATT 3’
Atp6v1d	5’ CGTTGATATCAATGTCGGGCAAAGACCGGA 3’	5’ CCTGCGGCCGCCTATTCGAACAGCAGGTCT 3’
Atp6v1e1	5’ CGTTGATATCAATGGCGCTCAGCGATGCAG 3’	5’ CCTGCGGCCGCTCAGTCCAGAAACTTCCTA 3’
Fkbp4	5’ CGTTGATATCAATGACCGCCGAGGAGATGA 3’	5’ CCTGCGGCCGCCTACGCTTCTGTCTCCACC 3’
Foxp1	5’ CGTTGATATCAATGATGCAAGAATCTGGGT 3’	5’ CCTGCGGCCGCTCACTCCATGTCCTCATTT 3’
Gldc	5’ CGTTGATATCAATGCAGCTGTGTGCCAGGG 3’	5’ CCTGCGGCCGCCTAAGAAGAAGCCCTCTTC 3’
Gys2	5’ CGTTGATATCAATGCTCAGAGGCCGCTCCT 3’	5’ CCTGCGGCCGCTCAGTTCTTATATTCTCCA 3’
Leprot	5’ CGTTGATATCAATGGCGGGCGTTAAAGCTC 3’	5’ CCTGCGGCCGCCTACCACTGTTCCCAACTG 3’
Leprotl1	5’ CGTTGATATCAATGGCGGGCATCAAAGCTT 3’	5’ CCTGCGGCCGCTCACCACTGCTGCCAGCTG 3’
Mcm4	5’ CAGGGATCCGTATGTCGTCCCCGGCATCCA 3’	5’ CCTGCGGCCGCTCAGAGCAGGCGGACAGTC 3’
Nipsnap1	5’ CGTTGATATCAATGGCTCCGCGGTTGTGCA 3’	5’ CCTGCGGCCGCTCACTGGAGAGGAGAAATC 3’
Pim3	5’ TCCGTCGACTTATGCTGCTGTCCAAGTTCG 3’	5’ CCTGCGGCCGCTCACAAGCTCTCACTGCTG 3’
Ppox	5’ CGTTGATATCAATGGGCCGGACTGTGATAG 3’	5’ CCTGCGGCCGCTCAGCTGTTAGATTCTGTG 3’
Psmb10	5’ CGTTGATATCAATGCTGAAGCAGGCAGTGG 3’	5’ CCTGCGGCCGCTCATTCCACCTCCATGGCC 3’
Pxmp4	5’ CGTTGATATCAATGGCCGCCCCACCGCAGC 3’	5’ CCTGCGGCCGCTTACTTGGAGGGGTGGCTC 3’
Ror1	5’ TCCGTCGACTTATGCACCGGCCGCGCCGCC 3’	5’ CCTGCGGCCGCTTACACTTCTGCAGAAATC 3’
S100a10	5’ CGTTGATATCAATGCCATCCCAAATGGAGC 3’	5’ CCTGCGGCCGCCTACTTCTTCCCCTTCTGC 3’
Serpinb9	5’ CAGGGATCCGTATGAATACTCTGTCTGAAG 3’	5’ CCTGCGGCCGCTTATGGAGATGAGAACCTG 3’
Stip1	5’ CGTTGATATCAATGGAGCAGGTGAATGAGC 3’	5’ CCTGCGGCCGCTCACCGAATTGCGATGAGA 3’
Usp2	5’ CGTTGATATCAATGTCCCAGCTCTCCTCCA 3’	5’ CCTGCGGCCGCCTACATACGGGAGGGTGGA 3’

Gene *(H. sapiens)*	Forward primer	Reverse primer
ADCK5	5'CGTTGATATCAATGTGGCGACCGGTGCAGC 3'	5'CCTGCGGCCGCCTAGGTCTCCAGGTACTGG 3'
BDH1	5'CGTTGATATCAATGCTGGCCACCCGCCTCT 3'	5'CCTGCGGCCGCTCAGCGGATGTAGATCATG 3'
BLCAP	5' CGTTGATATCAATGTATTGCCTCCAGTGGC 3'	5' CCTGCGGCCGCTTAGGTGCCCACAACGCCG 3'
CXADR	5'CAGGGATCCGTATGGCGCTCCTGCTGTGCT 3'	5'CCTGCGGCCGCCTACCTTAGCAGGTGGGAG 3'
DDX46	5' CGTTGATATCAATGGGTCGGGAGTCACGCC 3'	5' CCTGCGGCCGCCTATAAGACTTTGTATCTT 3'
DNAJB6	5'CAGGGATCCGTATGGTGGATTACTATGAAG 3'	5'CCTGCGGCCGCTTACTTGTTATCCAAGCGC 3'
ELOVL6	5'CGTTGATATCAATGAACATGTCAGTGTTGA 3'	5'CCTGCGGCCGCCTATTCAGCTTTCGTTGTT 3'
ETF1	5'TCCGTCGACTTATGGCGGACGACCCCAGTG 3'	5'CCTGCGGCCGCCTAGTAGTCATCAAGGTCA 3'
FKBP5	5'CGTTGATATCAATGACTACTGATGAAGGTG 3'	5'CCTGCGGCCGCTCATACGTGGCCCTCAGGT 3'
FMO1	5' CGTTGATATCAATGGCCAAGCGAGTTGCCA 3'	5' CCTGCGGCCGCTTATAGGAAAATCAGAAAA 3'
GLT25D1	5' CGTTGATATCAATGGCGGCGGCCCCACGCG 3'	5' CCTGCGGCCGCTCAGAGTTCATCCCGGGCA 3'
GSTM4	5' CGTTGATATCAATGTCCATGACACTGGGGT 3'	5' CCTGCGGCCGCTTACTTGTTGCCCCAGACA 3'
KLF15	5' CGTTGATATCAATGGTGGACCACTTACTTC 3'	5' CCTGCGGCCGCTCAGTTCACGGAGCGCACG 3'
LIN7C	5' CGTTGATATCAATGGCGGCGCTAGGGGAAC 3'	5' CCTGCGGCCGCTTAGGTCTGTTGCCTGCGT 3'
LSM6	5' CGTTGATATCAATGAGTCTTCGGAAGCAAA 3'	5' CCTGCGGCCGCTCACATCCGTCTCTTCTGT 3'
MAD2L2	5'CGTTGATATCAATGACCACGCTCACACGAC 3'	5'CCTGCGGCCGCTCAGCTGCCTTTATGAGCG 3'
MEF2A	5'CGTTGATATCAATGGGGCGGAAGAAAATAC 3'	5'CCTGCGGCCGCTTAGGTCACCCACGCGTCC 3'
MTHFD1L	5' CAGGGATCCGTATGGGCACGCGTCTGCCGC 3'	5' GATGATATCAATTAGAACAAGCCTTTAACT 3'
NEDD4L	5' TCCGTCGACTTATGGAGCGACCCTATACAT 3'	5' CCTGCGGCCGCTTAATCCACCCCTTCAAAT 3'
NUP62	5'CGTTGATATCAATGAGCGGGTTTAATTTTG 3'	5'CCTGCGGCCGCTCAGTCAAAGGTGATCCGG 3'
P2RX4	5' CGTTGATATCAATGGCGGGCTGCTGCGCCG 3'	5' CCTGCGGCCGCTCACTGGTCCAGCTCACTA 3'
PPM1B	5'CGTTGATATCAATGGGTGCATTTTTGGATA 3'	5'CCTGCGGCCGCTTAAAAAGCATTTGTCTTA 3'
PPP1R3B	5' CGTTGATATCAATGATGGCTGTGGACATCG 3'	5' CCTGCGGCCGCCTAGTAGTAGGGCCCTAGC 3'
PTER	5'CGTTGATATCAATGTCTTCCTTAAGTGGAA 3'	5'CCTGCGGCCGCCTATTTGAAAGTTAGCCAT 3'
RASL11B	5' CGTTGATATCAATGCGCCTCATTCAGAACA 3'	5' CCTGCGGCCGCTCAGACGGAGGTGACAGTC 3'
SLC37A4	5' CGTTGATATCAATGGCAGCCCAGGGCTATG 3'	5' CCTGCGGCCGCTCACTCAGCCTTCTTGGAC 3'
SOAT1	5'CGTTGATATCAATGGCTGTGCTGGCGGCA 3'	5'CCTGCGGCCGCCTACAGCTTCTGAATTAGC 3'
TCF7L2	5' CGTTGATATCAATGCCGCAGCTGAACGGCG 3'	5' CCTGCGGCCGCCTATTCTAAAGACTTGGTG 3'
TMED5	5'CGTTGATATCAATGGGCGACAAGATCTGGC 3'	5'CCTGCGGCCGCCTAAGCAGAGTTTGTATGT 3'
TMEM33	5'CGTTGATATCAATGGCAGATACGACCCCGA 3'	5'CCTGCGGCCGCCTATGGAACTGTTGGTGCC 3'
TSC22D3	5' CGTTGATATCAATGGATCTGGTGAAGAATC 3'	5' CCTGCGGCCGCTTACACCGCAGAACCACCA 3'
UGP2	5'CAGGGATCCGTATGTCTCAAGATGGTGCTT 3'	5'CCTGCGGCCGCTCAGTGGTCCAAGATGCGA 3'

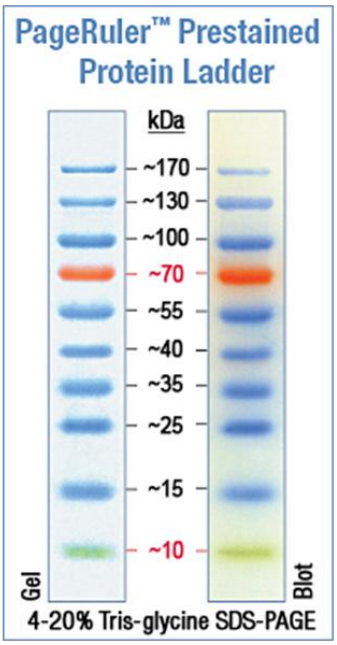
APPENDIX G: DNA and Protein Markers

DNA Molecular Weight Markers

Protein Molecular Weight Markers



Ladder 1 *Ladder 2*



APPENDIX H: Lab Equipments

Autoclaves	**:** CL-40S/SDP (60L) ALP autoclave
Centrifuges	**:** 4K15, Sigma Laboratory
	: 1-15, Sigma Laboratory
	: Microfuge 14-15, Sigma Laboratory
Deep freezes and refrigerators	**:** Heto Polar Bear 4410 ultra freezer, JOUAN Nordic A/S, catalog# 003431.
	: 2021 D deep freezer, Arcelik.
	: 1061 M refrigerator, Arcelik.
Electrophoresis equipments	**:** E-C Mini Cell Primo EC320, E-C Apparatus.
	: Mini-PROTEAN 3 Cell and Single-Row AnyGel Stand, Catalog# 165-3321, Bio-Rad.
Gel documentation system	**:** UVIpro GAS7000, UVItec Limited.
Ice Machine	**:** AF 10, Scotsman.
Shaker	**:** Innova 4300 incubator shaker
Magnetic stirrer	**:** Heidolph MR 3001
Pipettes	**:** Finnpipette F1 1-10µl, F1 10-100µl, F1 100-1000µl, ThermoScientific
pH meter	**:** Inolab pH level 1, order# 1A10-1113,
Power supply	**:** PowerPac Basic (300V,400mA,75W) Bio-Rad
Pure water systems	**:** DV25 PureLab Option ELGA
Spectrophotometer	**:** W-1700 PharmaSpec, Shimadzu Corporation.
Vortexing machine	**:** Reax Top, Heidolph2.2.
CO_2 Incubator	**:** Memmert INC108med
Semi-dry blotter	**:** Hoefer TE70XP
Incubator	**:** Incucell 111