

**INVESTIGATION OF THE INTERACTION BETWEEN
MOUSE CRYPTOCHROME-1 AND LEPTIN RECEPTOR
OVERLAPPING TRANSCRIPT LIKE-1**

by

Derya Kabacaoğlu

**A Thesis Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of
Master of Sciences
in
Molecular Biology and Genetics**

Koç University

August 2015

Koç University
Graduate School of Sciences and Engineering

This is

To certify that I have examined this copy of Master's thesis by

Derya Kabacaoğlu

and have found that it is complete and satisfactory in all respects,

and that any and all revisions required by the final

examining committee have been made.

Committee Members:

İ. Halil Kavaklı, Ph. D.(Advisor)

Nuri Öztürk, Ph. D.

Funda Şar, Ph. D.

Date: 27.07.2015

to my beloved family...

ABSTRACT

Circadian rhythms are oscillations in the biochemical, physiological, and behavioral functions of organisms that occur with a periodicity of approximately 24 h. Circadian rhythms affect many metabolic processes in body such as sleep/wake cycle, feeding, hormone secretion and many others. Most importantly it is known that dysfunction in circadian rhythmicity leads to various metabolic and psychological diseases such as jet-lag, depression, obesity and even cancer.

In mammals, circadian rhythms are maintained by autoregulatory transcriptional and translational feedback loops involving the basic helix loop helix PER-ARNT-SIM (bHLH/PAS) transcription factors BMAL1, CLOCK, and NPAS2. BMAL1 heterodimerizes with either CLOCK or NPAS2 and drive transcription through E-boxes located within the promoters of numerous target genes. Among the target genes are Period homolog (Per1-3), Cryptochrome (Cry1-2) and Rev-erba that encode repressors of the BMAL1: CLOCK/NPAS2 transcriptional activity. After a delay, the translated PER and CRY proteins heterodimerize, translocate to the nucleus, and repress BMAL1: CLOCK/NPAS2 heterodimers. The PER and CRY heterodimers are progressively degraded, allowing the circuit to start again. This negative feedback leads to a cycle in gene expression that takes approximately 24 h to complete. Studies showed that there are other genes that may participate in this molecular mechanism to regulate circadian rhythm. For example a study with machine learning strategy involving naïve Bayesian approach showed that there are several genes might participate in clock function either as modifiers or as a core clock component. As continuation of this study we observed that there is an interaction between Leptin Receptor Overlapping Transcript Like-1 Protein (Leprotl1) and CRY1 protein.

In this thesis, it is shown that both the mCRY1 and the mCRY2 interacts LEPROTL1 through the C-termini using the mammalian two-hybrid assay. This interaction is also confirmed with co-immunoprecipitation assay and Bi-molecular Fluorescence Complementation (BiFC).

LeprotL1 is a protein that has metabolic functions within organisms. It is already shown that LeprotL1 has role in leptin and growth hormone sensitivity of liver in mice, though its function in circadian rhythm is unknown. Here in, it is shown that LEPROTL1 interacts with both mCRY1 and mCRY2 and it is possible that such interaction may have a role in metabolic regulation of mammals.

ÖZET

Sirkadiyen ritimler organizmaların biyokimyasal, fizyolojik ve davranışsal fonksiyonlarında meydana gelen yaklaşık 24 saatlik salınımlardır. Sirkadiyen saatler uyku/uyanıklık döngüsü, beslenme, hormon salınımı ve başka birçok metabolic süreçleri etkilerler. En önemlisi, sirkadiyen ritimde görülen bozulmalar jet-lag, depresyon, obezite ve kanser gibi birçok metabolik ve fizyolojik hastalıklara sebebiyet verebilirler.

Memelilerde, sirkadiyen saatler otoregülasyonlu transkripsiyonal translasyonel geri besleme döğüleri ile sürdürölürler. Bu otoregülasyonlu transkripsiyonal translasyonel geri besleme döğüleri basit helix-loop-helix PER-ARNT-SIM (bHLH/PAS) transkripsiyon faktörleri BMAL1/CLOCK ve NPAS2 proteinlerini içerirler. BMAL1, CLOCK ya da NPAS2 ile heterodimerizasyon yapıp, hedef genlerdeki E-Box motifleri aracılığı ile bu genlerin transkripsiyonunu sağlarlar. Bu hedef genlerden olan Period homologlar (Per1-3), Cryptochrome'lar (Cry1-2) ve Rev-erba, BMAL1:CLOCK/NPAS2 transkripsiyonunu baskırlar. Bir sürelik gecikmeden sonra, üretilen PER ve CRY proteinleri zaman içerisinde parçalanırlar, böylelikle sirkadiyen devir ikinci devrine yeniden başlar. Bu negatif geri besleme gen ifadelerinde yaklaşık 24 saatlik devir oluştururlar. Yapılan çalışmalar bu geri besleme sistemini yöneten başka birçok bileşenin olduğunu göstermektedir. Örneğin Naif Bayez yaklaşımı ile yapılan bir çalışmada saat fonksiyonuna merkezi saat bileşeni ya da düzenleyici olarak katkıda bulunan birçok farklı bileşenlerin olduğu işaret edilmektedir. Bu çalışmanın bir devamında biz Leptin Receptor Overlapping Transcript Like-1 (Leprotl1) proteininin CRY1 proteini ile etkileşim halinde olduğunu gösterdik.

Bu tez çalışmasında, CRY1 ve CRY2 proteininin C-terminus ucu ile LEPROTL1 proteininin etkileşim halinde olduğunu memeli iki-hibrid analizi ile gösterdik. Bu etkileşim ayrıca co-immunopresipitasyon tahlili ve iki-moleküllü floresanlı tamamlama (BiFC) ile de doğrulandı.

LEPROTL1 organizmalarda metabolik fonksiyonları olan bir proteindir. Önceki çalışmalarda LEPROTL1'in hepatic leptin ve büyüme hormonu duyarlılığında görev aldığı gösterilmiş olup, bu proteinin sirkadiyen saat ile olan bağlantısı bilinmemektedir. Bu çalışmada gösterilen LEPROTL1'in fare CRY1 ve CRY2 proteinleri ile olan etkileşimi, memelilerde metabolik denetlenmenin sağlanmasında bir rol alabilir.

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the following people, who were instrumental in the writing and publication of this dissertation.

First, I would like to thank my thesis advisor Assoc. Prof. Halil Kavaklı for his guidance and support which made it possible to write this thesis. I am grateful to him since he shared his great and educative ideas and his endless patience. He inspired me about how to be a scientist, and I learnt lots from him.

I also want to propose my special thanks to Assoc. Prof. Ibrahim Barış for his support in this thesis work. His patience to answer all my questions helped me learn a lot.

I also want to present my gratitude to Selma Bulut, Hande Asımgil, Bengisu Seferoğlu and Darya Yarpavar for their help in both my professional and personal lives. They were all both my teachers and family in these two years. Their wisdom taught me a lot, and made my days very precious in this laboratory. My dear friend Berke Gürkan and his beard were always there to get along with my unexpected moods, by accompanying me. I had lots of fun with his taste of humor and companionship.

It wouldn't be enough how much I thank to my brothers Mehmet Tardu, Haşımcan Öner, Şeref Gül, and Fatih Rahim for their patience about the weird music that I play in the laboratory. With the funny nicknames I gave them, they were very helpful and kind in every aspect.

Of course, my dearest friends Eylem Külköylüoğlu and Sibel Çal with whom we started this 2 years of journey in this laboratory; I learnt what real patience from them. They were always very supportive in my both professional and personal lives. They showed me what real friendship is. I will always be very grateful to them for their guidance.

My best friend Burcu Özçimen, who has always been my soul mate since the day we met was always very kind and supportive to me in my personal life. She loved

me the way I am which I appreciate the most. Her valuable guidance and support made me pass through my miserable times in these years.

My friend Mohammad Haroon Qureshi with whom I met very recently was an inspiration for me about the way one should live. He is one of the most compassionate people I have ever met. I have learnt a lot from him, and hopefully will learn more. I also want to thank him lots for his valuable advice in every aspects of life.

My two years in this school couldn't be this much meaningful without my friend İbrahim Çağrı Kurt. I valued him the way he is, and dared the world. I want to propose my appreciation to him for revealing the real world to me and for helping me feel hope about this and other lives.

I want to propose special thanks to my friends Tugcem Yalçın, Beytullah Yılmaz, Barbaros Güngör, Utku Demir, Saliha Akbaş, Ali Doğan and Bekir Aksoy who I knew them since my undergraduate years in Koc University. Since the day that we became friends, no matter what they accepted and valued me as I am. Our friendship survived lots of fatal accidents; hopefully it will survive the distance of proceeding years.

A hearty thank to my dear family who supported me all these times of happiness and sadness. I am proud to be their child and always be. Their endless love made me who I am and I appreciate them for their unconditional love.

My dear teachers Funda Şar, Tugba Bağcı Önder, Tamer Önder, Nathan Lack, Nurhan Özlü, Gülayse İnce Dunn and Cory Dunn for their assistance in this journey. Especially, I am grateful to Tuğba Bağcı Önder who was always very kind and patient with my visits and questions regarding my career.

Finally I want to thank my dear friends especially Deniz Uğurlu and, Kerem Fidan, Uğur Gatfar, Ezgi Kaya, Gülnihal Kavaklıoğlu, Gülkız Baytek, Ayyub Ebrahimi, Hadi Nozari, Harun Çimen, Emel Akdoğan, Duygu payzın, Pelin İspir, Cansu Akkaya, Bahriye Erkaya, Gizem Güzelsoy, Ahmet Cingöz, Gökhan Güner, Ekin Güney and Filiz Şenbabaoğlu for their fun and beautiful friendship.

I acknowledge the financial support from the Scientific and Technological Research Council (TÜBİTAK) for the 2211 scholarships during my whole graduate study.

TABLE OF CONTENTS

ABSTRACT	iv
ÖZET.....	vi
ACKNOWLEDGEMENTS.....	viii
NOMENCLATURE	xv
CHAPTER 1: INTRODUCTION	17
CHAPTER 2: LITERATURE REVIEW	19
2.1 Biological Rhythms	19
2.2 Overview of the Circadian Rhythm and its history	19
2.3 Basic Concepts and Methodologies in Circadian Rhythm.....	21
2.5 Molecular Components of the Mammalian Circadian Rhythm.....	24
2.5.1 Transcriptional Feed Back Loops.....	24
2.5.2 Post-translational modifications of clock components.....	30
2.5.3 Epigenetic regulation of clock components.....	30
2.5.4 Structures of core clock components and their implications	34
2.6 Physiological Components of the Mammalian Circadian Rhythm	38
2.6.1 Perception of environmental stimulus	38
2.6.2 Suprachiasmatic Nucleus as central oscillator	40
2.6.3 Peripheral clocks	41
2.7 Circadian Rhythm and Metabolism	44
2.4 Circadian Rhythm Dysfunction Associated Diseases.....	47
2.8 Machine Learning and Bayesian Integration Strategy	50
2.9 Leptin Receptor Overlapping Transcript Like-1	52
CHAPTER 3: MATERIALS AND METHODS	56
3.1 Mammalian Two-Hybrid System	56
3.2 Chemically Competent DH5 α Preparation	56
3.3 Transformation into Chemically Competent DH5 α	57
3.4 Polymerase Chain Reaction (PCR)	57
3.5 Sub-cloning into pGEM-T-Easy vector.....	58

3.6 Gene Cloning into Target Vector	58
3.7 Cell Culture Maintenance	59
3.8 DNA Transfection and and Western Blot Analysis	60
3.9 Co-immunoprecipitation Assay	61
3.10 siRNA Transfection	61
3.11 RNA isolation	62
3.12 RT-qPCR Analysis (Real Time – quantitative Polymerase Chain Reaction)	63
3.13 Biolumicycle Analysis.....	63
3.14 Bi-molecular Fluorescence Complementation Analysis	64
CHAPTER 4: RESULTS	65
4.1 Mammalian Two Hybrid Assays	65
4.2 Cloning for Co-immunoprecipitation Assay.....	71
4.3 Over Expression of T5/T6 mCry1- pcDNA 4/myc-His A Clones	71
4.4 Co-immunoprecipitation Assay	72
4.5 BiFC (bi-molecular fluorescent complementation) system	74
4.6 The effect of LeprotL1 on circadian rhythm of U2OS and NIH3T3 cells.....	75
.....	77
4.7 Analyzing the Effects of LeprotL1 Knock Down on Core Clock Components.....	77
CHAPTER 5: DISCUSSION.....	80
VITA	83
APPENDIX I.....	84
List of the primer sequences.....	84
APPENDIX 2	86
Maps of Expression vectors	86
APPENDIX 3	87
Agarose Gel Images.....	87
APPENDIX 4	89
DNA and Protein Markers	89

APPENDIX 5	90
Lab Equipments	90
Vortexing machine : reax top, heidolph2.2.....	90
BIBLIOGRAPHY	92

LIST OF TABLES

Table 1 Locomotor activities associated with core clock component knock-outs.	27
Table 2 Core clock components and their epigenetic modifiers.	33
Table 3 Metabolic symptoms associated with core clock component mutations.	48

LIST OF FIGURES

Figure 1 Actogram of mouse locomotor activity	22
Figure 2 Transcriptional translational feedback loops	25
Figure 3 Locomotor activity in Rev-erb α/β knock out mouse.	26
Figure 4 Non-canonical model of transcriptional translational feedback loop	29
Figure 5 epigenetic modifications associated with circadian rhythm	32
Figure 6 Domains of core clock components are represented on scale bars. Retrieved from ⁶³	35
Figure 7 3D structures of PAS-A/B domains in BMAL1:CLOCK heterocomplex.....	36
Figure 8 Partial aminoacid sequence of CRY C-terminal tail from various species.....	37
Figure 9 Circadian photoreception in mammals	39
Figure 10 SCN neurons communicating through paracrine signaling	41
Figure 11 SCN synchronization of peripheral tissues	43
Figure 12 Effect of circadian rhythm on metabolites	45
Figure 13 Effect of Leprotl1 on leptin receptor localization	54
Figure 14 Oscillation of Leptin signaling components' transcripts.....	55
Figure 15 Leprotl1 transcript oscillation in liver of mice from CircaDB	55
Figure 16 Components of mammalian two-hybrid assay.....	67
Figure 17 Mammalian two-hybrid assay results	68
Figure 18 Identification of CRY1 domain interacting with LEPROTL1.....	69
Figure 19 Interaction between LEPROTL1 and CRY2 WT/501mut/503mut.....	70
Figure 20 Overexpression of FL/T5/T6 forms of CRY1	72
Figure 21 Co-immunoprecipitation western blot analysis.....	73
Figure 22 Cloned BiFC vectors	74
Figure 23 BiFC analysis results	75
Figure 24 Biolumicycle analysis of Leprotl1 knock down cells.....	77
Figure 25 qPCR analysis of core clock components in Leprotl1 knock down cells	78
Figure 26 qPCR analysis upon Leprotl1 knockdown upon synchronization.	79

NOMENCLATURE

Per	Period
Clock	Circadian Locomotor Output Cycles Kaput
Arntl/Bmal1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
CT	circadian time
ZT	zeitgeber time
TTFL	transcriptional translational feedback loops
bHLH	basic helix loop helix domain
PAS	per-arnt-sim domain
Cry	cryptochrome
ROR	RAR-related orphan receptor
Rev-erb	nuclear receptor subfamily 1, group D
RORE	RAR-related orphan receptor response element
HLF family	hepatic leukemia factor
TEF	thyrotroph embryonic factor
DBP	D site of albumin promoter (albumin D-box) binding protein
ParbZip	proline and acidic amino acid rich basic leucine zipper
CKIϵ	casein kinase 1 epsilon
DYRK1A	Dual-Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 1A
Gsk3β	glycogen synthase kinase 3 beta
HP1α/CBX	Chromobox protein homolog 5
MLL1	myeloid/lymphoid or mixed-lineage leukemia enzyme
SIRT1	sirtuin 1
NAD/NADH	Nicotinamide adenine dinucleotide
HAT	histone acetyl transferase
EZH2	Enhancer of zeste homolog 2
TAD	transactivating domain
FMN	flavin mononucleotide
FAD	flavin adenine dinucleotide
SCN	suprachiasmatic nucleus
RGC	retinal ganglion cells
RHT	retinohypothalamic tract
AVP	arginine vasopressin
GABA	Gamma-Amino Butyric acid
VIP	Vasoactive intestinal polypeptide
VPAC2	Vasoactive intestinal peptide receptor 2
CREB	cAMP response element-binding protein
CRE	cAMP response element
AcCoA	acetyl co-enzyme A
DSPS	delayed sleep phase disorder
ASPS	advanced sleep phase disorder

OB-R	leptin receptor
Leprotl1	lwptin receptor overlapping transcript-like 1
TGN	Trans-golgi network
GH	growth hormone
IGF1	insulin like growth factor
JAK	janus kinase
STAT	Signal Transducer and Activator of Transcription protein
SOCS	Suppressor of cytokine signaling
FGF21	fibroblast growth factor 21
BiFC	bi-molecular fluorescence complementation
ER	endoplasmic reticulum
siRNA	silencing RNA
RT-qPCR	realtime quantitative polymerase chain reaction

CHAPTER 1: INTRODUCTION

The circadian rhythm is biological processes showing an entrainable and endogenous oscillating profile with a period of approximately 24 hours. Evolutionary circadian rhythm helps organisms to adapt to daily rotation of earth and its changing conditions. Lots of biological outputs like sleep-wake cycle, reproduction, metabolism, locomotor activity and feeding cycle are under circadian rhythm control. Circadian rhythm is highly conserved among species from cyanobacteria to humans.

At the physiological level circadian rhythm is separated into three components named as input, clockwork and output components. Light is the strongest stimulus used to entrain innate rhythm to environment. In mammals upon the light stimulation, central oscillator suprachiasmatic nucleus (SCN) located in mid brain functions as the synchronizer of the peripheral clocks. Upon the synchronization, peripheral clocks generate physiological and behavioral outputs.

At the molecular level, mammalian circadian rhythm is mediated by transcriptional-translational feedback loops (TTFL). At the positive limb of the loop, BMAL1:CLOCK heterocomplex act as the transcriptional activator acting on E-Box motives of target genes. Among these genes are CRYs and PERs, which constitutes the negative limb of the TTFL. Accumulated PERs and CRYs interact with each other in cytoplasm and translocate back to nucleus where they act as a repressor complex on BMAL1:CLOCK complex. In time decrease in CRY and PER levels attenuates the repression activity on BMAL1:CLOCK complex leading to another cycle. This cycle is repeated within approximately 24 hours.

Up to date, many core clock components are identified, though there are still missing pieces of this complicated puzzle. Naïve Bayes machine learning strategy is an advantageous method with its ease of use and cost effective methodology. Basically, the machine learning strategy defines five criteria that are common among core clock components and computationally screens whole genome to find candidate clock components based on these criteria. Among the hits, a mammalian two-hybrid screen is performed to identify the candidates that interact with core clock component partners.

One of the hits, Leprotl1 is the main focus of this thesis. Our results showed that Leprot1 interacts with both CRY1 and CRY2 proteins. In addition, siRNA knockdown of Leprotl1 showed altered circadian activity in various cell lines.

In Chapter 2, detailed information about what circadian rhythm is, how it functions and what diseases associated with its dysfunction are explained.

In Chapter 3, materials and methods used during this thesis work are described in detail.

In Chapter 4, results obtained during this thesis work are presented showing that LEPROTL1 interacts with both mCRY1 and mCRY2.

The results are discussed in Chapter 5.

CHAPTER 2: LITERATURE REVIEW

2.1 Biological Rhythms

Chronobiology examines the biological rhythms of organisms. Various types of biological rhythms evolved throughout life that helped organisms to increase their survival chance by making them adapt to constantly changing conditions of earth ¹.

The geophysical cycle of earth is composed of daily (app. 24 hours), seasonal (365 days), lunar (29.53 days), tidal (12.8 hours), and circannual (12 months) cycles. Organisms synchronize their behavior by entraining their biological clock to these geophysical changes. Daily feeding patterns of most organisms, seasonal migration of birds and hibernation of some vertebrates and daily sporulation of some fungi species are the most obvious consequences of biological rhythms.

Biological rhythms are divided into three classes, which are infradian, ultradian and circadian rhythms ¹. Circadian rhythms repeat themselves within 24 hours. Sleep wake cycle, body temperature, hormone homeostasis, locomotor activity and feeding activities are under control of circadian rhythms ². Ultradian rhythms show shorter period of cycle than 24 hours with higher frequency than circadian rhythm. The cycle period can vary from seconds to hours. Appetite, breathing, hearth rate, urination and bowel activity are under control of ultradian cycles. On the other hand, infradian rhythms show longer periods with less frequency within 24 hours. Among the best examples of infradian rhythms are seasonal hibernation of animals, female menstrual cycles, seasonal migration of animals, and reproductive cycles of plants ¹.

2.2 Overview of the Circadian Rhythm and its history

With the sunrise, plants locate their leaves to benefit from sunlight, diurnal predators begin their hunt while the nocturnal bats fall asleep to recharge. As the sun sets, filamentous fungi produce their daily spores and cyanobacteria stop photosynthesis and start to fix nitrogen in a low oxygen environment. These biological processes are not generated by the solar cycle of the earth but are synchronized to daily rotation ³.

Circadian clock (circa: approximately dies: a day) is defined as any endogenous and entrainable biological process that shows approximately 24 hours of oscillation⁴. For a rhythm to be circadian three criteria are required. The first and most important property of a circadian rhythm is its ability to do “free run”. Without the environmental cues, circadian rhythms still show approximately 24 hours of oscillation, which shows its self-sustaining nature^{3,5}. Second property of circadian rhythm is the ability to sustain its buffered situation against strong changes such as daily temperature fluctuation. Due to this property, maintenance of rhythmicity with a relatively constant profile in wide range of temperature changes is achieved. Most of the biochemical reactions speed up with increased temperature within biological range. However, due to the compensatory nature of circadian rhythm, the effect of temperature is eliminated to maintain homeostasis⁶. Finally, circadian rhythms have ability to “entrain” themselves to environment by receiving stimulus-generating cues⁷.

The environmental cues that generate a circadian response are known as “Zeitgebers” (in German Zeit: time and Geber: giver). The strongest Zeitgeber is light that synchronizes circadian rhythm to earth’s daily rotation^{8,9}. Other than light many other cues influence circadian rhythm such as: social interactions and emotional stress^{10,11}, feeding behavior^{12–14} and temperature changes^{15,16} especially in poikilothermic organisms.

The first scientist that discovered circadian clock was a French astronomer, Jean Jacques d’Ortous deMarian. His curiosity upon light and its remarkable effects on biosystems made him realize the leaf movements of light sensitive plants, like *Mimosa Pudica*. The date was 1729, when deMarian decided to understand the connection between light and leaf movements. When mimosa flower is kept in its light-dark cycle (12 hours light-12 hours dark) leaves open during light stage and closes during dark stage. When deMarian places the flower under complete darkness, the leaves still stay open during the light stage. This observation of his was published as an academic article in Royal Academy of Science in Paris.¹ deMarian also speculated that these rhythms are correlated with sleep-wake cycle of organisms. After 30 years, three independent studies

performed by Hill in 1757, Duhamel duMonceau in 1759 and Zinn in 1759 verified these observations. They also showed the leaf movement was temperature independent glimpsing the second property of circadian rhythm ¹⁷.

Following the advances in molecular biology, molecular components of the circadian rhythm have started to be discovered. One of the most important clock component Period gene (Per) of *Drosophila melanogaster* was discovered by Seymour Benzer and Ron Konopka in 1971. They made observations upon the locomotor activity of *Drosophila melanogaster*, which analyzes the flying behavior. They isolated three mutants with aberrant period lengths which later on they realized that all three mutants were on the same gene of X chromosome ¹⁸. Another important clock component, circadian locomotor output cycles kaput (clock) is discovered in 1994 by Joseph Takahashi and his group. Basically, they performed a random mutagenesis screen on mice and found a mutant showing lengthened circadian period and abolished persistent rhythmicity in a time course experiment ¹⁹. Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL) which is also known as BMAL1 or MOP3 is discovered by John B. Hogenesch in 1997 ²⁰. All of these discoveries paved the way to identify new molecular clock components.

2.3 Basic Concepts and Methodologies in Circadian Rhythm

Rhythmic events have several parameters including amplitude, period, frequency, phase and phase angle. To understand these concepts lets focus on a simple sea wave. Amplitude is the change in single period giving the difference between highest and lowest magnitude. So, the height of the sea wave referring to the ground gives the amplitude. Period is defined as the required time interval for a wave to pass from a reference point to target fixed point or traveled distance per time unit. On the other hand, frequency is the number of cycles per unit of time. Consider two parameters, which are mutually synchronized to each other like circadian rhythm and earth's light cycle. The relationship between these two parameters gives the phase angle difference ¹.

The story becomes much more complicated when it comes to understand waves of circadian rhythm. Measuring the output of circadian rhythm in multicellular organism becomes shattering for the viability of the cells, causing the disruption on the feasibility of the results. In mammals suprachiasmatic nucleus (SCN) located in the hypothalamus is basically the central pacemaker. To make output records in form of action potentials, the individual neurons are invaded with electrodes generating lethal results for the cells in few hours. Hence, use of indirect methods rather than direct ones becomes much more feasible to generate measurable observations ¹.

Among the indirect methods, generation of a measurable modal for behavioral phenotypes is important. This measure must not be invasive for the organism while reflecting the real clock very closely. Locomotor activity is one of the easiest observable phenotype used as an indirect measure. Rodents show voluntary wheel running activity at night reflecting their nocturnal living. This phenotype is so robust that change in their circadian rhythm becomes detectable with proper locomotor activity recording ^{1,21}. Nocturnal mouse's locomotor activity which is wheel-running at night in constant darkness is shown as an actogram in Figure 1.

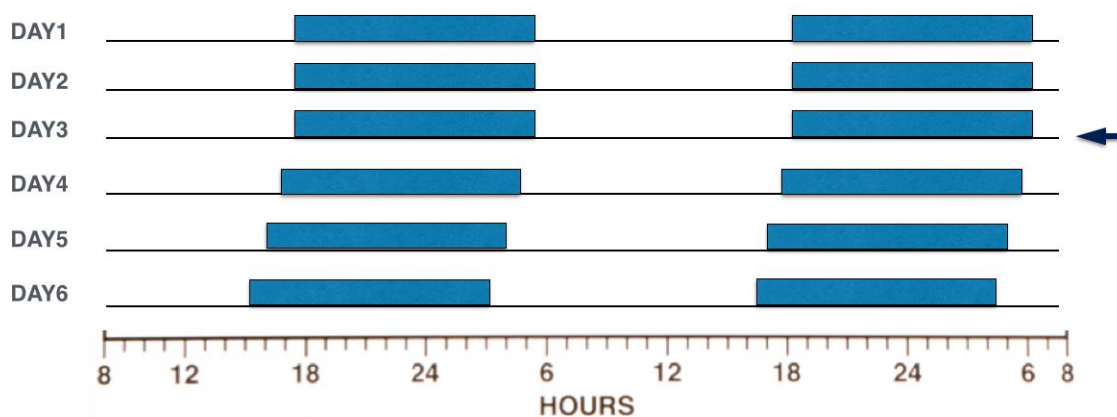


Figure 1 Actogram of mouse locomotor activity

The thin black lines indicate the absence of locomotor activity, whereas thick blue lines show the presence. Before the arrowhead mice is put in 12D/12L cycle. After arrowhead mice is put in constant darkness. Adapted from ⁸

For the unicellular organisms, measurable behavioral activities vary. Single celled photosynthetic organism *Gonyaulax*'s light glowing rhythm is recorded whereas in *Paramecium* mating behavior is used to track rhythm ^{22,23}. Also, the reproductive stages of *Neurospora Crassa* are used to follow its circadian timing ^{22,24,25}. *Drosophila melanogaster* have many physiological circadian outputs, because of their ease of use eclosion (aka. pupal hatching) behavior is preferred ^{18,26,27}. In a limited space, individual animals hatch at a limited time that is best for their survival. Bang bottles are developed to estimate the number of hatched flies in a given time interval. Basically, the bang bottles are bumped to drop flies down away from the sides where they hatch. Then each bottle is poured into a collection tube where each time the number of hatches is counted ^{1,28}.

Circadian time is a very common term in chronobiology, which differs from regular time zone. Circadian time is subjective to endogenous organism time. In a free running animal under constant conditions, innate rhythm is not exactly 24 hours as in earth. For example an organism with 23 hours free running circadian time equals to 24 hours of regular time zone. Conventionally, circadian day starts at subjective sunrise corresponding to circadian time 0 (CT 0) and respectively subjective sunset is CT 12 ¹. For rodents (nocturnal animal) activity onset starts at CT12, while in humans activity onset starts at CT0. Zeitgeber time is the timing in a light entrained system in which ZT0 is the beginning of the day as the light phase starts while ZT12 is the beginning of the night as dark phase starts ¹.

Free running rhythms are seen when endogenous rhythms are isolated from environmental cues. The period of the free run is not equal to the environmental period; it runs either faster or slower than 24 hours. Though, free runs still maintain their entrainable properties. Strikingly, endogenous free running rhythms are innate with their genetic background rather than being developed by learning.

2.5 Molecular Components of the Mammalian Circadian Rhythm

2.5.1 Transcriptional Feed Back Loops

To date molecular mechanism of circadian clock is understood as a transcriptional translational feedback loop (TTFL) containing at least ten genes as shown in Figure 2³. The positive arm of the TTFL is composed of CLOCK and BMAL1 proteins. Clock and Bmal1 genes encode bHLH (basic Helix-Loop-Helix) and PAS (Per-Arnt-Single-minded) domains, which are essential for their proper functioning. With their PAS domains, CLOCK and BMAL1 heterodimerize in nucleus to initiate transcriptional activation. BMAL1:CLOCK heterodimer binds to either E-Box (5'-CACGTG-3') or E'-Box (5'-CACGTT-3') on promoters of target genes²⁹⁻³¹. Per1, Cry1 and Cry2 are controlled by BMAL1:CLOCK heterodimer complex since they contain cis regulatory E Box elements³²⁻³⁴. It is important to note that Per2 gene is controlled by a non-canonical E Box motif named as E'box³⁵. The set of genes that contain E/E'-box regulatory elements constitute the negative arm of the TTFL. In the canonical view PER and CRY proteins dimerize in cytoplasm and translocate back to nucleus in order to inhibit BMAL1:CLOCK transcriptional activation³⁶⁻³⁸. The PERs and CRYs are regulated also by post-translation modifications altering their localization and degradation. To date the identified modifications are performed by kinases, phosphatases³⁹⁻⁴¹ and E3 ubiquitin ligases⁴²⁻⁴⁴.

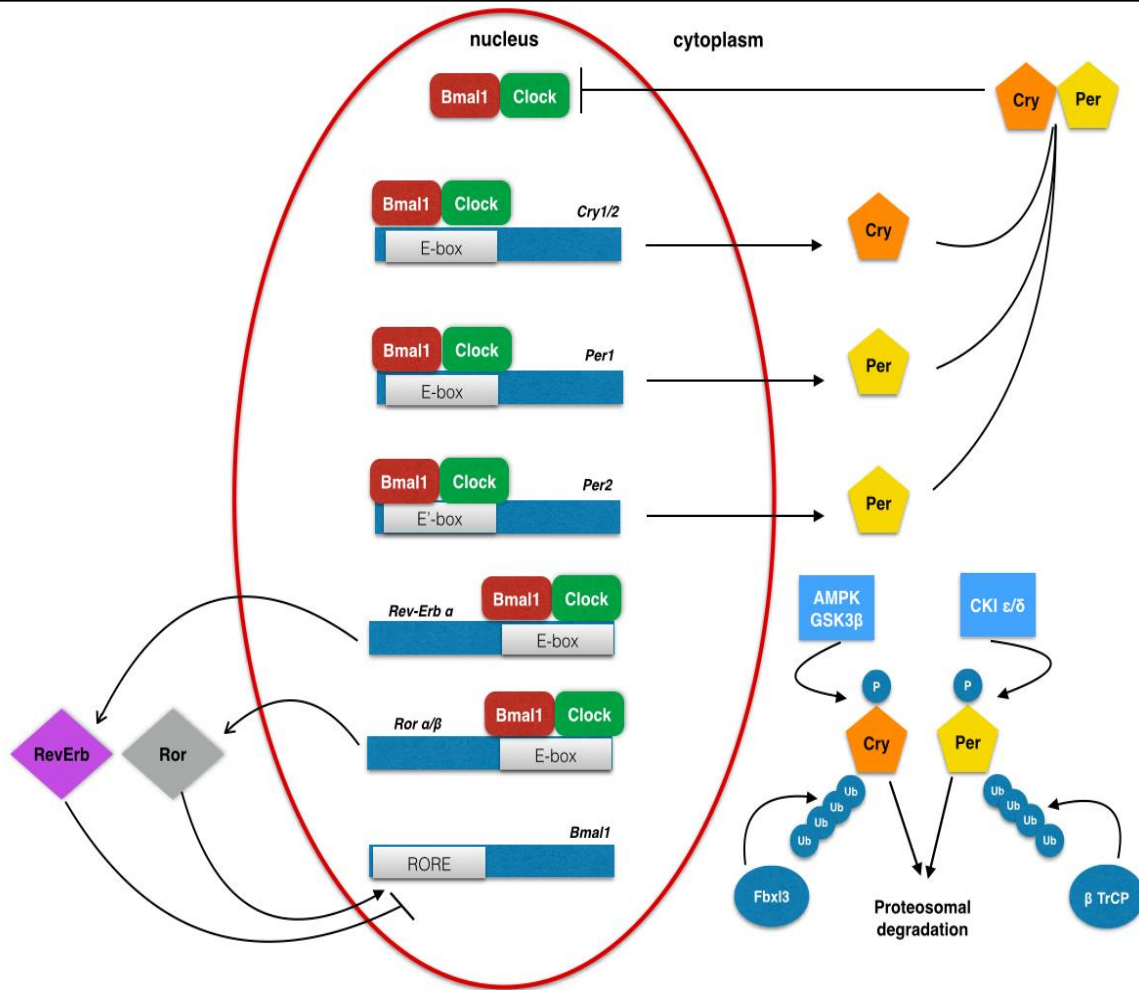


Figure 2 Transcriptional translational feedback loops

BMAL1:CLOCK complex (red:green) bind E-E' Boxes on the promoters of target genes to initiate their transcription. Produced PER and CRY proteins (yellow:orange) dimerize in the cytoplasm and translocate back to nucleus to inhibit BMAL1:CLOCK transcriptional activation. Retrieved from ³

Cryptochromes have two paralogs in mammals as Cry1 and Cry2 whereas Periods have three as Per1, Per2 and Per3. Partial paralog compensation within these families is observed. A decrease in CRY1 and PER1 production is partially compensated by an increase in CRY2 and PER2 production respectively. However, reduction in CRY2 and PER2 is not compensated by an increase in CRY1 and PER1 production.⁴⁵ Considering this, different locomotor activity outputs observed in Cry1 and Cry2 knock out mice is not unexpected (Table 1)³.

A second TTFL contains orphan nuclear receptors ROR (2 homologs α and β) and REV-ERBs (2 homologs α and β) that regulate directly the transcription of Bmal1 gene and cycling of other clock controlled genes^{46–48}. The expression of ROR and REV-ERBs are also mediated by E-Box elements. ROR and REV-ERB proteins compete to bind Retinoic acid related Orphan receptor Response Element (RORE) binding sites on Bmal1 promoter, where RORs activate and REV-ERBs inactivate Bmal1 transcription^{47,49,50}. Deletion of both Rev-erb α/β genes is lethal for mice, however deletion of single genes show mild changes in the rhythm (See Table 1), (Figure 3). Generation of inducible Rev-erb α/β knock out mice revealed that core clock components' expression and lipid homeostasis are regulated by Rev-erbs⁵¹.

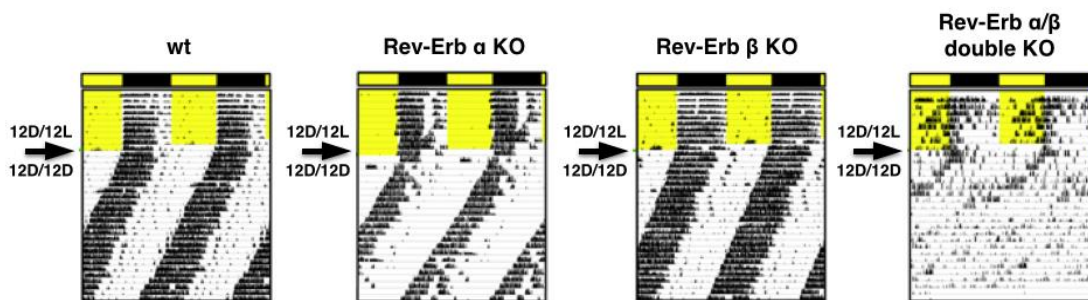


Figure 3 Locomotor activity in Rev-erb α/β knock out mouse.

As seen in the figures, single knock out of either Rev-erb genes doesn't affect the locomotor activity. Inducible knock outs of both genes revealed loss of circadian rhythm. Retrieved from⁵¹.

Another set of genes containing D-Box binding domains have probable roles in TTFL. These genes are HLF family proteins (hepatic leukemia factor), TEF (thyrothroph embryonic factor) and DBP (albumin D-site-binding protein), which belong to PARbZip (proline and acidic amino acid rich basic leucine zipper) transcription factor family. Their expression are also regulated by BMAL1:CLOCK activity, which in turn expression PARbZip transcription factor family proteins regulate genes with D-box motif on their promoter⁵²⁻⁵⁴.

The cycling of the TTFL generates circadian oscillations in the expression of both core clock components and clock-controlled genes. In addition, they regulate oscillation of output genes in a tissue specific manner⁵⁵. It is speculated that the three DNA binding elements provide necessary time to generate approximately 24 hours of cycling: E-Box (morning), D-Box (day), and RORE (evening)⁵⁶. Still, gene families containing D-box accessory loops are not required for the functional rhythm but it is shown that they make oscillations robust and precise³.

Table 1 Locomotor activities associated with core clock component knock-outs.

Mutation	Locomotor Activity in mice	Ref. No
Clock	Longer period, attenuated persistence of rhythm in constant conditions	19
Per1	Decreased amplitude, shorter period, loss of rhythm	33
Per2	Shorter period, loss of rhythm	33
Per3	Modest shortened period	57
CK1ε	Shortened period in hamsters	
Cry1	Shorter period	58
Cry2	Longer period	58
Cry1/2	Complete loss of rhythm	58
Bmal1	Loss of rhythm	31
Dbp	Modestly shorter period	59
Npas2	Shorter period	60
Rev-erba/β	Loss of rhythm	51

Chip-Seq analysis revealed that CRY1/2, PER1/2, BMAL1, CLOCK are recruited to 1444 sites commonly within genome of mouse liver ^{61,62}. According to canonical model, PER:CRY complex inhibit the activity of BMAL1:CLOCK heterodimer after translocating to nucleus in between CT12 (evening) to CT20. In time, degraded CRYs and PERs can no longer inhibit BMAL1:CLOCK activity which reaches its acme at CT8. The recruitment of these complexes to clock controlled genes such as *Dbp* obeys to canonical view where PER:CRY complex inhibit BMAL1:CLOCK activity (Figure 4) ⁶³. First it was proposed by Natali Ozber and her colleges that CRY might not need PER for repressing BMAL1:CLOCK complex's transcriptional activator role ⁶⁴. After the emergence of the crystal structure of CRY:PER interaction ⁶⁵, another non-canonical model for the TTFL system is proposed. On the light of recent findings, BMAL1:CLOCK activity can also be repressed by only CRY binding without PER activity ^{64,66}. According to this model, there are three phases of BMAL1:CLOCK and CRY:PER complexes as early, late and active states. Based on the ChIP and proteomics data, in the active stage transcriptional activators such as p300/CBP are also recruited to BMAL1:CLOCK heterocomplex to enhance transcription. With the recruitment of PERs and CRYs, BMAL1:CLOCK activity is repressed in the early phase. In the late stage, BMAL1:CLOCK activity is repressed by CRY in a poised manner between CT0-CT4 time period (Figure 4) ^{63,66}. This model presents an explanation for requirement of a delayed *Cry1* expression for circadian oscillation ⁵⁶.

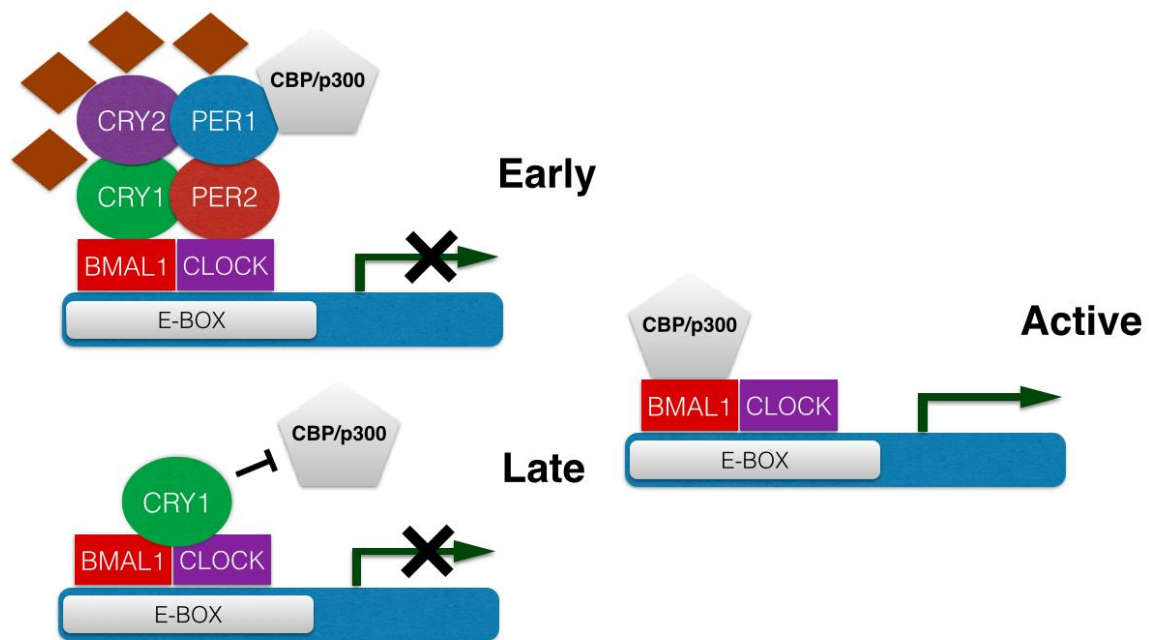


Figure 4 Non-canonical model of transcriptional translational feedback loop

Scheme representing the non-canonical repression of BMAL1:CLOCK activity in early, active and late forms. Retrieved from ⁶³.

2.5.2 Post-translational modifications of clock components

CRY and PER turnover rate is associated with period length determination. Several mechanisms are illuminated to date revealing the post-translational modifications of CRY and PER proteins affecting CRY and PER stabilization.⁶⁷ PERs are targeted for ubiquitination after their hyper-phosphorylation by Casein Kinase I ϵ on PER's phosphodegron site. Following ubiquitination by β -TrCP, PERs undergo proteosomal degradation^{67,68}. CRY2 is phosphorylated on Ser557 by DYRK1A (dual specific tyrosine-phosphorylation-regulated kinase 1A) in a rhythmic manner. This phosphorylation leads another phosphorylation by GSK-3 β (glycogen synthase kinase 3 β) on Ser553. This two-step phosphorylation subsequently causes the proteosomal degradation of CRY2⁶⁹. Another study published in 2008 revealed BMAL1 regulation by SUMOylation. Under physiological conditions, BMAL1 is conjugated to poly-SUMO2/3 whose attachment to BMAL1 is also showing oscillation. Differential SUMOylation patterns of BMAL1 results in ubiquitination of BMAL1 subsequently affecting its turnover rate⁷⁰.

CRY1/2 proteins are ubiquitinated by SCF complex (Skp1-Cul1-FBXL3) who functions as E3 ubiquitin ligases^{71,72}. Phosphorylation of CRYs by AMPK (AMP activated protein kinase) causes their interaction with FBXL3, subsequently causing proteosomal degradation of CRYs⁴¹. In another pathway FBXL21 also ubiquitinates CRYs, though this ubiquitination leads CRY stabilization. Recent studies show that, the antagonistic function of FBXL3 and FBXL21 is important for the robustness of the oscillation^{42,44}.

2.5.3 Epigenetic regulation of clock components

Besides transcriptional translation feedback loop, circadian rhythm is also regulated by epigenetic modifications (Figure 5). Transcriptional activation by BMAL1:CLOCK complex is also supported by modifications on H3/H4 histones. Acetylation and deacetylation of H3/H4 histones show circadian oscillation⁷³. It was

shown on Dbp promoter that Dbp transcription is activated by BMAL1:CLOCK heterodimer along with acetylation of Lys9 on H3, trimethylation of Lys4 on H3 histones. On the other hand, daily repression is supported by dimethylation of Lys9 on H3 and binding of HP1 α (heterochromatin protein 1 α) subsequently lead to an elevated histone density⁷⁴. BMAL1:CLOCK complex further recruits MLL1 methyltransferases JARID1 (a histone deacetylase inhibitor protein) to increase transcription^{75,76}. The deacetylation of H3 on promoters is mediated by SIRT1 (sirtuin 1) protein. SIRT1 activity is regulated by cellular NAD⁺:NADH ratio, which is also shown to affect BMAL1:CLOCK binding to E-Boxes *in vitro*⁷⁷⁻⁷⁹.

CLOCK protein itself has a HAT domain (histone acetyl transferase), which only CLOCK HAT domain transfection is shown to rescue Clock knockout phenotype in fibroblast cells⁸⁰. CLOCK as a bona fide HAT, which acetylates clock controlled gene promoter on H3K14 residues⁷⁸. Other than its histone acetyltransferase activity, CLOCK can also acetylate its dimerization partner BMAL1 on its Lys537, which shows daily oscillation pattern in mouse liver⁸¹. EZH2 a polycomb group protein is also associated with Per promoter methylation on H3K27^{82,83}. Various epigenetic modifiers and their interacting core clock partners are shown in Table 2. The interplay of molecular circadian rhythm and epigenetics is a fact though it still required to be elicited⁸⁴.

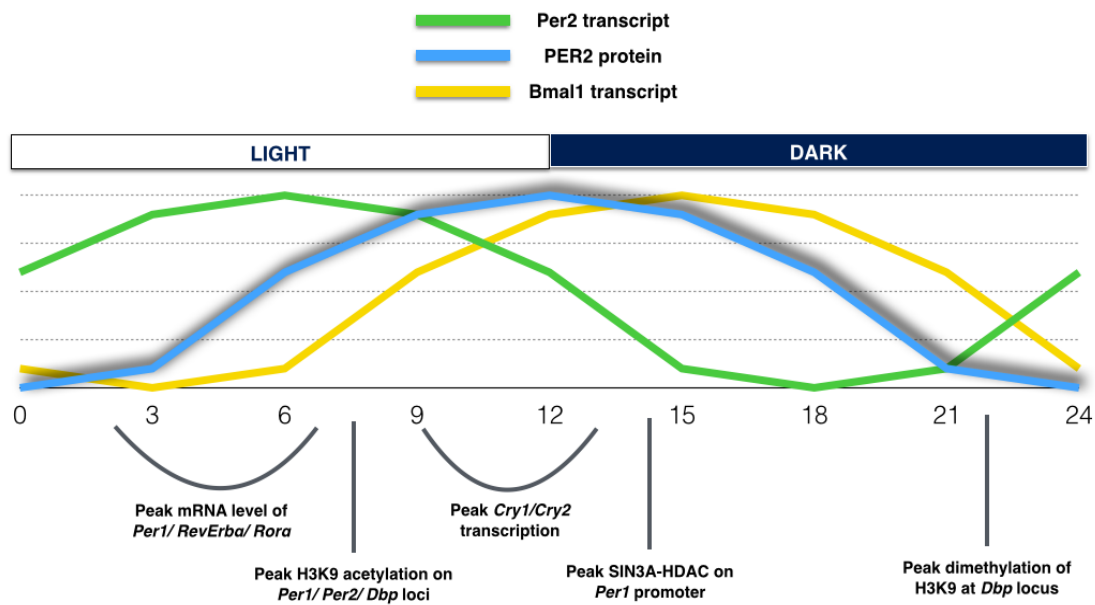


Figure 5 epigenetic modifications associated with circadian rhythm

Epigenetic modifications, transcript- and protein abundance of core clock components are shown in the schematic. Yellow, orange and blue waves show the Per2 protein amount, Per2 mRNA and Bmal1 mRNA level in mouse SCN respectively. Different epigenetic modifications on Per and Dbp genes are also illustrated within 24 hours of time period. Adapted from ³

Table 2 Core clock components and their epigenetic modifiers.

Adapted from 85.

CLOCK COMPONENT	ASSOCIATED CHROMATIN MODIFIER	TARGET	REF. NO
BMAL1:CLOCK	CLOCK	H3K9, H3K14, Bmal1 (K537), GR	80,86,87
	p300		88,89
	PCAF		89
	CBP		88,89
	SIRT	H3K9, H3K14, Bmal1 (K537), Per2	77,90
	MLL1	H3K4	75
	EZH2	H3K27	82
	JARID1a		76
BMAL1:NPAS2	P300	H3,H4	89
	PCAF	H3, H4	89
	ACTR		89
PER	PSF-SIN3A-HDAC1	H3, H3K9, H4K5	91
	WDR5	H3K4	92
CRY	SIN3B-HDAC1/2	H3, H4	93
REV-ERB α/β	NCoR-HDAC3	H3, H4, H3K9	94-97

2.5.4 Structures of core clock components and their implications

The molecular structures of the core clock components just started to be revealed in the last few years. As the models emerge, it became remarkable that the domains required to interact with DNA and other core clock components are highly conserved among clock components⁶³.

Mammalian core clock components share homology in between their domains as positive and negative regulators. As shown in Figure 6 positive regulators BMAL1 and CLOCK proteins share their bHLH (basic helix loop helix), PAS (Per-Arnt-Sim) and TAD (trans-activating) domains. On the other hand, PER as a negative regulator also contains PAS domain^{63,98}.

PAS domains have 120-150 amino acids as 5-antiparallel β sheets and 3 flanking α helices. The α helices form a cavity on the one face of β sheets to interact with cofactors such as heme, FMN and FAD^{99,100}. In fruit fly and mammals PAS domains show a conserved localization as tandem repeats (PAS-A and PAS-B). The PAS-A and PAS-B domains are important for homo-heterodimer formation in PER proteins; whereas in bHLH-PAS transcription factors BMAL1 and CLOCK, PAS-A and PASB domains are required for heterocomplex formation^{98, 101}. The DNA binding ability of BMAL1 and CLOCK proteins are mediated by E-Box binding motif named bHLH domain located at the C-terminus of PAS domains. Mammalian CLOCK also contains a HAT (histone acetyl transferase) domain, which acetylates K537 of BMAL1 and H3K14^{80,81}. PAS-B domain of CLOCK protein is also essential for CRY1 binding to BMAL1:CLOCK heterodimer, in which release of CRY1 allows CBP-p300 complex to bind BMAL1 on the same domain¹⁰². The domain organization of PAS-A and PAS-B along with BMAL1:CLOCK heterocomplex determined upon crystallization experiments are shown in Figure 7.

+ regulators



- regulators



Figure 6 Domains of core clock components are represented on scale bars. Retrieved from ⁶³

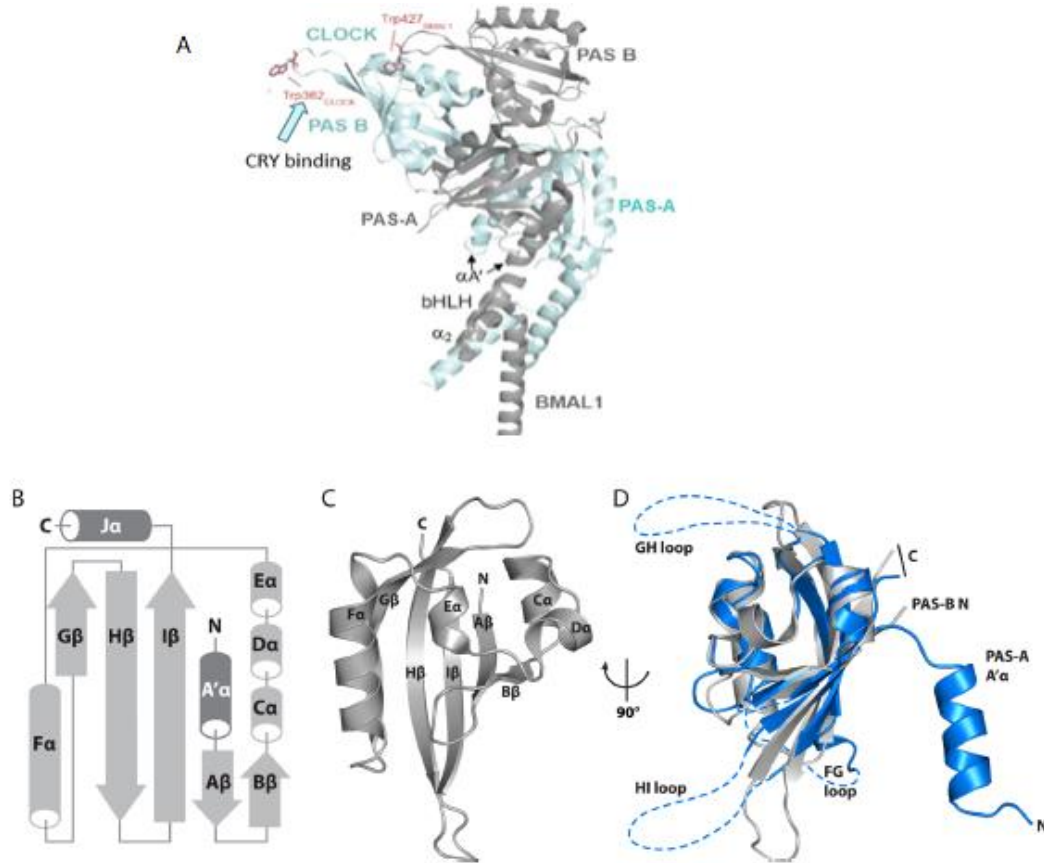


Figure 7 3D structures of PAS-A/B domains in BMAL1:CLOCK heterocomplex.

A) Crystal structures of CLOCK (top) and BMAL1 (bottom) proteins ²²². They are organized in such a way that each domain interacts with counterpart domain of other partner. Trp427 of PAS-B in BMAL1 interacts with PAS-B in CLOCK. Trp362 of CLOCK holds a cavity for CRY1 binding. Adapted from ⁹⁸.

B) Secondary structures of PAS-A and PAS-B domains. C) Tertiary structure of a PAS domain. Secondary structure names are taken from a mouse BMAL1-PAS B model. D) Tertiary structure of both BMAL1 PAS-A (blue) and PAS-B (grey) domains. The loops GH-HI and FG are unstructured. Adapted from ⁶³

CRYs contain 2 domains named PHR (photolyase homology region) with N-terminal α/β domain and a C-terminal α -helical ($\alpha 22$) region, and an unstructured C-terminal domain (Figure 6) ¹⁰³. CRY1-PER2 and CRY2-PER2 interacting domains are stabilized by a Zinc ion which interacts with Cys1210 and Cys12313 of Per2 and conserved His437 of CRYs (on CRY1 zinc is also stabilized by Cys414) ^{65,104}. Arg501 and Lys503 mutations on $\alpha 22$ region localized where PHR and C-terminal domains of CRY2 meet is important for Per2 binding (Figure 8) ⁶⁴. Also, luciferase complementation and fluorescence two-hybrid assays revealed that, mutations on CRY1 protein localized onto $\alpha 22$ region (K485D/E and G336D) are also important for mCRY1:PER2 and mCRY1:BMAL1:CLOCK interaction. However, this loss of interaction between mCRY1 and BMAL1:CLOCK complex doesn't affect the transcription activity of BMAL1:CLOCK heterocomplex ¹⁰⁵.

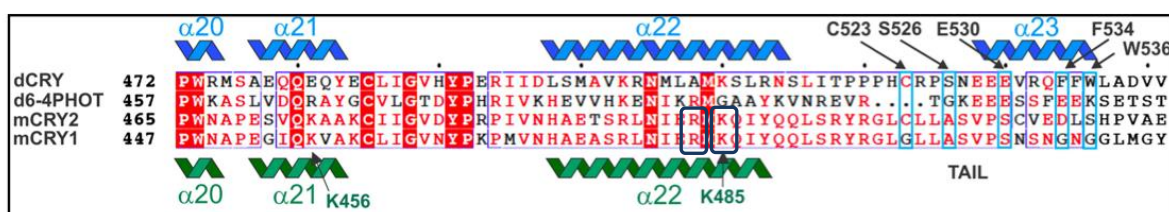


Figure 8 Partial amino acid sequence of CRY C-terminal tail from various species

dCRY (Drosophila m.), d6-4PHOT (Drosophila M. 6-4 Photolyase), mCRY2 (mouse CRY2) and mCRY1 (mouse CRY1). Retrieved from ¹⁰⁵

2.6 Physiological Components of the Mammalian Circadian Rhythm

Physiological components of the mammalian circadian system are classified into three components named input, clockwork and output. Though there are no sharp boundaries between these components since they are highly integrative¹⁰⁶. The input part is achieved by light perception through eye which sends signals to SCN (suprachiasmatic nucleus) via retinohypothalamic tract^{8,107}. SCN is the central oscillator of the mammals which synchronizes the peripheral clocks⁶. The output component appears as direct neuronal connections of SCN with other regions of brain or through sent endocrine signals to other brain parts and peripheral regions of the body^{22,108}. Perception of environmental signals, integration of these stimuli in SCN and generation of synchronized outputs will be analyzed in subsequent chapters.

2.6.1 Perception of environmental stimulus

Environmental stimuli important for circadian perception are feeding, emotional stress, and ambient temperature; though the strongest zeitgeber synchronizing the circadian rhythm to environment is light⁸. Depending on the type of the organism, light perception can be achieved by several organs including eyes, pineal gland and mid brain. For instance, non-mammal vertebrates perceive light by photoreceptors located in their brain, pineal gland and retina¹⁰⁹. On the other hand, in drosophila and zebrafish, photoreceptors are localized in whole cells of the organism; since their body allows the light passage, multiple organs achieve perception of the light^{8,110}.

Mammals perceive light only through their eyes. Supporting this, mice having *math5*^{-/-} mutation (impaired optic nerve development) show no entrainment to daily light cycle, but sustain their innate rhythm¹¹¹. Conventional rod and cone photoreceptors are not responsible for circadian entrainment but the other like melanopsin is important for photic entrainment (Figure 9)⁶. It was shown in *rd/rd* mice (impaired rod specific signal transduction), even if the visual ability is completely gone, mice can still sustain entrainment to light cycle^{112 113}. Retinal ganglion cells (RGCs) in eye can depolarize in response to light unlike other rod-cone dependent ganglion cells and send signals to SCN

supporting their importance for photic entrainment^{8,114}. Melanopsin, located in RGCs, is the photopigment important for circadian photoreception as a photoactivatable protein^{107,115,116},¹¹⁷. It was shown that melanopsin knock out mice can entrain light dark cycles and doesn't show any unusual circadian behavior in constant darkness. Still, they show impaired phase resetting in response to light pulses relative to wild type animals¹¹⁸. This indicates an important role for melanopsin in circadian photoreception¹⁰⁷.

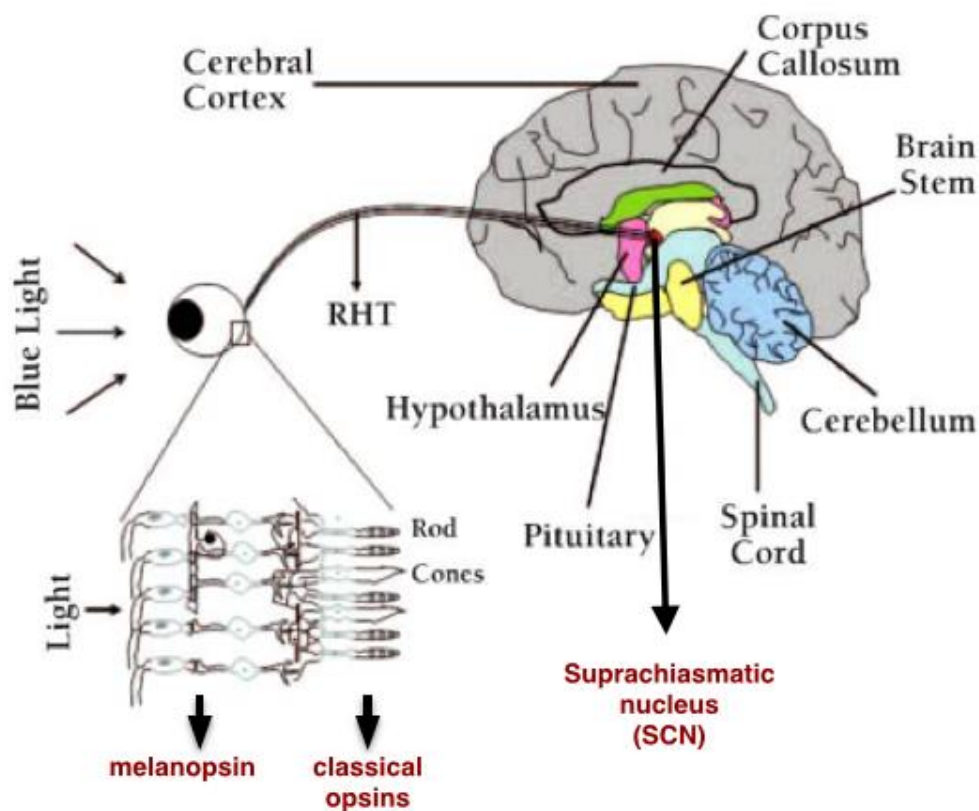


Figure 9 Circadian photoreception in mammals

Light signals are received by photoreceptors of eye and sent to SCN via RHT. Retrieved from⁸

2.6.2 Suprachiasmatic Nucleus as central oscillator

SCN (suprachiasmatic nucleus) located in hypothalamus is the center for circadian timing synchronization. SCN has spontaneous daily rhythm, but signals taken by RGCs subsequently help SCN for synchronization. Based on this, SCN stimulates output pathways via neural and endocrine pathways^{119,120}.

SCN is composed of around 10,000 GABAergic neurons located in ventral core region and a dorsal shell region. Based on their localization, they show distinct properties. The neurons in ventral core region can be stimulated directly by RGCs and produce Vasoactive Intestinal Polypeptide (VIP). Dorsal shell region cells producing Arginine Vasopressin (AVP) is responsible for the generation of output signals^{119,121}. SCN mediates sleep wake cycle by making direct neuronal connections with sleep-wake centers of brain and subsequently stimulates secretion of prolactin and growth hormone at night¹⁰⁸. GABA and VIP are known as the primary synchronizer within SCN neurons^{122,123}. In VPAC2 knock out mice (VIP receptor sub-type 2) synchronization of SCN neurons is abolished with disruption of oscillating gene expressions and reduced spontaneous firing in SCN neurons^{124,125}. The balance of counteracting hormones is important for proper synchronization of SCN neurons.

Core clock components constituting positive and negative arm of the TTFL, are also expressed in SCN showing circadian oscillation. *Per* and *Cry* transcripts reach maximum in SCN at late circadian day¹²⁶. Transcripts and proteins belong to *Clock* is constitutively expressed in SCN cells, while *Bmal1* transcript peaks in the middle of the circadian night^{46,127}. Mutations in core clock components cause changed stability, amplitude and period length in circadian oscillations¹²¹.

Light entrainment is achieved in molecular level via kinase cascades and Ca^{+2} signaling. Light exposure induces electrical firing in SCN neurons activating a Ca^{+2} dependent kinase signaling. Subsequently, CREB (cyclic AMP response element binding protein) gets activated binding to CRE sequences on *Per* gene^{6,128}. Light induced *PER* production is also activated on shell of SCN via sustained action potential generation in SCN neurons by light¹²⁹. *CRY* production is also induced by light induction but with a

delay¹²¹. Per expression falls, shutting the negative arm of the loop with the sun set. In a very simplistic model, neuropeptidergic coupling of SCN neurons upon light stimulation is achieved by VIP secretion as a result of TTFL loop within a neuron. Simultaneous production of VIP is sensed by VPAC2 receptors on other neurons stimulating another cascade of TTFL (Figure 10). Though, it is known that VIP is the not only peptide responsible for synchronization of SCN neurons¹¹⁹.

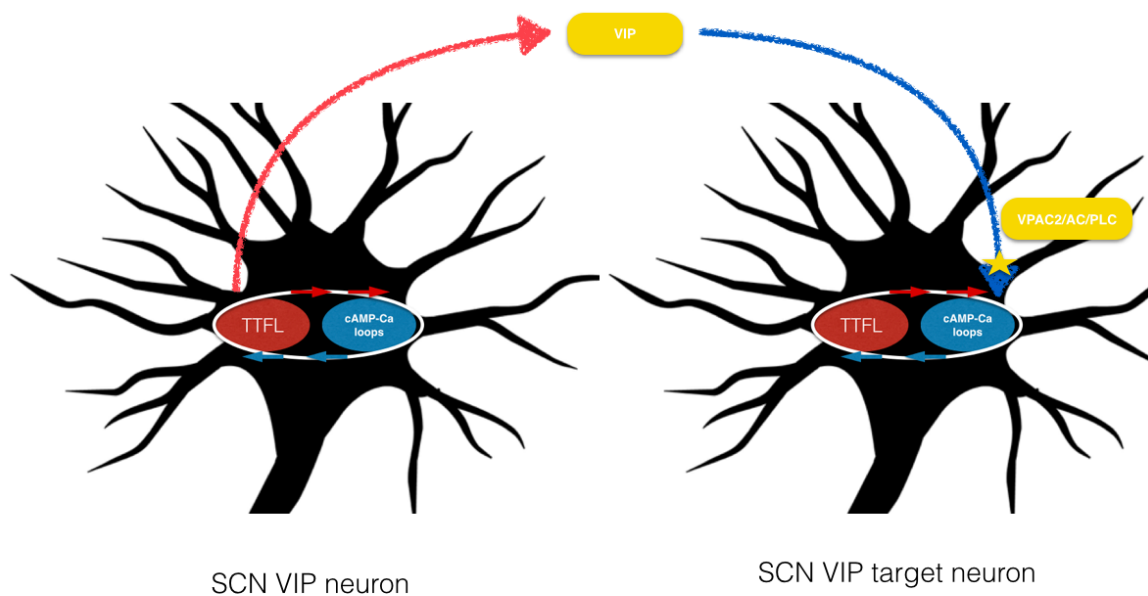


Figure 10 SCN neurons communicating through paracrine signaling

A very simplistic model representing how SCN neurons communicate with each other through paracrine signaling. VIP acting on VPAC2 (VIP receptor) is produced under TTFL control can subsequently activate other neurons inducing another TTFL.¹¹⁹

Peripheral tissues such as liver, kidney heart also express core clock components in a rhythmic profile. However, the synchronization of these cells is achieved via SCN stimulation¹²¹. Genome-wide profiling experiments revealed that 5-9% of the transcriptome is under circadian regulation in liver and heart. Among the oscillating genes, approximately 10% is common constituting the genes that are close to core clock signaling^{55,130}.

Depending on the organ, cycling pathways show divergence. For example in retina, around 300 genes have rhythmic expression in darkness, which are involved in photoreception, synaptic transmission and some metabolic pathways. However, strikingly rhythmic expression of these genes is lost upon Bmal1 knock out ¹³¹. A similar type of experiment performed on liver in which BMAL1:CLOCK activity is diminished in a tissue specific manner. The same study in liver showed that around 400 genes oscillate in a circadian profile. Though unlike retina, in Bmal1 knock out cells 31 of the genes didn't diminish rhythmic expression ¹³².

With the effect of SCN synchronization of peripheral clocks, several physiological events get synchronized in mammals including core body temperature adjustment, release of hormones such as prolactin, growth hormone and melatonin, serum cortisol level, bowel movement and immune system (Figure 11) ¹²¹. However, feeding can reset peripheral clocks without affecting the central pacemaker. For instance, decreased food availability can shift circadian timing of liver without affecting the SCN and overall behavior ¹³³. Glucocorticoid signaling and ADP-ribosylation are important pathways for liver resetting by altered food uptake ^{134,135}. Combined with the results obtained with Bmal1 knock out experiments, these results show that hepatic circadian rhythm is regulated at multiple levels within the boundaries of SCN control.

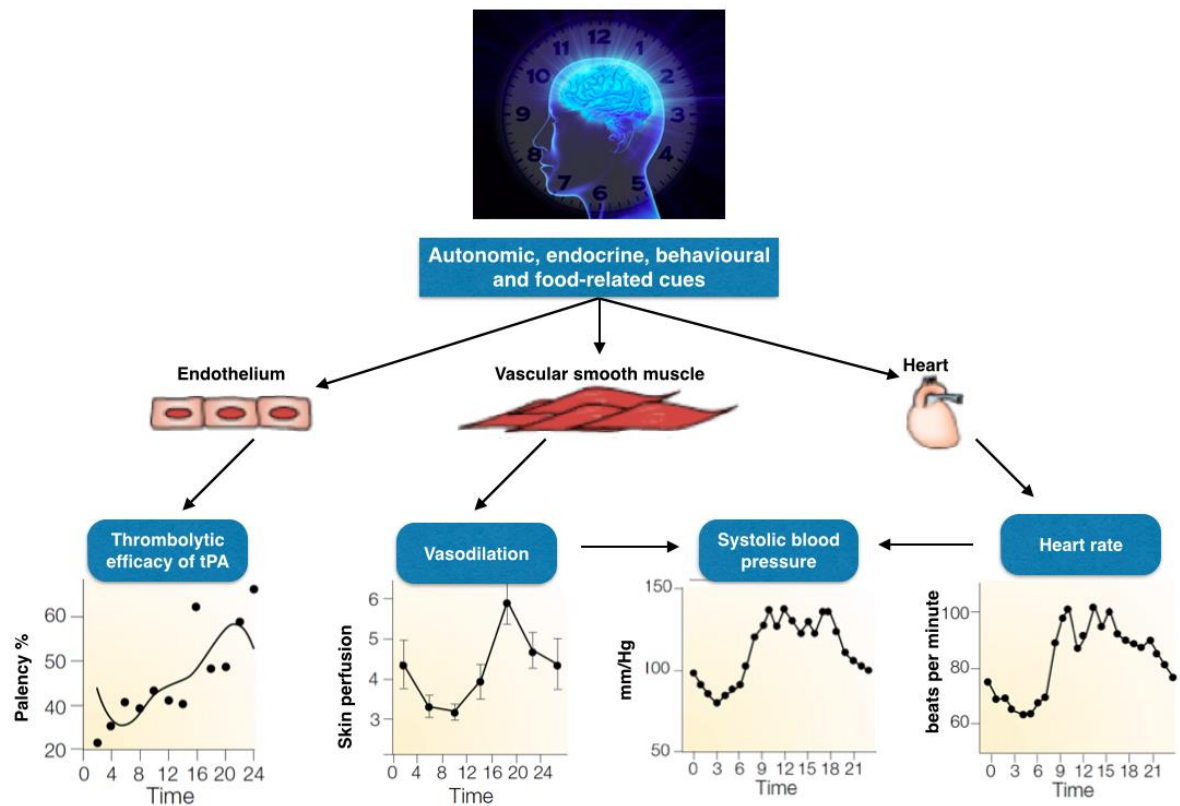


Figure 11 SCN synchronization of peripheral tissues

Upon synchronization, CCG (clock controlled genes) subsequently causes oscillation of thrombolytic activity, vasodilation, systolic blood pressure and heart rate. These physiological outcomes are interacting which means they also affect each other. Retrieved from ¹²¹

2.7 Circadian Rhythm and Metabolism

Circadian rhythm regulates body metabolism in a tightly manner in order to synchronize anticipated activities with proper metabolic functioning⁸⁵. Misalignment of circadian rhythm vs metabolism can enhance the risk for metabolic diseases. For instance, night shift workers demonstrate higher risk to develop metabolic diseases including diabetes, obesity, cardiovascular diseases and metabolic syndromes^{136,137}. Based on these results it is clear that metabolism and circadian clock are interconnected. Mammalian molecular circadian clock composed of TTFL (transcriptional translational feedback loops) is also fine-tuned by metabolic cues including hormones and metabolites.

Environmental cues such as nutrition, exercise, and stress can have effect on metabolic hormones including insulin, glucagon and leptin, and also on metabolites like NAD⁺ (nicotineamide adenine dinucleotide), ATP, AcCoA (acetyl coenzyme A), SAM (s-adenosylmethyonine) (Figure 12)^{138,139}. A direct link between metabolism and circadian rhythm is established by the example of peroxiredoxin proteins which are found in archaeabacteria, plants and some other eukaryotes. Peroxiredoxin is regulated by redox state of the cells since it has a thiol group on the active site which has role in electron transport from ROS (reactive oxygen species)¹⁴⁰. Most importantly, it shows rhythmic activity when there is no transcription in the cells as it is shown in erythrocytes. This result also indicates that circadian oscillations can proceed without transcription¹⁴¹.

In mammals, regulation of leptin and ghrelin hormones is circadian rhythm associated. Leptin release is regulated by SCN, which stimulates adipose tissue for the hormone release.¹⁴² On the other hand, negative regulation of leptin hormone is mediated by ghrelin hormone secreted from stomach^{143,144}. Leptin production peaks at early night whereas ghrelin amount peaks during resting in nocturnal rodents¹⁴⁴. It was also shown that, via feedback mechanism in fasting state in which ghrelin production is stimulated, ghrelin directly affects the core clock components in SCN. This shows a possible link between stomach and central clock oscillation^{145,146}.

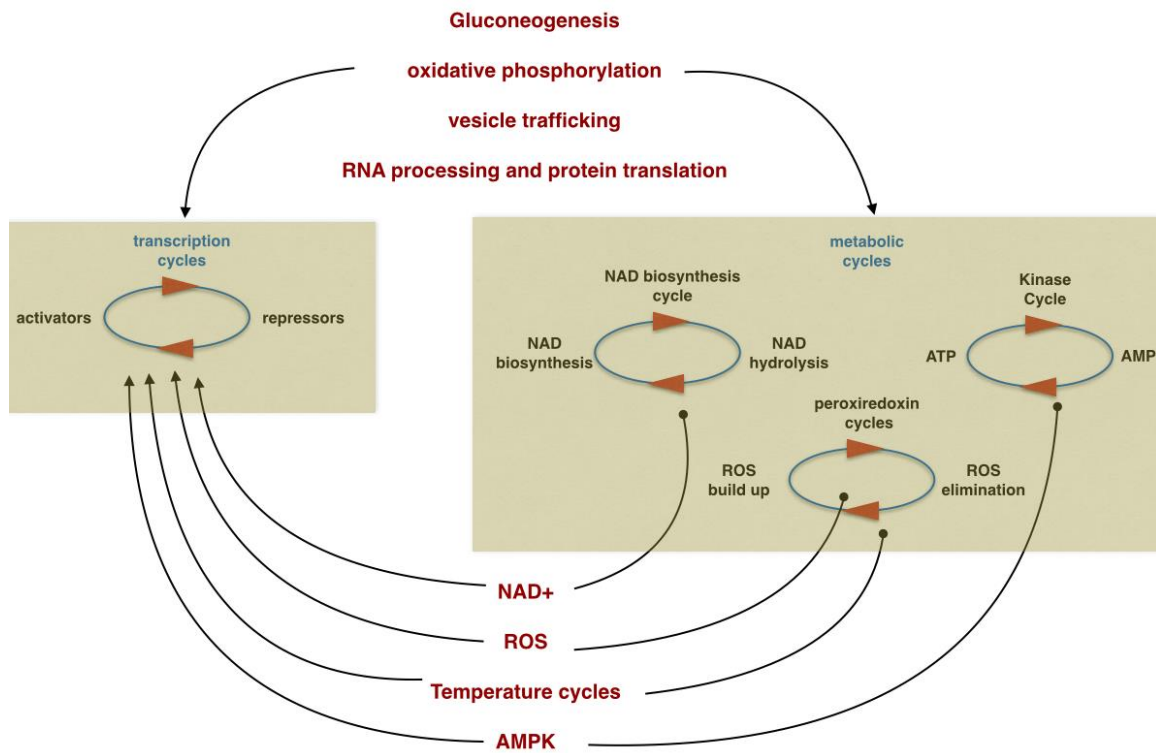


Figure 12 Effect of circadian rhythm on metabolites

Cycling of core clock components subsequently affect gluconeogenesis, oxidative phosphorylation, vesicle trafficking and RNA processing and translation. On the other part of the cycle, these metabolic processes affect levels of several metabolites including NAD^+ , ROS and ATP. Several forms of the metabolites can again in-turn affect the core oscillation. Adapted from ¹³⁹

While most of the hormones' circadian oscillation shows opposite oscillation patterns in nocturnal and diurnal animals, melatonin production shows similar patterns¹⁴⁷. Upon light stimulation, GABA is rhythmically released from SCN. This release subsequently inhibits the pineal gland activation for melatonin secretion. In dark phase on the other hand, glutamatergic stimulation takes place which subsequently stimulates pineal gland for melatonin production^{142,148}. In the physiological level, disruption of melatonin oscillation by suppressing the melatonin production at night causes impaired glucose homeostasis¹⁴⁹. Considering this, it is important to avoid light stimulation at night, which inhibits melatonin secretion through GABA in order to maintain glucose homeostasis.

Normally, it is known that insulin and glucagon levels alter with food intake, which means their level is in the same phase with the food uptake. The regulation of glucose homeostasis via SCN is directly proved first by performed feeding experiments on rats. Rats are forced to eat 6 meals per day with 4 hours intervals. These animals still showed higher glucose responses in insulin production just before their active phase anticipating for the nocturnal activities¹⁵⁰.

At the molecular level, CLOCK regulates a rate-limiting enzyme of glycogen synthesis pathway named Gys2 (glycogen synthase 2). In a particular mutation of Clock gene, oscillation of hepatic glycogen and glycogen synthase 2 productions is attenuated¹⁵¹. Glucose metabolism is also impaired by mutations on Cry genes. Overexpression of a mutant version of Cry1 in mice generates symptoms of diabetes¹⁵². Also, overexpression of Cry1 in db/db mice (Leptin receptor deficient) is associated with insulin sensitivity¹⁵³. In hepatocytes CRY1 represses the transcription of PEPCK (phosphoenolpyruvate kinase) an enzyme that has important roles in gluconeogenesis¹⁵⁴.

2.4 Circadian Rhythm Dysfunction Associated Diseases

It is a fact that circadian rhythm provides a selective advantage for organisms to better adapt and survive in the fluctuating conditions by earth's daily rotation. On the other hand, faulty circadian rhythms induce various disorders. The most conspicuous diseases are mostly related to sleep disorders such as Delayed Sleep Phase Syndrome (DSPS) and Advanced Sleep Phase Response (ASPS) causing insomnia or hypersomnia respectively in patients ^{155–157}. In these patients, innate sleep cycle misaligns with ordinary sleep behavior. Timing of hormonal cycle, body temperature fluctuation and peak mental alertness timing doesn't collide with usual rhythm. The familial ASPS (the disease is inherited) problem originates from a point mutation on phospho-acceptor site of Per2 (S662G) ¹⁵⁸ or a loss of function mutation in Per2 phosphorylating CK1 δ (T44A) ¹⁵⁹. Associated with the sleep disorders, jetlag and shift work disorders also stem from misalignment of innate rhythm to the environment ³.

Other than sleep disorders, metabolic diseases intersects with dysfunctional circadian timing. There are various examples to this as loss of BMAL1 function in pancreatic beta cells cause diabetes and hypoinsulinaemia ^{160,161}. A homozygous *Clock^{Δ19}* mutant mice expressing a loss of function CLOCK protein showed aberrant sleep cycle but most importantly they developed metabolic dysfunctions such as hyperlipidemia, hyperleptinemia, hyperphagia and hyperglycemia ¹⁶². Metabolic syndromes and circadian rhythms are coupled from primitive organisms to humans ¹⁶³. Mutations on core clock components impair glucose, lipid and hormone homeostasis accumulatively causing metabolic syndromes. The metabolic symptoms in mice associated with mutations on some core clock components are shown in Table 3.

Table 3 Metabolic symptoms associated with core clock component mutations.This table is adapted from ¹⁶³

Mutation type	Metabolic defects on mice	Ref.
Clock/Clock mutant	Metabolic syndrome	164
Clock^{-/-} mutant	Reduced arterial blood pressure, impaired renal function, excretion of dilute urine	53
Bmal1 KO only in liver	Fasting hypoglycemia	165
Bmal1 KO only in pancreas	Diabetes mellitus	166 161
Bmal1 mutation	Attenuated blood glucose and triglyceride oscillation	167
Per1 mutation	Elevated urinary sodium excretion	168
Per2 mutation	Decreased body weight and impaired lipid metabolism	169
Cry1 and Cry2 mutation	Hyperglycemia and salt-sensitive hypertension	153 170
Rev-Erbα KO	Elevated blood triglyceride concentration	171
Rorα mutation	Decreased blood triglycerides and high density lipids, atherosclerosis	172
Pgc-1α KO	Enhanced insulin sensitivity and altered thermogenesis	173 174
Nocturnin mutation	Diet-induced obesity resistance and altered lipid metabolism	175,176

Cancer, among the pathological conditions that are driven by dysfunctioning circadian clock holds a conflicting position^{177,178}. Disruption of the clock by mutations on *Per2* makes mice liable into cancer^{179,180}, but reverse affects are observed in *Cry1/2* knock out mice¹⁸¹. Hence, the mechanism of impaired circadian rhythm looks more relevant to carcinogenesis rather than the effect of the impaired circadian clock itself¹⁷⁸. There are many studies revealing the interplay between circadian clock and cancer. Wee1 protein as mitotic kinase is regulated by BMAL1-CLOCK complex^{182,183}. On the other hand in a study conducted in 2006 showed that overexpression of *Per1* gene sensitizes cells to DNA damage induced apoptosis. In the study it was noted that *Per1* protein interacts with ATM and Chk2 cell cycle checkpoint proteins¹⁸⁴. In embryonic fibroblasts, a proto-oncogene *N-myc* is shown to be regulated by NPAS2-BMAL1¹⁸⁰, *RORα* and *REV-erbβ*¹⁸⁵ at transcriptional level.

In another study it was shown that *Cry1/2* and *p53* knock out mice show decreased period of cancer progression than only *p53* knock mice¹⁸¹. The decrease was shown to be related with enhanced sensitivity of the cancer cells to both extrinsic and intrinsic apoptotic signals¹⁸⁶. In the light of this observation, only *p53*^{-/-} cells treated with oxaliplatin are arrested in cell cycle slower than *Cry1/2*^{-/-}*p53*^{-/-} knock out cells subsequently leading to apoptosis¹⁸⁷. Also immunotherapy with TNFα (an extrinsic apoptosis inducer) of the NOD-SCID mice strains containing xenografts of only *p53*^{-/-} or *Cry1/2*^{-/-}*p53*^{-/-} tumor cells revealed that *Cry1/2*^{-/-}*p53*^{-/-} tumor cells are more sensitive to extrinsic apoptotic signals than only *p53*^{-/-} cells¹⁷⁸.

Other than the molecular links, epidemiologic studies also signify the relevance between circadian clock and cancer. By the international Agency for Research on Cancer, shift work that causes circadian dysfunction is considered as a probable Group2A carcinogen¹⁸³. It is indicated that circadian impairment enhances the risk of breast, colon, prostate, lung and ovarian cancer.^{188–191}

It is known that molecular circadian signaling events are incorporated into various signal transduction networks^{138,178,192}. Consequently, it is expected for clock to affect

metabolic, pathologic and physiological events. Even if the link between circadian rhythm and cancer is well realized, the mechanism how this link works is not very clearly known. Understanding how this connection is established is important to develop better chemotherapeutic, radiotherapeutic and immunotherapeutic agents or even combinational therapies with well-controlled delivery timings¹⁷⁸.

2.8 Machine Learning and Bayesian Integration Strategy

Identification of clock components is achieved by several methods including forward and reverse genetics, and behavioral phenotyping (locomotor activity). In the forward genetics approach, random mutagenesis is applied on the organism showing detectable circadian phenotype. Based on the changes in the locomotor activity, mutant organisms were selected and via genetics tools the mutant gene is identified. Identification of core clock components PER and CLOCK are made upon drosophila and mice forward genetics respectively^{18,19}. Discovery of BMAL1 is achieved while searching for proteins containing bHLH-PAS domains in humans. For this purpose, EST tags of conserved bHLH and PAS are used as probes. Later on in BMAL1 knock out mice loss of circadian rhythm showed its role in clockwork³¹. Discovery of CRYs actually firstly made upon *Arabidopsis Thaliana* plant in which it shows blue light photoreactivation¹⁹³. Sequence analysis revealed its resemblance to a highly conserved protein named photolyase which is responsible for UV induced DNA damage repair¹⁹⁴. Later, analysis of its human homologs CRY1 and CRY2 revealed that they do not show photoreactivation. After the observation of diminished circadian rhythm in *Cry1/2^{-/-}* mice, its role in circadian rhythm became absolute¹⁹⁵.

Even if forward and reverse genetics combined with phenotyping techniques are very beneficial, they still contain some major drawbacks such as their expense and time consuming nature. Identification of candidate clock components speed up with the use of Naïve Bayes Machine Learning strategy. With the use of Naïve Bayes Machine Learning strategy publicly available datasets are generated by combined genome-wide analysis and probable core clock components are identified¹⁹⁶.

Core clock components in general share 5 main properties, which are also used in Bayesian Integration Strategy. These properties are as given below:

- 1) Expression of core clock components oscillates with a frequency of approximately 24 hours. For this purpose, high-resolution micro array data for liver, pituitary and NIH3T3 cell lines are used.
- 2) There is a functional genetic interaction among core clock components. Core clock components likely have functional connections with other genes. To analyze this, radiation hybrid mapping datasets are used.
- 3) Knock down of core clock components cause alterations on circadian phenotypes *in vitro*. For this purpose, a genome-wide RNAi screen is performed to understand their effects on the rhythmicity.
- 4) Core clock components are ubiquitously expressed in many tissue types. For this purpose, EST tags designed for individual genes are used to understand their expression patterns.
- 5) Core clock components are phylogenetically conserved. Homologene Database¹⁹⁷ is used to understand whether a *Drosophila melanogaster* homolog of the gene is present.

The probability for being a clock component is estimated distinctly for all five properties by a “circadian evidence factor”. Based on the estimated combined evidence factors of already known core clock components, a cumulative distributive function to be a core clock component became learned by the machine learning algorithm which is stated as “machine learning”. Based on this data, candidate genes’ circadian evidence factor is estimated and combined to estimate “combined evidence factor”. Then candidate genes are ranked based on their likelihood of being a core clock component¹⁹⁶. On the light of these collected results, in our lab by Gulsum Elif Muku candidate clock components are screened in order to understand their interaction with already known core clock components. Among the candidates, Leptin Receptor Overlapping Transcript like-1 (Leprotl1) was a hit.

2.9 Leptin Receptor Overlapping Transcript Like-1

Leptin signaling is associated with mostly appetite control and body weight, but also with regulation of hematopoiesis and metabolism^{198–200}. Strikingly, in ob-ob mice it was shown that recombinant leptin implementation diminishes the hereditary obesity phenotype²⁰⁰. Among the major components of leptin signaling are leptin and leptin receptor proteins.

Leptin receptor protein is a membrane protein with single transmembrane domain belonging to class-I cytokine family. Through alternative splicing, leptin receptor isoforms with different cytoplasmic tails are produced²⁰¹. Among the short isoforms are OB-Ra, OB-Rc and OB-Rd and their functions are still ambiguous. Long splice variant OB-Rb senses leptin stimulus through activation of JAK-STAT pathway^{202–204}. OB-Rb protein has 2 domains namely JAK and STAT binding domains. Other short forms of OB-Rs contain only the potential JAK binding domains²⁰⁵. It is also known that, C-terminus truncated version of OB-Rb protein has role in leptin insensitivity due to inability to perform JAK-STAT signaling in db-mice^{205,206}. LeprotL1 (endospanin 2) is expressed from OB-Rb gene sharing 2 exons with it²⁰⁷. This gene is conserved among species including yeast (Vps55 family), mouse and humans^{208–210}. Leprotl1 is composed of 131 amino acids showing great homology with another transcript named OB-RGRP (aka. Endospanin 1 and Leprot) also spliced from OB-Rb gene²⁰⁷.

Leprotl1 protein is a ubiquitously expressed membrane protein located at TGN (trans-golgi network) but also to late endosome in a lesser extent. Leprotl1 has 4 transmembrane domains as both termini project toward the cytoplasm. It is responsible for membrane trafficking including post internalization of leptin receptor from cellular membrane²¹¹.

Leprotl1 is also associated with hepatic growth hormone (GH) resistance²¹². Growth hormone regulates postnatal growth in mammals. Basically, GH induces lipolysis in peripheral organs, inhibits insulin driven glucose disposal and stimulates protein

storing²¹³. With restrictive food, liver becomes GH resistant. Hepatic GH induced IGF1, which is a negative feedback regulator of GH release from pituitary gland, is produced less leading increased GH release^{214–216}. Hepatic growth hormone stimulates subsequent STAT5 signaling which becomes desensitized to growth hormone in time with elevated SOCS level (negative regulator of STAT5 signaling). This desensitization is also supported further by Leprotl1 function, which takes role in the post-internalization step of GH receptor endocytosis leading to lysosomal degradation²¹².

In a similar manner, Leprotl1 is also shown to take role in Leptin receptor endocytosis leading to leptin resistance in liver. Chronic food restriction causes FGF21 production, which induces Leprotl1 production in liver. Subsequently, LEPROTL1 induces internalization and lysosomal degradation of leptin receptor (Figure 13)²¹⁷.

Leptin signaling shows a circadian rhythmicity as demonstrated in a microarray analysis revealing the daily transcription of leptin signaling components (Figure 14). The transcription level of Leprotl1 in liver is also demonstrated in CircaDB database (Figure 15)²¹⁸. Database results also indicate that in Clock knock out mice Leprotl1 oscillation is attenuated.

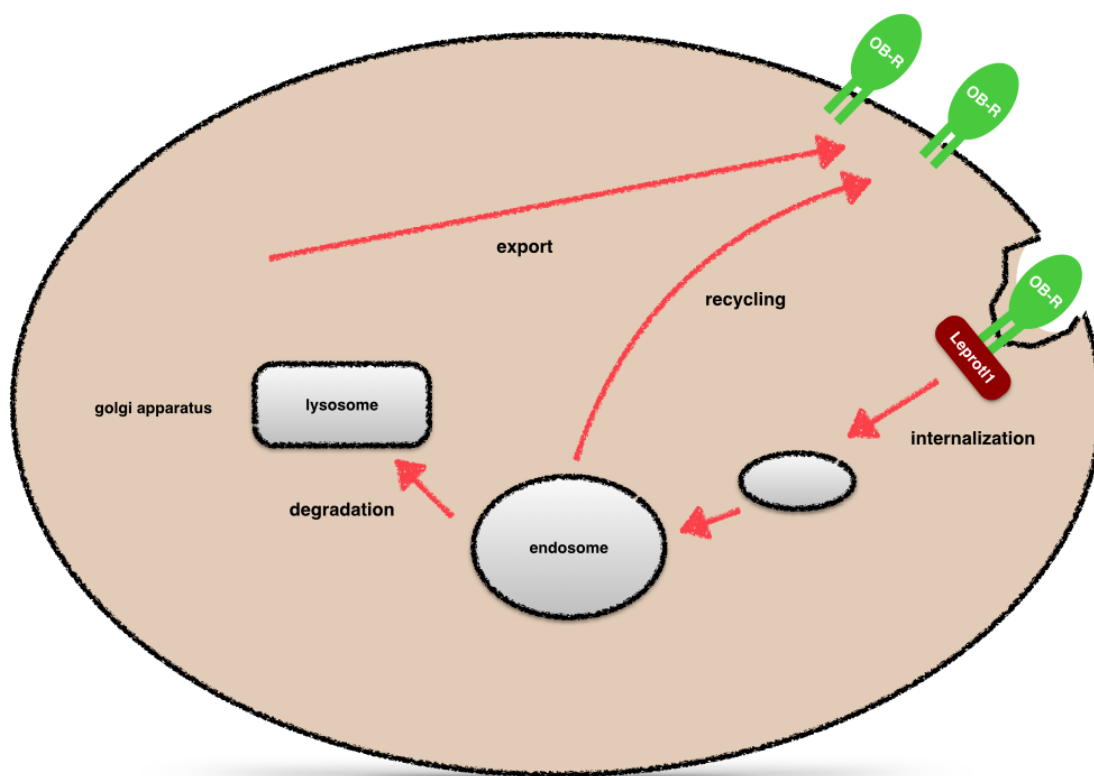


Figure 13 Effect of Leprotl1 on leptin receptor localization

Leprotl1 has role in post internalization step of leptin receptor endocytosis. Upon endocytosis, leptin receptor is either degraded in lysosomes or recycled back to cell membrane.

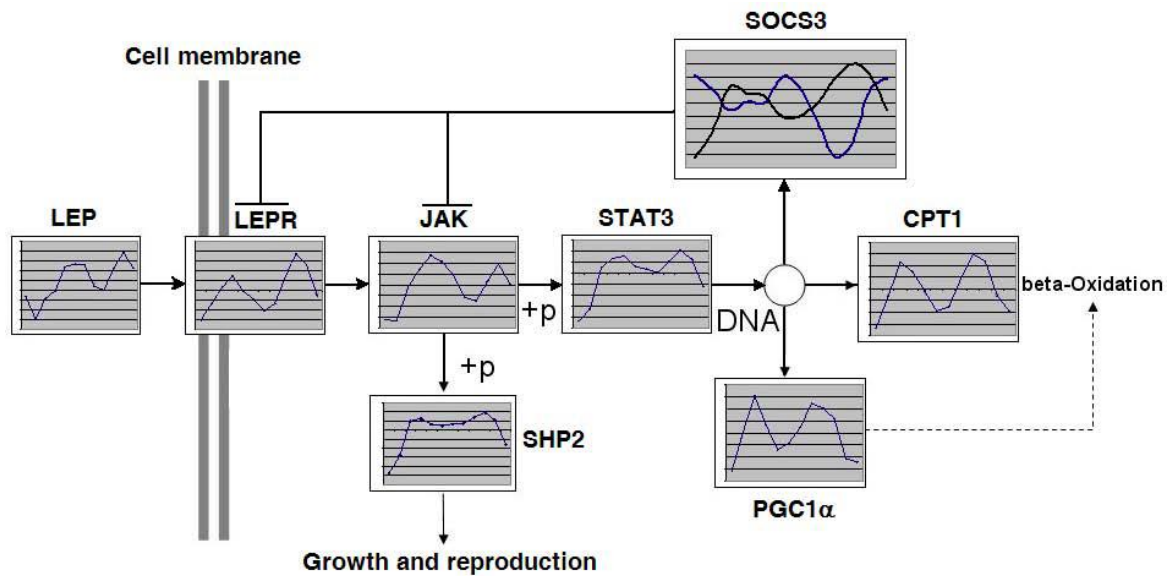


Figure 14 Oscillation of Leptin signaling components' transcripts

KEGG pathway analysis of leptin signaling and the expression levels of leptin signaling components. The transcripts were collected for two days. As can be deduced from the figure, all of the components show daily rhythmic expression whereas SOCS3 as the negative regulator of the system shows 2 transcripts oscillating in counter phase. (LEP: leptin, LEPR: leptin receptor). Retrieved from ²²³.

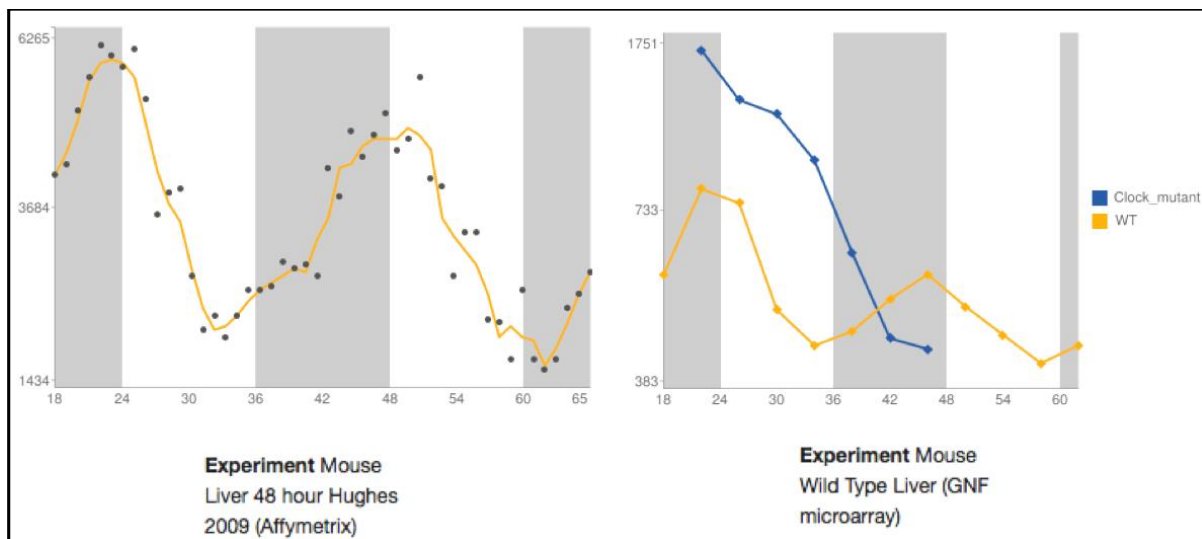


Figure 15 Leprotl1 transcript oscillation in liver of mice from CircaDB .

As can be deduced from the left graph, **LeprotL1** transcript level oscillates with approximately 6 fold change. On the other hand, in Clock knock out mouse oscillating profile is lost (right graph).

CHAPTER 3: MATERIALS AND METHODS

3.1 Mammalian Two-Hybrid System

HEK293T cells were split into 10cm plate 1 day before the transfection. As the cells reach to 70-80% confluency, reverse transfection is made with TurboFect Transfection Reagent (Thermo Scientific). Reaction mixture is prepared as 50ng *LeptotL1*-pACT, 50ng either one of the clones of Full Length-T1-T2-T3-T4-T5-T6-T7 mCRY1 pBIND or mCRY2-pBIND, 50ng of pG5-Luc, 1ng of PGL4 (renilla luciferase) and finally with empty pCMV-sport6 vector up to 300ng in 50μL of DMEM. Per reaction 0,9 μL of TurboFect is added and the reaction mixture is incubated at room temperature for 20 minutes. Each reaction is made as triplicates. Meanwhile cells are trypsinized (GIBCO-Life Technologies) for 1 minute and re-suspended in 2X Medium (DMEM supplemented with 20% fetal bovine serum, 4mM L-Glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin). Per well 40.000 cells are seeded in 96 well plates. After 20 minutes of incubation reaction mixture is put on seeded cells. 24 hours after transfection, luciferase activity is estimated via Dual-Glo Luciferase Assay System kit (Promega). Luciferase activity is measured with Fluoroskan Ascent FL (Thermo Scientific, USA) after 5 minutes of 75μL Dual-Glo® Luciferase Reagent addition. After taking few measurements, on each well 75μL Dual-Glo® Stop & Glo® Reagent is added to estimate the renilla luciferase activity. After addition of Dual-Glo® Stop & Glo® Reagent, cells are incubated for 10 minutes to let buffer quench previous luciferase activity. Then up to 3 measurements are taken by Fluoroskan Ascent FL (Thermo Scientific, USA). Obtained luciferase activity values were normalized with renilla and background negative control values.

3.2 Chemically Competent DH5α Preparation

2 days before competent cell preparation, DH5α cells from -80°C stock are streaked on no-antibiotic LB-agar plate. Next day, single colony is picked and inoculated into 5mL LB medium without antibiotics. 1 day before the preparation, required buffers are prepared and autoclaved. These buffers are: 500 mL of Buffer I

(80mM CaCl_2 + 50mM MgCl_2) and 50mL of Buffer II (10mM PIPES + 15% Glycerol + 60mM CaCl_2). Also, clean eppendorf tubes, centrifugation tubes and 500mL LB are autoclaved 1 day ago. In the morning, grown 5mL DH5 α cells are inoculated into 500mL LB with 1:100 dilution. Cell suspension is incubated at 37°C for 3-4 hours until the OD reaches 0.9 at 600nm. Afterwards, cells are incubated on ice for 20 minutes. Cells are centrifuged at 4°C for 15 minutes at 4000rpm. After the removal of supernatant, cells are dissolved in 100mL Buffer I on ice. Again cells are centrifuged and this step is repeated with 25mL Buffer I. Next, cells are suspended in 7mL Buffer II on ice, and then 3mL of 80% glycerol is added. Then cells are aliquoted in eppendorf tubes as 50-100 μL . In order to understand if the cells became competent, their transformation efficiency is estimated. To make sure there is no contamination, they are streaked on LB agar plates with different antibiotics.

3.3 Transformation into Chemically Competent DH5 α

Both 50 μL DH5 α cell from -80°C stock and construct to be transformed are incubated on ice for 15-20 minutes. Then 50ng of the construct is put in 50 μL DH5 α . Mixture is incubated on ice for 10 minutes. Cells are put at 42°C for 45 seconds to generate heat shock. After heat shock, cells are incubated on ice for 5 minutes. Fresh 1mL LB medium without antibiotic is put on cells. Cells are grown at 37°C for one hour on shaker. Desired amount of cells are spread on LB-agar supplemented with required antibiotic.

3.4 Polymerase Chain Reaction (PCR)

Truncation-5 (T5) and Truncation-6 (T6) forms of mCry1 are cloned in to pcDNA4/myc-His A vector. For both constructs, forward primer contains EcoRV and reverse primer has NotI restriction cut site. Constructs were amplified by polymerase chain reaction which uses 50ng of Full Length mCry1-pBIND plasmid as template. In 50 μL reaction volume, 10 μL of 5X Phusion HF Buffer (Thermo Fisher Scientific-Life Technologies), 1 μL of 10mM dNTP mix, 2.5 μL of 10 μM forward primer, 2.5 μL of 10 μM reverse primer, 0.5 μL of Phusion High Fidelity Enzyme (2U/1 μL) and 32.5 μL

MilliQ water is put. For the cloning of T5-mCry1, mixture is supplemented with 1 μ L DMSO. The polymerization protocol is made as: 94°C- 30 seconds, 55°C- 30 seconds, 72°C 90 seconds with 30 cycles. PCR products were run on 0,8% agarose gel, and isolated with QIAquick Gel Extraction Kit (QIAGEN).

3.5 Sub-cloning into pGEM-T-Easy vector

Both PCR products of T5-T6-mCry1 are TA cloned into pGEM-T-Easy vector (promega). To add TA overhangs, 30 μ L gel extracted PCR product is put in 3.5 μ L 10X DreamTaq Buffer (Life Technologies), 0.2 μ L DreamTaq and 1 μ L of 10mM dNTP mix. Reaction mixture is incubated for 30 minutes at 72°C. Then, PCR purification is made by Gene JET PCR Purification Kit (Life Technologies). Afterwards, ligation reaction is performed as follows: 5 μ L of 2X Rapid Ligation Buffer, 1 μ L of 50ng/1 μ L pGEM-T-Easy Vector, 3 μ L of PCR product (depends on the concentration of it), and 1 μ L of T4 ligase. All of the components of the ligation reaction belong to Promega pGEM®-T Easy Vector Systems. Reaction mixture is incubated at room temperature for 1 hour. 4 μ L of ligation product is transformed into chemically competent 100 μ L DH5 α . Transformed cells are plated on X-Gal/IPTG plates for Blue-white selection. For the preparation of X-Gal/IPTG plates, per plate 100 μ L of 0.1M of IPTG (dissolved in water) and 20 μ L of 50mg/mL X-Gal (dissolved in DMSO) is spread. Then plates are left in 37°C incubator for drying. Afterwards, transformed DH5 α cells are spread. Blue colonies represent the ones without insert, whereas white colonies represent the transformed ones with insert. White colonies are inoculated into 5mL LB medium supplemented with 100 μ g/mL ampicillin for further experiment.

3.6 Gene Cloning into Target Vector

Positive T5 and T6 mCry1-pGEM-T-Easy plasmids and empty pcDNA4/myc-His A are restriction digested first with EcoRV in reaction mixture as follows: 8 μ L 10X FastDigest Buffer (Life Technologies), 4 μ g of target plasmid, 2 μ L of FastDigest Eco321 (FD-EcoRV), up to 80 μ L MilliQ water. Reaction mixture is incubated at 37°C

for 2-3 hours. Cut products are isolated with Gene JET PCR Purification Kit (Life Technologies). Concentrations of purified constructs are estimated by Thermo Scientific NanoDrop™ ND-2000 1-position Spectrophotometer.

Purified constructs are then cut with NotI restriction enzyme in reaction mixture as follows: 6μL of Buffer O (Life Technologies), all elute of purified template DNA, 2μL NotI (Life Technologies) and up to 60μL MilliQ water. Reaction mixture is incubated at 37°C for 4 hours. In last 30 minutes, 1μL FastAP Thermosensitive Alkaline Phosphatase (Life Technologies) is added to pcDNA4/myc-His A reaction mix. Then product is run on 0.8% gel and gel extraction is performed on respective bands by QIAquick Gel Extraction Kit (QIAGEN). Concentrations of purified constructs are estimated by Thermo Scientific NanoDrop™ ND-2000 1-position Spectrophotometer.

For the ligation reaction 100ng of EcoRV-NotI digested pcDNA4/myc-His A, 90ng T5 or T6 mCry1 insert, 4μL of 5X FastLigase Buffer (Life Technologies) and 1μL T4-FastLigase is mixed and incubated at room temperature for 8-10 minutes. 5μL of the mixture is transformed into 100μL DH5α cells. Cells are spread over LB-agar plates supplemented with 100μg/mL ampicillin. Few colonies are inoculated and miniprep is done with GeneJET Plasmid Miniprep Kit (Life Technologies). Isolated constructs are cut with BamHI which cuts each of T5-T6 insert and pcDNA4/myc-HisA vector 1 time for diagnostic purposes. The ones that gave correct size are sent for sequencing.

3.7 Cell Culture Maintenance

In tissue culture experiments, three types of cells are used. These are NIH3T3, U2OS, and HEK293T cells. All of the cells are grown in 1X Medium supplemented with Dulbecco's modified Eagle's Medium (DMEM, Gibco), 10% FBS (Gibco), 100 μg/ml streptomycin and 100 μg/ml penicillin (Gibco), and 2 mM L-Glutamine (Gibco). Cells are grown in humidified 37°C, 5% CO₂ incubator. Before each passage, medium is aspirated and then washed twice by 5mL 1X Phosphate Buffered Saline

(PBS). Cells are detached by 1mL Trypsin-EDTA (Gibco) and resuspended by addition of 4mL-1X medium. 1mL of resuspended cell is poured over 9mL of 1X medium in fresh 10cm plates (Nest-ABC Scientific).

3.8 DNA Transfection and Western Blot Analysis

24 hours prior to transfection, 200,000 HEK293T cells are seeded in 6 well plates (Nest-ABC Scientific). Per well 2µg DNA is transfected. Reaction mixture is prepared as follows: 2µg DNA (either T5 or T6 mCry1- pcDNA4/myc-His A or LeprotL1-pCMV-Sport6), 4µL of TurboFect Transfection Reagent (Life Technologies) in 200µL Dulbecco's modified Eagle's Medium (DMEM, Gibco). Mixture is incubated at room temperature for 20 minutes and poured homogeneously over 6-well plate. 40 hours after transfection, cells per well are collected with 1.5mL pre-chilled 1X PBS. Collected cells are precipitated with centrifuge at 3000rpm for 10 minutes at 4°C. Supernatant is taken off while cell pellet remained intact.

For expression check, collected cell pellet is dissolved in 100µL MilliQ water by pipetting up and down. Afterwards, 20µL of 6X protein loading dye is added and mixture is boiled at 95°C for 10 minutes. Immediately after boiling cell lysates are put on ice and incubated on ice for 5 minutes. Lysates are centrifuged at 14200 rpm for 10 minutes. From supernatant, 10-20 µL is loaded to SDS-PAGE gel with respective percentage. For mCry1-HIS tagged protein with 69kD weight, 8% acrylamide gel is used whereas for LeprotL1-FLAG tagged protein with 15kD weight, 15% acrylamide gel is used. Afterwards proteins on gel are transferred to 0.45 µm pore size Hydrophobic PVDF Transfer Membrane (Merck-Millipore) via wet transfer technique. PVDF membrane is blocked with 2.5% fat free milk powder for 1 hour, and then incubated with primary antibody at room temperature for 1 hour. For mCry1-HIS tagged protein, Anti-6X His tag® antibody (HRP) (ab1187) antibody is used whereas for Flag tagged LeprotL1 protein Monoclonal ANTI-FLAG® M2-Alkaline Phosphatase antibody produced in mouse (Sigma) is used. Membranes are washed 3 times with 0.1% TritonX-TBS for 10 minutes. For HRP visualization ECL buffer

system is used. For AP visualization 1-Step™ NBT/BCIP Substrate Solution (Life Sciences) is used.

3.9 Co-immunoprecipitation Assay

For Co-IP assay, for 1 well of Full-Length or T5 or T6 mCry1- pcDNA4/myc-His A transfection, 2 wells of LeprotL1-pCMV-Sport6 transfection is made in 6 well plates with 2µg of DNA per well. After 40 hours of incubation, cells are collected as explained in DNA transfection and Western Blot Analysis section. Precipitated cells are lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 450 mM NaCl, %1 NP-40, 1mM EDTA, 1mMNaF, 1 mM Na3VO4) supplemented with Protease Inhibitor Cocktail, DDT and PMSF. The lysates are rotated for 45 minutes at 4°C. Meanwhile, ANTI-FLAG ® M2 Affinity Gel (Sigma) is equilibrated. Per reaction 20µL of ANTI-FLAG ® M2 Affinity Gel is used. Affinity gel is washed twice with ice-cold 1mL TBS-200 and twice with ice-cold 1mL Lysis Buffer (without PIC, DDT, and PMSF). While washing, affinity gel is centrifuged at 4°C, 3000rpm for 8 minutes. Cell lysates are centrifuged at 14.200rpm for 30 minutes at 4°C. From cell free extract, 10% of volume is separated as input. Cell free extracts are mixed with affinity gel and rotated at 4°C overnight. Mixture is centrifuged at 4°C for 8 minutes at 5000rpm. Supernatant is saved as flow through. Affinity gel pellet is washed 3 times with TBS-0.1mM CaCl₂. Then affinity gel is dissolved in 20µL of MilliQ water and load on gel. Western blot analysis is performed as explained in DNA Transfection and Western Blot Analysis section.

3.10 siRNA Transfection

24 hours before the siRNA transfection, 200,000 either U2OS or NIH3T3 cells are seeded into 6 well plates with siRNA medium supplemented with Dulbecco's modified Eagle's Medium (DMEM, Gibco), 10% FBS (Gibco), and 2 mM L-Glutamine (Gibco). siRNA mix contains four types of siRNAs whose sequences are given as in Appendix 1?? Each siRNA is 10 µM in their stocks, which are diluted to 2µM in a mixture. 6µL of siRNA mix is dissolved in 250µL Opti-mem (Life

Technologies) while 5 μ L of Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies) is dissolved in 250 μ L Opti-mem. After 5 minutes incubation of the mixes at room temperature, the solution are mixed into same tube and incubated for another 20 minutes at room temperature. Then the mix is added on wells dropwise. 2 days after transfection, cells are used for subsequent experiments such as RNA isolation.

3.11 RNA isolation

RNA extraction is performed from 6 well plates. 1mL of Trizol reagent (peqGOLD Trifast) per well is used to lyse and extract nucleic acids of cells. After incubation for 15 minutes at room temperature, 200 μ L chloroform is added to mixture and the mixture is incubated at room temperature for 5 minutes. The solution is then centrifuged at 12.000 rpm for 15 minutes at 4°C. On ice, upper phase is mixed with pre-chilled 100% isopropanol. The mix is incubated on ice for 15 minutes and centrifuged at 4°C for 10 minutes at 14.000rpm. After precipitating the RNA, isopropanol on the upper phase is poured away. On ice 1mL 75% EtOH is added gently in order to wash to pellet following centrifugation at 4°C, 14.000rpm for 5 minutes. The EtOH is poured and RNA is air-dried. With 50 μ L DNase RNase free water (DEPC water), RNA is dissolved. To analyze the quality of the isolated RNA, 8 μ L of the solution is run on 1% agarose gel. With nanodrop2000 (Thermo Scientific) the concentration of the RNA mix is estimated. Isolated RNA then is treated with DNase I (Thermo Scientific). For DNaseI treatment, 1 μ g of RNA is supplemented with 1 μ L 10X DNase reaction buffer with MgCl₂, 1 μ L of DNase I and upto 10 μ L with DEPC-treated water. After the incubation of reaction mixture at 37°C for 30 minutes, 1 μ L EDTA (Thermo Scientific) is added and incubated at 65°C for 10 minutes in order to inhibit further DNase I activity. On the mix, 1 μ L of Oligo(dT)₁₈ (Thermo Scientific) is added and incubated at 65°C for 10 minutes. For the synthesis of cDNA, RevertAid RT Reverse Transcription Kit (Thermo scientific) is used. The reaction mixture is prepared as follows: 4 μ L of 5X Reverse Transcription Buffer, 10 μ L of 10mM dNTP mix, 1 μ L of Ribulock RNase inhibitor, 1 μ L of Revert Aid Reverse

Transcriptase and 1 μ L of DEPC treated water. The mix is then incubated at 42°C for 1 hours and then 70°C for 15 minutes. Obtained cDNA is diluted with 80 μ L DNase RNase free water for subsequent reactions.

3.12 RT-qPCR Analysis (Real Time – quantitative Polymerase Chain Reaction)

For qPCR analysis TIANGEN SuperReal PreMix SYBR Green is used. qPCR analysis is performed in reaction mixture with ingredients as follows: 10 μ L of 2X SYBR Green, 2 μ L of primer mix (5pmole of both forward and reverse primers), 2 μ L of template cDNA and 6 μ L of MilliQ water. List of the primer sequences can be found in appendix 1. The cycling protocol is performed on LightCycler 2.0 Instrument (Roche). The cycling protocol is performed as 95°C 10 seconds denaturation, 57 to 62°C for 20 seconds annealing (the degree is determined by primer type) and 72°C for 30 seconds of elongation. The quality of the PCR product is analyzed by T_m curves and agarose gel imaging. Normalization of the values is made with GAPDH values.

3.13 Bioluminescence Analysis

Prior to siRNA transfection, 200,000 U2OS cells were seeded on 35mm cell culture plates. 24 hours after seeding when the confluency of the cells reach 60-80%, siRNA transfection is made as mentioned previously. 48 hours after siRNA transfection, cell medium is changed with 1X medium (DMEM, 10% FBS, 100 μ g/ml streptomycin and 100 μ g/ml penicillin and 2 mM L-Glutamine) supplemented with 0.1 μ M Dexamethasone (Sigma-Aldrich). After 2 hours of incubation at 37°C, medium is changed with 2mL Lumicycle Medium. 1L lumicycle medium contains DMEM powder (sigma D-2902, 10X 1L), 0.35gr sodium bi-carbonate (tissue culture grade, sigma S5761), 3.5gr D(+) glucose powder (tissue culture grade, sigma G7021), 10mL 1M HEPES buffer (Gibco 15140-122), 2.5 mL Pen/Strep, 50mL 5% FBS and upto 1L sterile milliQ water. Luciferin is added freshly with 0.1mM concentration. After medium change, in order to eliminate air passage, perimeters of the plate caps are

coated with autoclaved grease oil. Meanwhile, it is important to eliminate light exposure of the plates. Plates are placed in LumiCycle 32 incubator (ActiMetrics). For 8 days, luminometric measures are taken. For each sample type, plates are prepared as triplicates. One of the plates are used for RNA isolation and validation of siRNA knock down as described previously. The other plates are used for bioluminescence analysis. The analysis of the results were made by LumiCycle Data Analysis Software (ActiMetrics).

3.14 Bi-molecular Fluorescence Complementation Analysis

Cry1-VC155 vector clone was already generated by Yool Lee in University of Pennsylvania. LeprotL1 was cloned into BiFC-VN173 vector as described previously by restriction digestion with EcoRI and KpnI enzymes. The list of the primers used can be found in appendix 1. 40,000 of U2OS cells were seeded on coverslips in 24 well plates 24 hours before the transfection. 500ng of each Cry1-VC155, LeprotL1-VN173, mCh-Sec61b beta clones were co-transfected with FuGene HD Transfection reagent (Promega). mCh-Sec61 beta is an endoplasmic reticulum marker whose localization marks ER with red fluorescent color. The transfection is made by mixing the DNAs in 100 μ L DMEM with 4.5 μ L FuGene Transfection Reagent. 36 hours post-transfection cells are prepared for confocal imaging. Each well is gently washed twice with 1X PBS buffer for 3 minutes. The coverslips are then placed on petri-dish to eliminate mechanical distortions. Cells are fixed by 100 μ L 4% paraformaldehyde for 10-12 minutes. Then coverslips are washed three times with 100 μ L of 1X PBS supplemented with 0.1% Triton-X for 5 minutes. 300 μ L of 300ng/ μ L DAPI is put on cover slips and incubated for 15 minutes at dark. Coverslips are washed with 1X PBS supplemented with 0.1% Triton-X twice and only 1X PBS for one time. Coverslips are placed on glass slides with mounting media and dried. Imaging is made by confocal microscope (Nikon, 90i).

CHAPTER 4: RESULTS

4.1 Mammalian Two Hybrid Assays

Mammalian two-hybrid assay is a method based on quantification of the interaction between two different proteins. Because of its cost effective results for screening purposes it is widely used. The assay uses two different fusion proteins in which one of the candidates is fused to transcriptional activator domain and the other candidate is fused to a DNA binding domain. The candidate genes were cloned into 2 different vectors named as pACT and pBIND (CheckMate™ Mammalian Two-Hybrid System / Promega).

The pACT vector encodes a transcriptional activator protein named herpes simplex virus VP16. On the other hand, pBIND vector is designed as it contains a yeast GAL4 DNA binding domain. Whenever candidate genes are properly cloned into these vectors, fusion proteins are formed respectively. The reporter is a firefly luciferase gene whose promoter region contain GAL4 binding site. This reporter system is encoded within PG5luc vector. Basically, if there is an interaction between the two candidate genes, their fused partners GAL4 and VP16 comes in close proximity. Hence, RNA polymerase II is recruited to TATA box that is in the promoter of luciferase gene. Consequently, transcriptional expression of luciferase gene increases yielding increased firefly luciferase protein production. In order to eliminate the variances in transfection efficiency, another vector named PGL4 encoding a renilla reniformis luciferase gene under SV40 promoter control is used. Independent from the interaction between the two candidate genes, renilla luciferase gene is transcribed constitutively due to strong SV40 promoter. Renilla luciferase's quantified activity is used for normalization of firefly luciferase activity, so the variances in transfection efficiency and also the changes that are originated from altered cellular viability are attenuated (Figure 16).

24 hours after the transfection of HEK293T cells, the firefly luciferase activity is quantified by Dual-Glo® Luciferase Assay System. The easiness of the systems

comes from the usage of both renilla and firefly luciferase substrates on the same well. The first buffer added causes immediate cell lysis followed by luminescent recording of firefly luciferase activity. After taking the record of luminescent activity of firefly luciferase, second buffer that has the ability to quench firefly luciferase activity is used. The second buffer contains the renilla luciferase substrate.

From the previous work done in our laboratory, Gulsum Elif Muku showed that among the several core clock components, LeprotL1 interacts with mCry1/2 (mouse cryptochrome 1 and 2). In her work, she cloned already identified core clock components into pBIND vector, and 52 candidate clock components into pACT vector (Figure 16). In the screen, LeprotL1 was one of the hits significantly interacting with mCry1 and mCry2 (Figure 17A). Based on this work, I confirmed these interactions via mammalian two-hybrid system with renilla luciferase activity normalization. In the assay, mPer2-pBIND is used as positive control since it is already known that it strongly interacts with mCry1 and mCry2 (Figure 17).

In order to analyze the interaction between mCry1 and LeprotL1 further, we performed another mammalian two hybrid assay that composed of C-terminal truncated versions of mCry1. In our laboratory by Dr. Hande Asimgil, truncated versions of mCry1 gene were cloned into pBIND vector. These truncations are designed such that in each step 20 amino acids are deleted from C-terminus of mCry1 (Figure 18 A/B). Based on the results of this assay, the interaction between mCry1 and LeprotL1 is completely diminished in truncation 6 region (T6) where 120 amino acids from C-terminus region are deleted (Figure 18C).

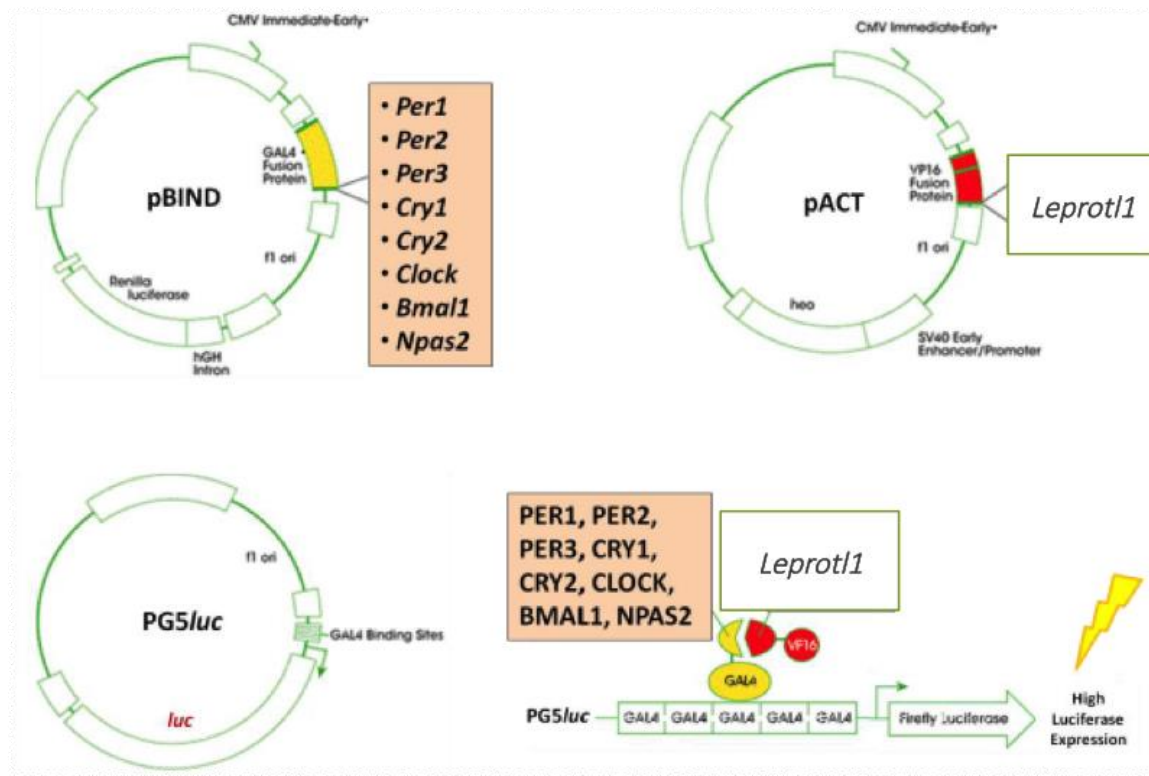


Figure 16 Components of mammalian two-hybrid assay

Core clock components were already cloned into pBIND vector encoding GAL4 DNA binding domain protein. *Leprotl1* was cloned into pACT vector encoding fused VP16 transcriptional activating domain. Another construct containing luciferase gene with GAL4 binding site is also used as reporter system.

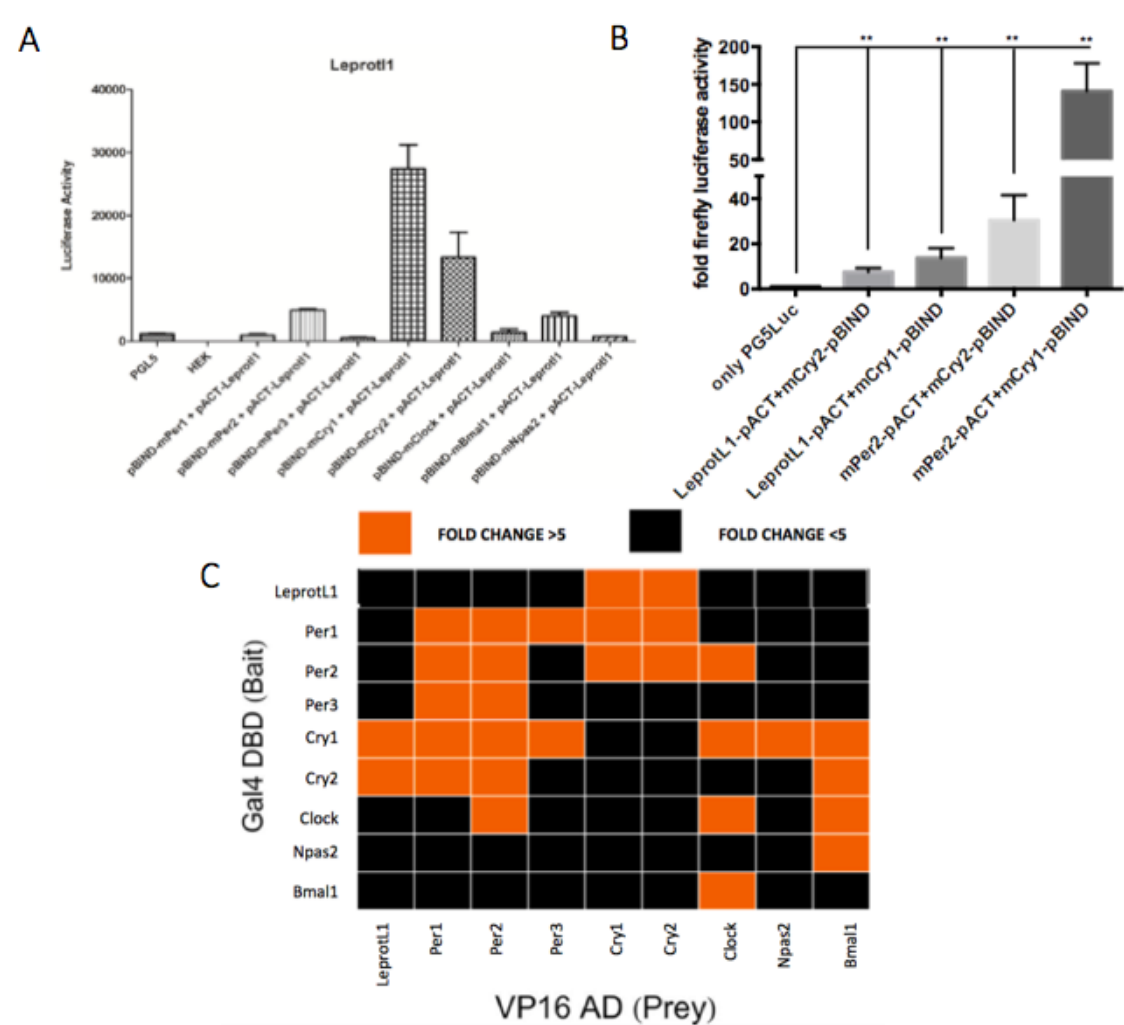


Figure 17 Mammalian two-hybrid assay results

A) Luciferase assay performed by Gulsum Elif Muku showed that Leprotl1 interacts with Cry1 and Cry2 among other clock components. B) This interaction is validated by also renilla activity normalization. C) Figure shows the fold changes in luciferase activities with different clock components' combinations.

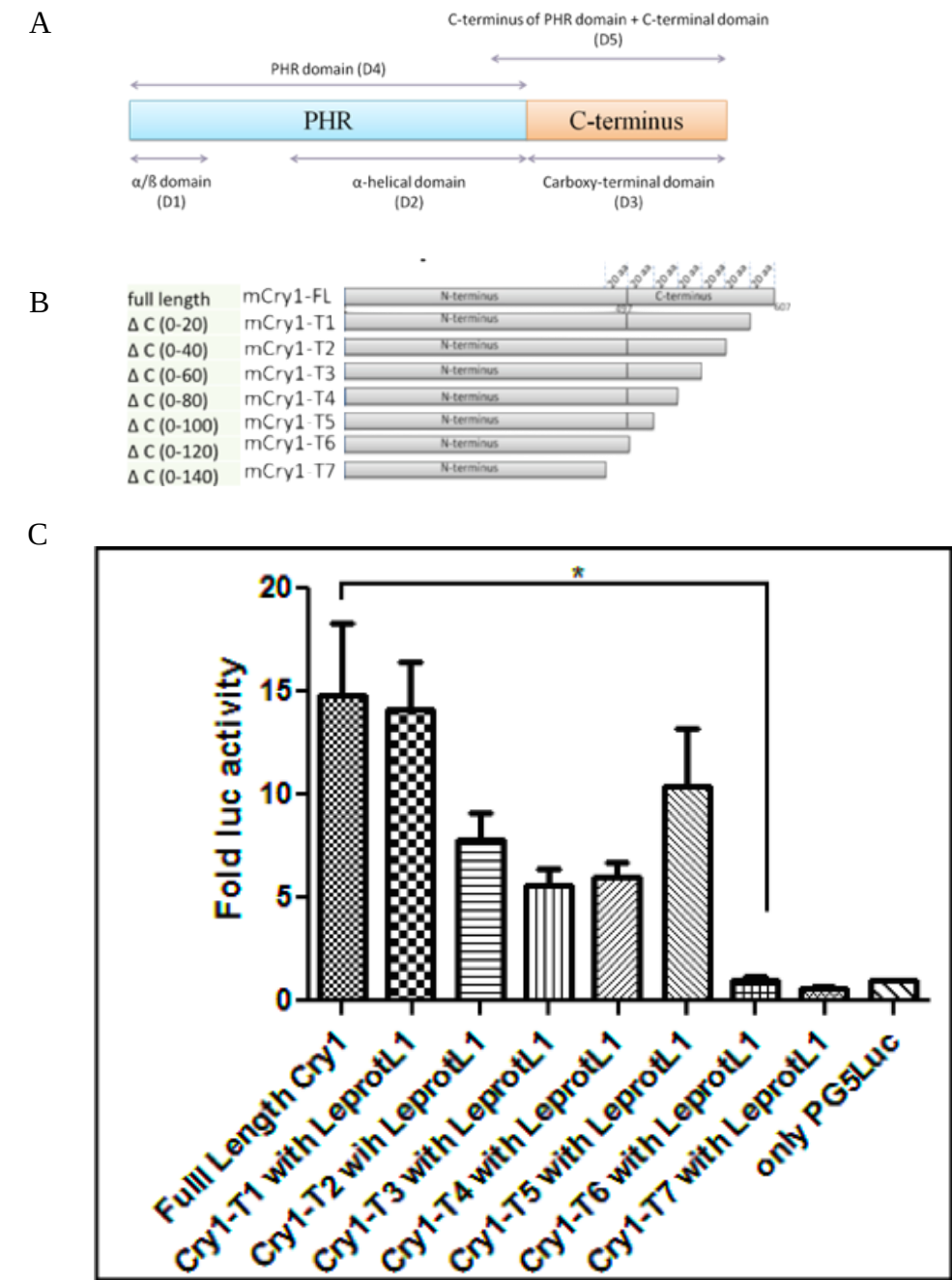


Figure 18 Identification of CRY1 domain interacting with LEPROTL1

A) domains of mouse Cry1 protein. B) C-terminus truncated versions of mCry1 cloned into pBIND vector. C) Mammalian two-hybrid results show that at T6 region interaction is completely lost. Values are normalized with renilla activity. ($p < 0.05$)

The T6 region of mCry1 where the interaction is cut encodes an α -helix named as α -22. This region is highly conserved between mCry1 and mCry2. α -22 is important for both mCry1 and mCry2's interaction with mPer2 and Bmal1 protein. In the laboratory, we already had the mutant versions of mCry2 carrying point mutations on 501st and 503rd amino acids (corresponding to α -22 region) in pBIND vector. Mammalian two-hybrid assay confirmed that α -22 region of mCry2 which is also conserved in T6 region of mCry1 is important for mCry2 and LeprotL1 interaction (Figure 19).

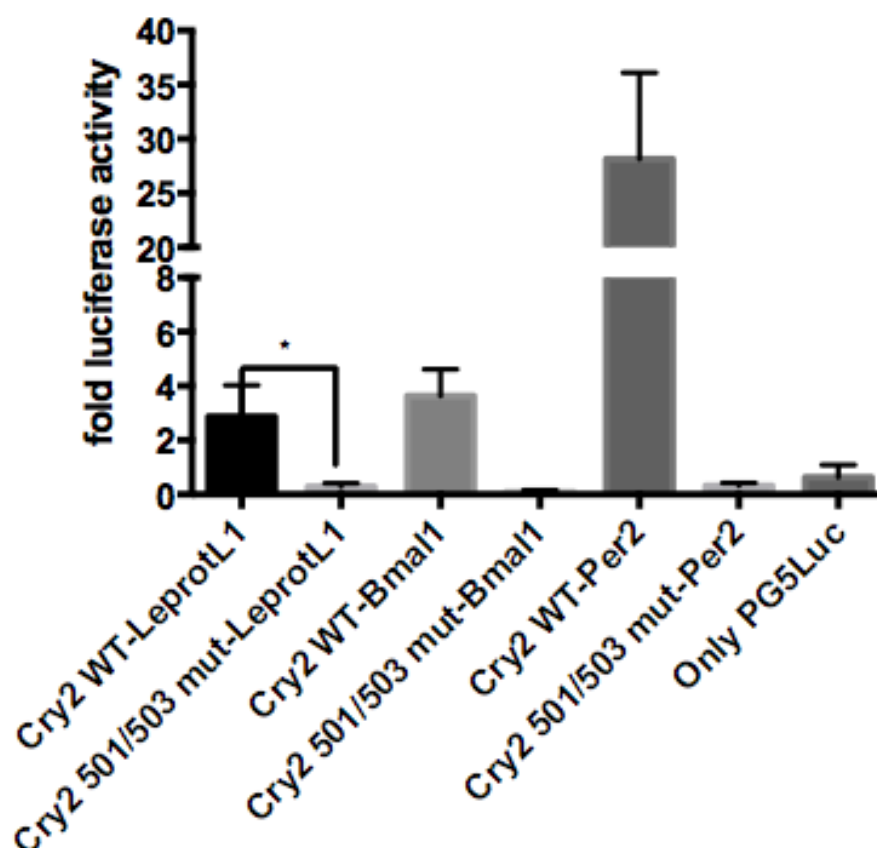


Figure 19 Interaction between LEPROTL1 and CRY2 WT/501mut/503mut

The wt-mCry2 shows luciferase activity indicating the presence of an interaction. However, the mutant mCry2 doesn't show luciferase activity. Values are normalized with renilla activity. (p<0.05)

4.2 Cloning for Co-immunoprecipitation Assay

In order to validate the interaction between LeprotL1 and mCry1, co-immunoprecipitation assay was performed. Gulsum Elif Muku already cloned LeprotL1 into pCMV-sport6 vector. In order to over express mCry1-T5 and mCry1-T6 protein, these genes were subcloned first into pGem-t-Easy vector (Promega) and then cloned into pcDNA 4/myc-His A vector. With proper forward and reverse primers, T5 and T6 versions of mCry1 were amplified from full-length mCry1-pcDNA vector by PCR (polymerase chain reaction). Agarose gel photos of the PCR products can be seen in Appendix 3. The length of full length-mCry1 is 1821 bp, T5-mCry1 is 1521 and T6-mCry1 is 1461 bp. Whole PCR product was run on 0.8% agarose gel following gel extraction. Designed forward and reverse primers contain EcoRV and NotI restriction cut sites respectively. The inserts are then TA cloned into pGem-t-Easy vector. Double digestion of empty pcDNA 4/myc-His A vector and pGem-t-Easy vector containing the insert is performed. Digested pcDNA 4/myc-His A vector was treated with alkaline phosphatase to avoid self-ligation. With T4 ligase, T5 and T6 mCry1 were cloned into pcDNA 4/myc-His A vector. Ligation product was transformed into competent DH5 α strain, and plated on ampicillin plates. Selected colonies were grown in LB medium overnight, and via mini-prep kit plasmid isolation was performed. Positive colonies were selected by diagnostic cut with NotI and EcoRV. Agarose gel results confirmed that desired genes were cloned into target vector.

4.3 Over Expression of T5/T6 mCry1- pcDNA 4/myc-His A Clones

Clones of T5/T6 mCry1-pcDNA have HIS tag on their C-terminus. Constructs were transfected into HEK293T cells on 6 well plate 40 hours after transfection, cells were collected and lysed by 6X protein loading dye following 5 minutes boiling at 95 °C. After centrifugation, soluble part was run on 10% SDS-PAGE gel, and then transferred to PVDF membrane. Subsequently, 1 hour of 5% milk blocking and 45 minutes of anti-HIS antibody incubation is performed. Since anti-HIS was already HRP (horseradish peroxidase) conjugated, secondary antibody is not used.

The expected molecular weight of full length-mCry1, T5, and T6 clones were 69kDa, 60kDa and 58kDa.

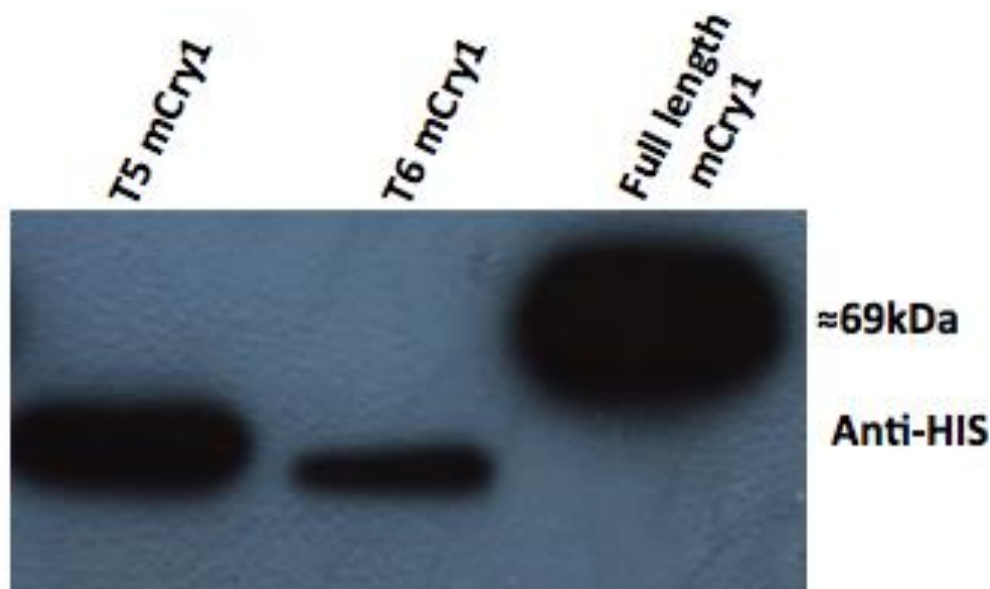


Figure 20 Overexpression of FL/T5/T6 forms of CRY1

Western Blot analysis of overexpressed FL/T5 and T6 mCry1 proteins. The molecular weight of FL mCry1 is around 69kDa, whereas that of T5 and T6 mCry1 are around 58 and 60kDa respectively.

4.4 Co-immunoprecipitation Assay

With the use of mCry1 clones, co-immunoprecipitation with anti-FLAG agarose microbeads is performed. LeprotL1-pCMVsport6 which was already cloned by Gulsum Elif Muku encodes LeprotL1 with FLAG tag on its C-terminus. Co-transfection of LeprotL1-pCMVsport with full length, T5 or T6 mCry1-pcDNA separately, or transfection of full length, T5 or T6 mCry1-pcDNA alone into HEK293T cells were done on 6 well plates. Anti-FLAG micro beads bind specifically to FLAG tagged leprotL1 in total cell lysate. Meanwhile, if there is also an interaction between different mCry1 forms, it is expected to see the precipitation of mCry1 forms with anti-FLAG micro beads. After performing optimized washing step, which removes the unbound proteins in total cell lysate, western blot analysis is performed. The washing step is critical since if the wash buffer is too stringent the present

interaction get lost. Also, if the stringency of the buffer is too weak, nonspecific binding of other proteins can decrease the quality of western blot. After few optimizations, 1mM CaCl₂ (Calcium chloride) in TBS (Tris-buffered saline) buffer gave best result. Co-immunoprecipitation results validated the mammalian two hybrid results as expected. Full length and T5 mCry1-HIS co-immunoprecipitated with LeprotL1-FLAG, though T6 mCry1-HIS did not despite its expression is higher than T5 mCry1 as seen in input part (Figure 24).

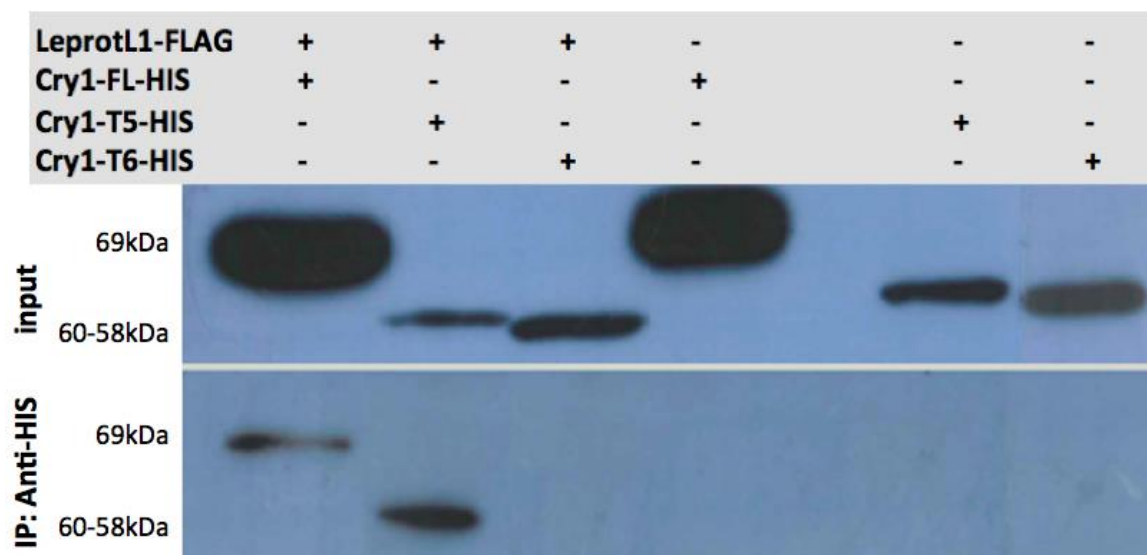


Figure 21 Co-immunoprecipitation western blot analysis

As can be detected from the figure, immunoprecipitation of LeprotL1 pulls down FL and T5 mCry1 but not T6 mCry1. Western blot is performed with anti-HIS-HRP.

4.5 BiFC (bi-molecular fluorescent complementation) system

BiFC is a cell biology method simply aiming to understand the localization of interacting proteins. Basically, system works with 2 plasmids encoding 2 different domains of Venus green fluorescent protein. With the proper cloning of two candidate genes into BiFC vectors, whose interaction is going to be investigated, fluorescent complementation is observed. Two vectors named pBiFC-VC155 and BiFC-VN173 were used whose vector maps are shown in Figure 22.

mCry1 gene was already cloned into VC155 vector by Yool Lee from University of Pennsylvania. LeprotL1 was cloned into VN173 vector with EcoRI and KpnI enzymes with the cloning protocol as mentioned before (Appendix 3). Both of the vectors encode the Venus GFP part (either N or C-terminus of Venus GFP) on C-terminus of the cloned gene. If there is an interaction between cloned genes, the N-terminus and C-terminus of Venus protein comes into close proximity giving fluorescence signal.

BiFC is performed on U2OS cell line, which shows robust circadian rhythmicity. 24 hours after transfection, cells are fixed on glass slides via 4% PFA (paraformaldehyde) following DAPI staining. Sac61b-RFP is used as an ER (endoplasmic reticulum) marker. The confocal imaging results can be seen in Figure 23. The results indicated that LeprotL1 and mCry1 interact both in nucleus and ER.

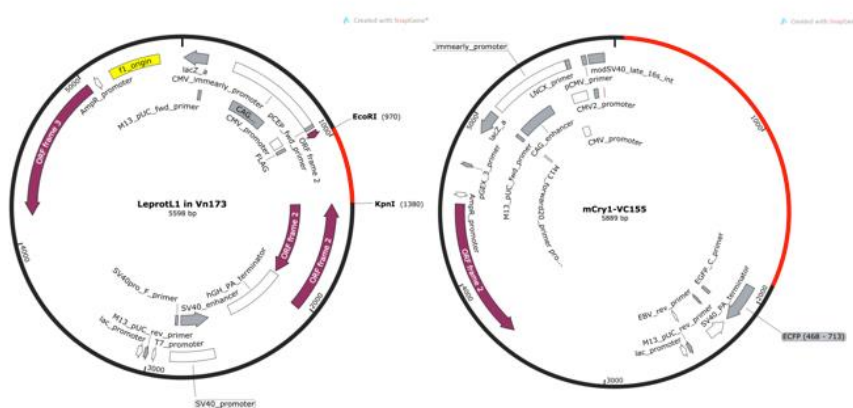


Figure 22 Cloned BiFC vectors

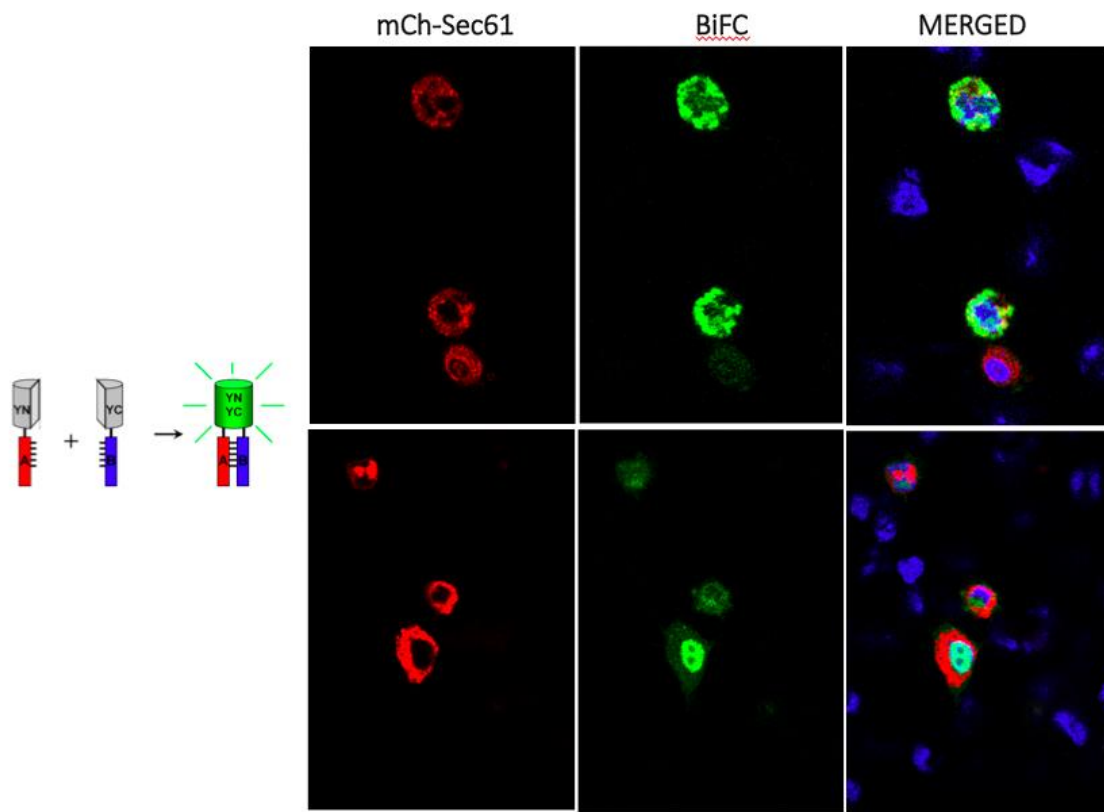


Figure 23 BiFC analysis results

According to the results, bi-molecular fluorescence complementation is achieved both in ER and nucleus. This indicates that LeprotL1 and mCry1 interaction localizes both in ER and nucleus.

4.6 The effect of LeprotL1 on circadian rhythm of U2OS and NIH3T3 cells

In order to understand how LeprotL1 affects the rhythmicity of the U2OS and NIH3T3 cell lines, siRNA knock down of LeprotL1 is performed. The importance of U2OS and NIH3T3 cell lines originates from their robust circadian rhythm. Manipulations on these cell lines do not easily attenuate their rhythmicity. Most importantly, these cell lines contain genome integrated luciferase reporters, which help us understand the circadian timing. Bmal1-Luc reporter system containing Bmal1 promoter at the upstream of luciferase gene is virally integrated into U2OS cell line,

whereas mPer1-luc reporter is inserted into NIH3T3 cell line. Dr. Halil Kavakli already showed the effect of siRNA knock down of LeprotL1 in U2OS and NIH3T3 cell lines in University of Pennsylvania. In this study, the phenotype in U2OS cell line is confirmed after 2 biological repeats.

U2OS cell lines are seeded on 32mm plates as duplicates 24 hours before the siRNA transfection. As negative control, all-star siRNA provided by QIAGEN is transfected. The all-star siRNA shows no homology with any known mammalian transcript, therefore, it is not expected to down-regulate a transcript. 48 hours after siRNA transfection, cells are pulse-chase treated with 0.1 μ M dexamethasone for 2 hours to reset the circadian timing of all individual cells. Dexamethasone is a glucocorticoid receptor agonist whose treatment robustly induces Per gene transcription down-stream. Later on, cells' medium is changed with lumicycle medium containing luciferin as luciferase substrate. Since the promoters of Bmal1 and Per1 (controlling luciferase production) are robustly under control of circadian timing, luminescent signal indicates the circadian timing of the cells. The siRNA knock down of U2OS cells were also validated by RT-qPCR.

The observed phenotypes when well-characterized core clock components are knocked down are shown in Figure 24. As can be seen from the figure, knock down of LeprotL1 in U2OS cell lines cause decreased amplitude and phase shift, in NIH3T3 cell lines showed decreased amplitude. These types of phenotypes are also observed in when core clock components are knocked down. For example, when Cry2 is knocked down, decreased amplitude and phase shift are observed. Knock down of Bmal1 and Per1 diminished rhythmicity whereas Clock knock down cause damp amplitude and phase shift. Cry1 knock down shows a very quick damping in the amplitude, with shortened period. On the light of these results, LeprotL1 seems to act as a clock component in U2OS and NIH3T3 cell lines.

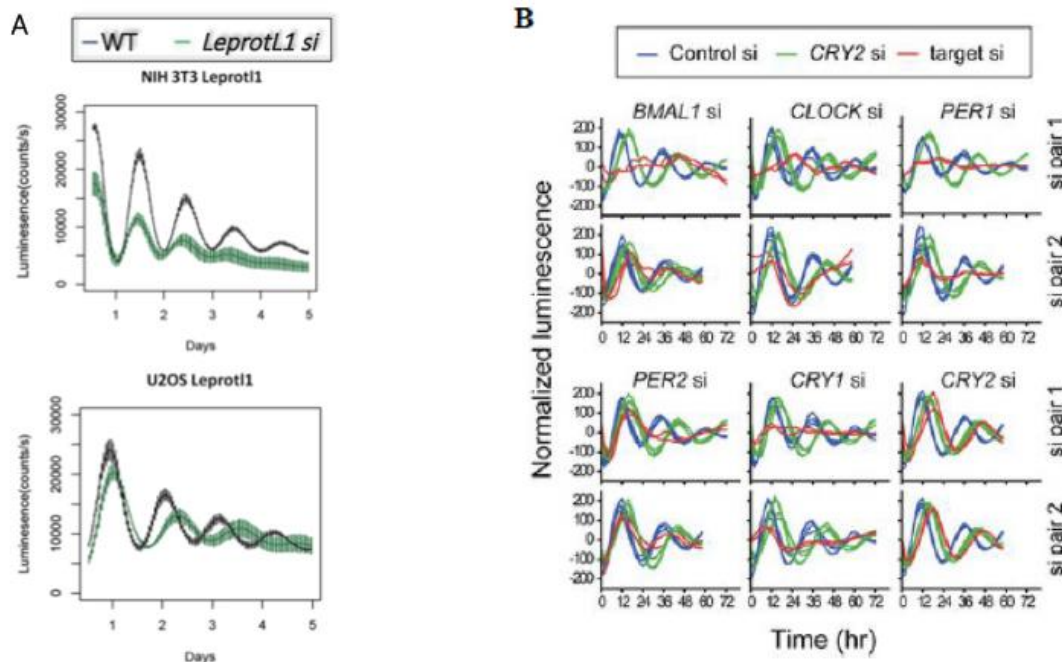


Figure 24 Bioluminescence analysis of LeprotL1 knock down cells

A) Upon LeprotL1 knockdown with siLeprotL1, NIH3T3 cells show damped amplitude whereas in U2OS cells both damped amplitude and phase shifts are observed. B) Similar phenotypes are also observed in core clock component knock down cells.

4.7 Analyzing the Effects of LeprotL1 Knock Down on Core Clock Components

U2OS cell lines are seeded on 6 well plates as they reach 80% confluence next day just before the siRNA transfection. Cells are transfected with siLeprotL1 and All-star siRNA. 48 hours after transfection, total RNA extraction is performed following cDNA synthesis. For RT-qPCR experiments, GAPDH (glyceraldehyde 3-phosphate) is used as an internal control since its expression is not affected by altered circadian rhythm. siRNA knock down of LeprotL1 was very successful as can be seen from the Figure 28. The efficiency of the siRNA knock down was around 98%. All of the core clock components showed decreased expression level except Bmal1. Dbp (D-box

binding protein) is a clock-controlled gene and the observed decrease in its expression level means that circadian output is also well affected by LeprotL1 knock down. Change in the expression level of LeprotL1 in Cry1 knock down cells was also quantified by RT-qPCR. Results indicate that siRNA knock down was 90% efficient. Change in the expression level of LeprotL1 was not very significant (Figure 25).

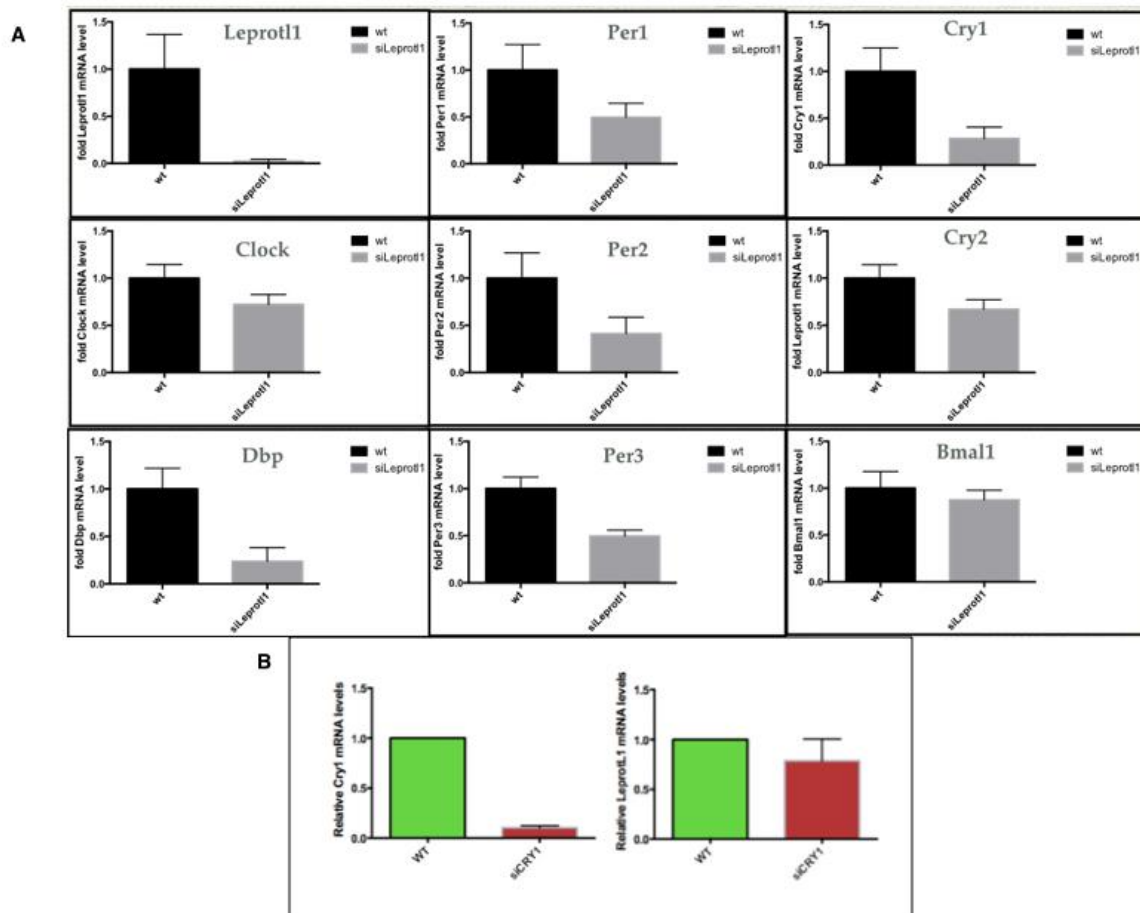


Figure 25 qPCR analysis of core clock components in Leprotl1 knock down cells

A) In Leprotl1 knock down cells Cry1/2, Per1/2/3 and Dbp levels decreased very significantly. Though, Bmal1 and Clock mRNA levels didn't change significantly. B) In Cry1 knock down U2OS cells, expression levels of Leprotl1 didn't change significantly.

The effect of LeprotL1 knock down was also examined time dependently on reset U2OS cells. Basically, after siRNA transfection cells are treated with dexamethasone to reset the circadian timing of all cells at the same point. 24 hours after resetting, total RNA extraction from cells is performed following cDNA synthesis in each 4 hours. Via RT-qPCR analysis, changes in the expression levels of core clock components are quantified (Figure 26).

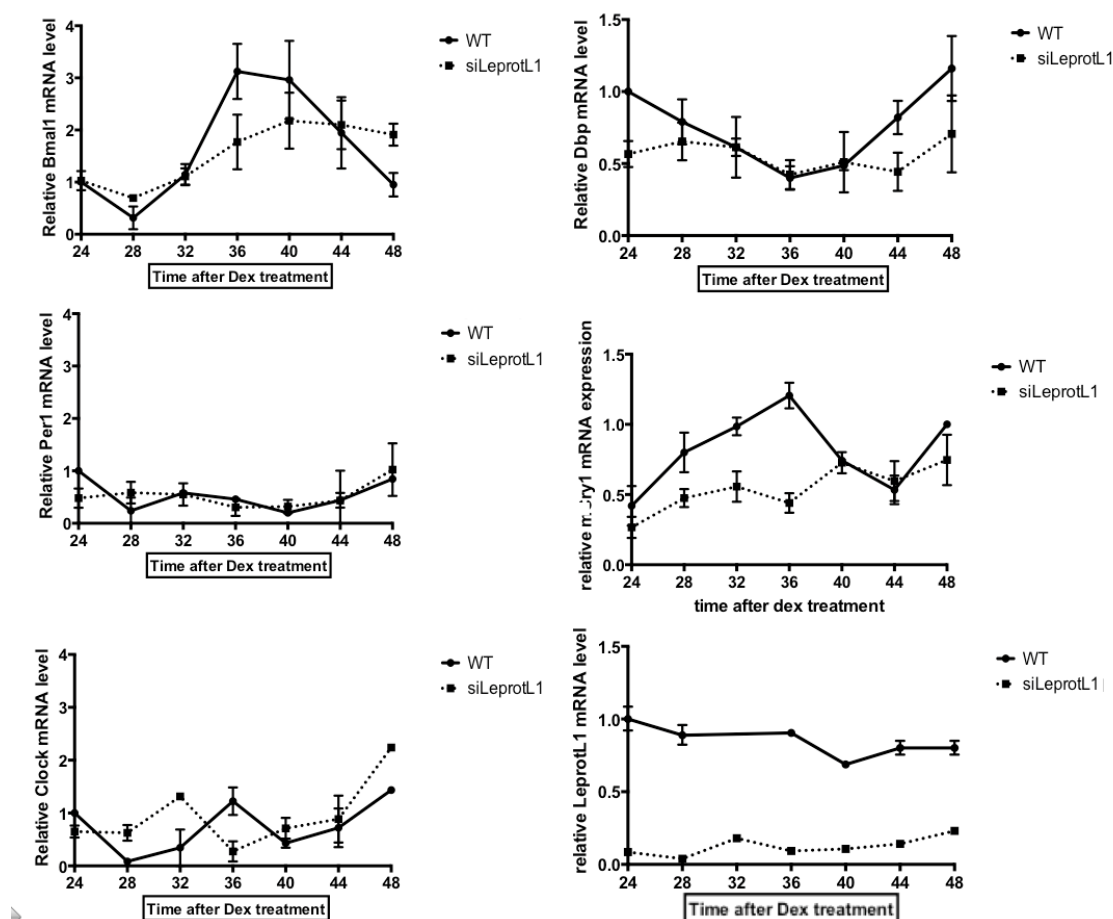


Figure 26 qPCR analysis upon LeprotL1 knockdown upon synchronization.

Solid lines represent WT cells whereas dashed lines show the LeprotL1 knock down cells.

CHAPTER 5: DISCUSSION

Naive Bayes Machine Learning strategy is a very powerful method to identify the clock components based on computational analysis seeking for proteins that show common properties of already known core clock components. The strength of the strategy is also proven by identification of Chrono gene as a core clock component which was also a hit obtained through Naïve Bayes Machine Learning strategy.¹⁹⁶ 55 candidate clock components obtained by this method are screened via mammalian two-hybrid assay for their probable interaction with well-defined core clock components namely CRY1/2, PER1/2/3, CLOCK, BMAL1 and NPAS2 by Gulsum Elif Muku in our lab. Among the hits LEPROTL1 showed interaction with both CRY1 and CRY2.

In this thesis work, via mammalian two-hybrid analysis interaction between CRY1/2 and LEPROTL1 is confirmed. Also, the CRY1 domain that is essential for LEPROTL1:CRY1 interaction is analyzed by mammalian-two hybrid assays. For this purpose truncated clones of Cry1 gene is used such that in each truncation 20 amino acids were deleted. According to the results, after LeprotL1 showed interaction until T5 region though the interaction is diminished in the T6. The T6 region constitutes the place where C-terminus domain of CRY1 meets with PHR domain. An α -helix (α 22) which is also located at T6 region was already identified as an important region for CRY2:PER2 and CRY1:PER2 interaction.^{64,105} Considering that α 22 sequence is conserved between CRY1 and CRY2, another mammalian two hybrid assay is performed with CRY2 501/503 mutants to further validate the specificity of the interaction between LEPROTL1 and CRYs. CRY2 501/503 mutations are located on α 22 region and it was already showed that these mutants show attenuated interaction with PER2.⁶⁴ According to mammalian two hybrid results, LEPROTL1 interaction with CRY2 501/503 mutant is completely diminished with approximately 9-fold difference between CRY2 WT version.

To further validate the interaction between LEPROTL1 and CRY1, co-immunoprecipitation analysis is performed. According to the results, LEPROTL1

Chapter 5: Discussion

pulled down full length and T5 CRY1 proteins, but not T6 CRY1. Another method named Bi-molecular Fluorescence Complementation (BiFC) analysis is performed in order to validate and analyze the localization of the interaction. In order to mark the compartments of the cells, Sec61b-mCherry transfection along with DAPI staining is performed. Sec61b is a protein specifically localized in ER (endoplasmic reticulum). BiFC analysis revealed that the localization of the interaction is both nucleus and perinuclear area.

To analyze the effect of LEPROTL1 on circadian rhythm, biolumicycle analysis on LeprotL1 knock down cells is performed. For this purpose, U2OS and NIH3T3 cell lines were used. RT-qPCR analysis validated that knock down efficiency of siLeprotl1 was around 95%. According to the results obtained by Dr. Halil Kavakli, Leprotl1 knock down causes damped amplitude and phase shift in U2OS cells, and damped amplitude in NIH3T3 cells. The phenotype observed in U2OS cells is also validated in this thesis work. The different phenotypes observed in U2OS and NIH3T3 cells indicates a modulator role of Leprotl1 in central molecular oscillation rather than being a core clock component.

Via RT-qPCR analysis, the effect of Leprotl1 knock down on transcription of core clock components is analyzed both in synchronized and unsynchronized U2OS cells. In unsynchronized cells, knock down of Leprotl1 significantly decreased core clock components' transcription including Cry1/2, Per1/2/3, and Clock. Surprisingly, Bmal1 expression is not significantly changed. Dbp as a clock-controlled gene is also downregulated significantly upon Leprotl1 knock down.

After the synchronization of U2OS cells' circadian times with dexamethasone treatment, via RT-qPCR analysis the effects of Leprotl1 knock down core clock components are analyzed by extracting total RNA for 24 hours with 4 hours intervals. According to the results, Leprotl1 knock down caused decreased amplitude in Cry1, Bmal1, Dbp transcript levels. On the other hand, phase change in the clock transcript is observed. An oscillation of LeprotL1 transcript level in wild type cells is not observed. However, this doesn't necessarily mean that Leprotl1 function is not associated with circadian rhythm. Use of tissue culture systems is handy to study the

Chapter 5: Discussion

molecular circadian clock, though this method becomes limited for researching on clock-controlled genes and pathways.¹⁷⁸ *In vivo* robust circadian rhythms are generated by inter-talk between autonomous central oscillator and systematic cues such as feeding. But these properties of robust rhythms are hard to replicate *in vitro*.²¹⁹ According to CircaDB database, *Leprotl1* robustly oscillates in liver, but this oscillation isn't observed in U2OS cell lines probably due to lack of some other external factors.

Hepatic growth hormone response while food restriction is attenuated in order to decrease the energy expenditure. This attenuation is achieved by FGF21 (fibroblast growth factor 21) action which increases^{152,153} *Leprot* and *Leprotl1* transcription in liver. *Leprot* and *Leprotl1* in turn take role in post-internalization step of GHR (growth hormone receptor). Upon endocytosis of GHR, liver becomes insensitive to GH action.²¹⁷ Also, it was shown that hepatic FGF21 transcription oscillates which might have a role in hepatic oscillation of *Leprotl1* transcript.²²⁰ It was also shown that, GHR mRNA production oscillates within 24 hours.²²¹ The role of *Leprotl1* as a link between GH metabolism and circadian rhythm can be related with post-translational control of GHR since it is known that *Leprotl1* action on GHR leads lysosomal degradation.^{212,217}

Leptin signaling is also associated with circadian control.¹⁶⁰ In db-db mice in which leptin receptor gene is deleted, *Cry1* overexpression enhances insulin sensitivity.^{152,153} Regulation of leptin metabolism by circadian rhythm might also be achieved through *CRY1:LEPROTL1* interaction.

To understand whether *Leprotl1* is a core clock component or not, locomotor activity analysis on *Leprotl1* knock out mice must be observed as a future perspective. It is known that metabolism and circadian rhythm are intertwined and feeding is important for hepatic clock resetting. Even if *Leprotl1* is not a core clock component, based on the results indicating the interaction between *CRY1* and *LEPROTL1*, *LEPROTL1* might be an intermediate pathway protein having role both in metabolism and circadian rhythm.

VITA

Derya Kabacaoğlu was born in Manisa, Turkey, December 22, 1990. She received her B. Sc. Degree in Molecular Biology and Genetics from Koc University, Istanbul, Turkey in 2013. Later, She joined the MSc. Program in Molecular Biology and Genetics at Koc University with full scholarship from TUBITAK-2211 where she studied “Investigation of the interaction between mouse cryptochrome-1 and Leptin Receptor Overlapping Transcript Like-1” under the supervision of Assoc. Pro. Dr. I. Halil Kavaklı. She also worked as a teaching assistant during her MSc years.

APPENDIX I

List of the primer sequences

PRIMER NAME	SEQUENCE
FL-mCry1-pBIND-fwd	GCGTTGATATCAGGGGTGAACGCCGTGC
FL-mCry1-pBIND-rev	ACCTGCGGCCGCTCAGTTACTGCTCTGCCGCTG
T1-mCry1-pBIND-rev	ACCTGCGGCCGCTCAACGCTTCCCACTGCTGAG
T2-mCry1-pBIND-rev	ACCTGCGGCCGCTCACTGCTGACTGTCCCCGTG
T3-mCry1-pBIND-rev	ACCTGCGGCCGCTCAGCTACAGCTCGGGACGTT
T4-mCry1-pBIND-rev	ACCTGCGGCCGCTCAGCTACAACTCGGGACATT
T5-mCry1-pBIND-rev	ACCTGCGGCCGCTCAGTTAGAAGGGACCGAGGC
T6-mCry1-pBIND-rev	ACCTGCGGCCGCTCACTGCTTCATTCTTTCAAT
T7-mCry1-pBIND-rev	ACCTGCGGCCGCTCAGTAATTAACCTCCTATCAA
LeprotL1-pACT-fwd	CGTTGATATCAATGGCGGGCATCAAAGCTT
LeprotL1-pACT-rev	CCTGCGGCCGCTCACCCTGCTGCCAGCTG
FL-mCry1-pcDNA-fwd	TGCAGATATCCAATGGGGGTGAACGCCGTGCAC
FL-mCry1-pcDNA-rev	GACTCGAGCGGCCGCCATCAGTTACTGCTCTGCCGCTGG
T5-mCry1-pcDNA-rev	GAGCGGCCGCCACTGGCTACAACTCGGGACATTCTC
T6-mCry1-pcDNA-rev	GAGCGGCCGCCACTGGTTAGAAGGGACCGAGGCGAG
LeprotL1-sport6-fwd	TCCCGGGATATCATGGCGGGCATCAAAGCTTTAA
LeprotL1-VN173-fwd	CCGCGAATTCAATGGCAGGCATCAAAGC
LeprotL1-VN173-rev	GTCGACTGGTACCGATCCACTGCTGCCAGCT
RT-QPCR PRIMER NAMES	SEQUENCE
HS-mCry1-RT-fwd	ACAGGTGGCGATTTTTGCTTC
HS-mCry1-RT-rev	TCCAAAGGGCTCAGAATCATACT
HS-mCry2-RT-fwd	CTACCGGGGACTCTGTCTACT
HS-mCry2-RT-rev	ACTGGGTAGTGGTCTTGGGC
HS-Per1-RT-fwd	CCTCTTCCAGGATGTGGATGAA
HS-Per1-RT-rev	CAACTGCAGAATCTTCTTGTGGA
HS-Per2-RT-fwd	GCGTGTTCCACAGTTTCACC
HS-Per2-RT-rev	GGCTTTTCCGGACACTGACA
HS-Per3-RT-fwd	CAAAGAGCTGCACGGTATGAA
HS-Per3-RT-rev	ATGATCCGGAATGGGGAGTG
HS-Bmal1-RT-fwd	GCCATTGAACATCACGAGTAC
HS-Bmal1-RT-rev	CCTGAGCCTGGCCTGATAGTAG
HS-GAPDH-RT-fwd	TGCACCACCAACTGCTTAGC

Appendix

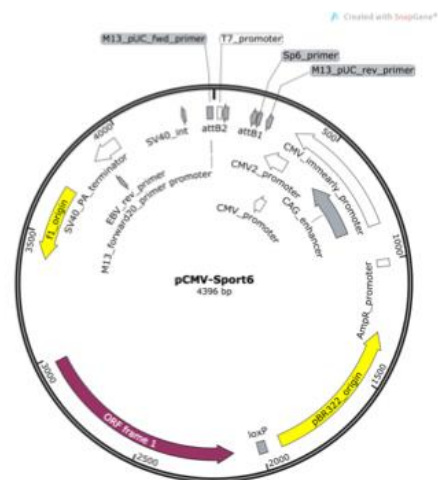
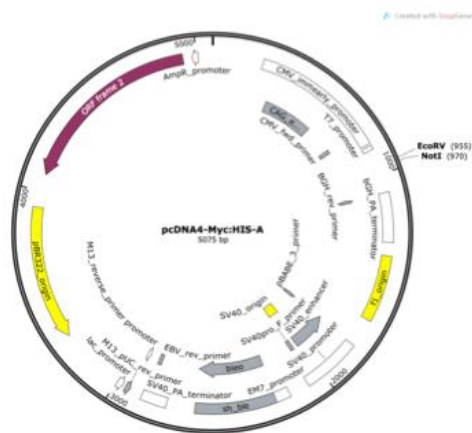
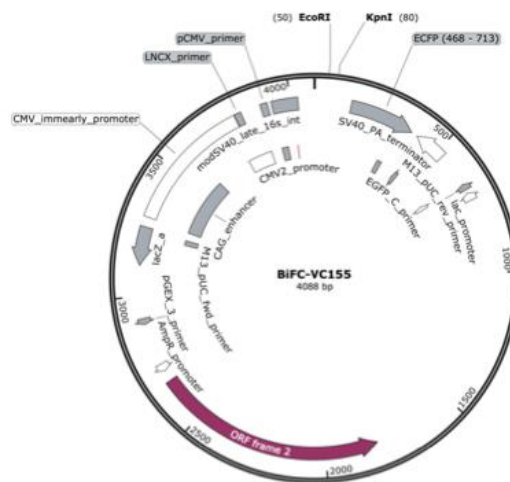
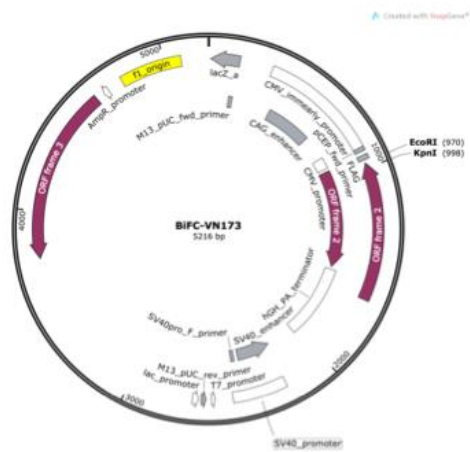
HS-GAPDH-RT-rev	ACAGTCTTCTGGGTGGCAGTG
HS-Dbp-RT-fwd	GAGGAACCTTAAGCCCCAGCC
HS-Dbp-RT-rev	CTCGTTGTTCTTCTACCGCC
HS-Clock-RT-fwd	GGCACCACCCATAATAGGGTA
HS-Clock-RT-rev	TGTTGCCCCCTTAGTCAGGAAC
HS-LeprotL1-RT-fwd	GGCATTGTCGTGTCAGCTTT
HS-LeprotL1V1-RT-rev	CTGCCAGCTGAAGTCGTCAT
HS-LeprotL1V2-RT-rev	ACAATCTTCTCATGGCGGCT

siRNA NAMES

HS_LEPROTL1_8
HS_LEPROTL1_9
HS_LEPROTL1_10
HS_LEPROTL1_11
Mm_LeprotL1_1
Mm_LeprotL1_2
Mm_LeprotL1_3
Mm_LeprotL1_4
HS_CRY1_5
HS_CRY1_7
HS_CRY1_6
HS_CRY1_3

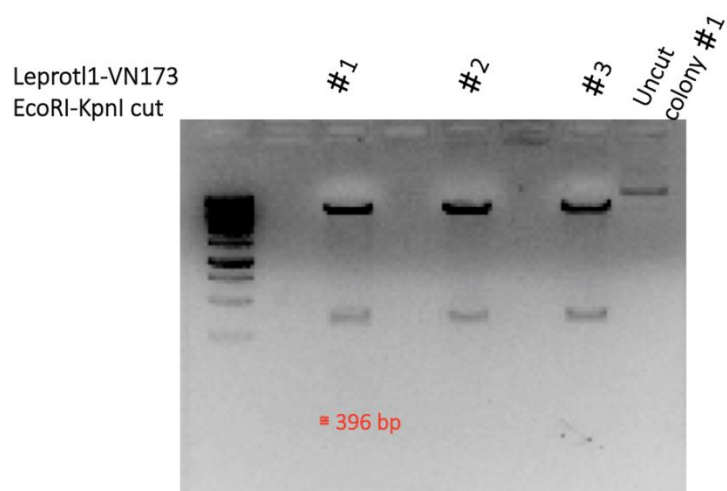
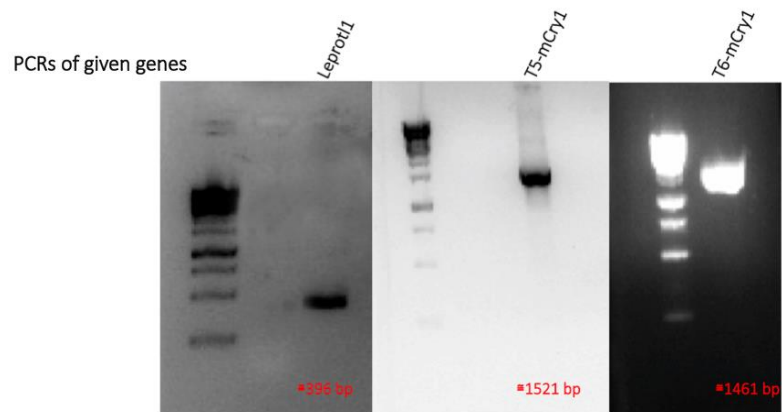
TARGET SEQUENCE

ATGAGTAACGCTTGTAAGGAA
CAAGAAGATTAGTGGATGATA
CCCTTCCAATATACAACAAAT
CAGGCATCAAAGCTTTGATTA
TACATTGTATATATTAAGTAA
CAGCAGTGGCTTGTTGAGTAA
AAGAATTTATGTTAGACTTTA
TCAGTTAATCTCCATTATTAA
CTGTTTGTGATTTCGTGGACAA
CTCCTCCAATGTGGGCATCAA
CGAGACGCAGCTATTAAGAAA
ATCAGCAGCTTTCACGATATA

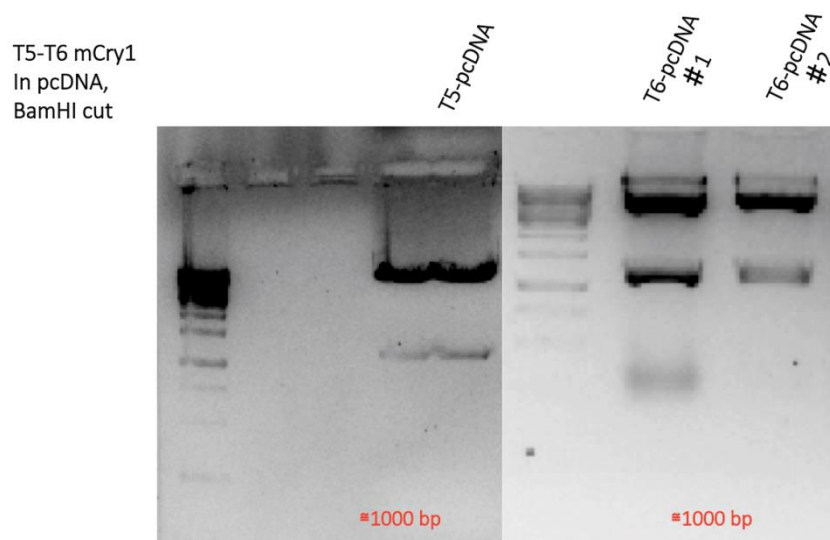
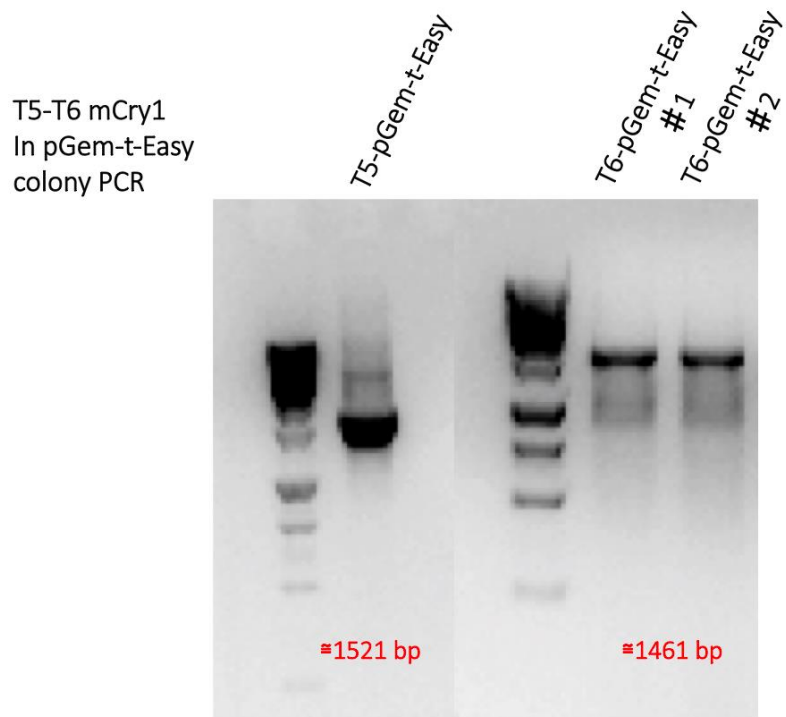


APPENDIX 3

Agarose Gel Images



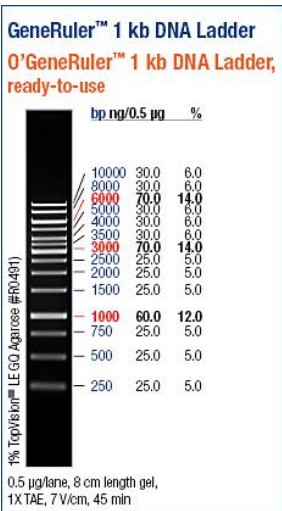
Appendix



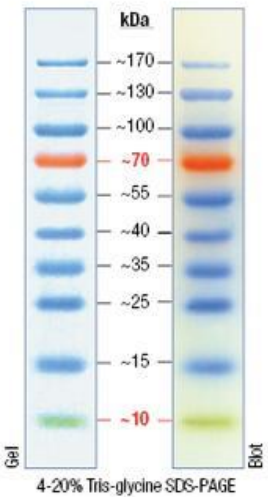
APPENDIX 4

DNA and Protein Markers

DNA Molecular Weight Marker



The ladder is a mixture of chromatography-purified individual DNA fragments.



PageRuler Prestained Protein Ladders

APPENDIX 5**Lab Equipments**

Autoclaves	: CL-40S/SDP (60L) ALP autoclave
Centrifuges	: 4K15, 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	: Pipetteman P10, P 100, P1000, Eppendorf
pH meter	: Inolab pH level 1, order# 1A10-1113,
Power supply	: PowerPac Basic (300V,400mA,75W) Biorad
Pure water systems	: DV25 PureLab Option ELGA
Spectrophotometer	: W-1700 PharmaSpec, Shimadzu Corporation.
Vortexing machine	: reax top, heidolph2.2

BIBLIOGRAPHY

- 1 Dunlap JC, Loros JJ, DeCoursey PJ. *Chronobiology: Biological Timekeeping*. Sinauer Associates, Inc.: Sunderland, MA 01375 U.S.A, 2004.
- 2 Mohawk JA, Green CB, Takahashi JS. Central and Peripheral Circadian Clocks in Mammals. *Annu Rev Neurosci* 2012; **35**: 445–462.
- 3 Buhr ED, Takahashi JS. Molecular components of the mammalian circadian clock. *Handb Exp Pharmacol* 2013; **217**: 3–27.
- 4 Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol* 2014; **24**: 90–99.
- 5 Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. *Hum Mol Genet* 2006; **15**: 271–277.
- 6 Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature* 2002; **418**: 935–41.
- 7 Challet E. Minireview: Entrainment of the suprachiasmatic clockwork in diurnal and nocturnal mammals. *Endocrinology*. 2007; **148**: 5648–5655.
- 8 Kavakli IH, Sancar A. Circadian photoreception in humans and mice. *Mol Interv* 2002; **2**: 484–92.
- 9 Partch CL, Sancar A. Cryptochromes and circadian photoreception in animals. *Methods Enzymol* 2005; **393**: 726–45.
- 10 Landgraf D, McCarthy MJ, Welsh DK. Circadian Clock and Stress Interactions in the Molecular Biology of Psychiatric Disorders. *Curr. Psychiatry Rep.* 2014; **16**: 483.

- 11 Lamont EW, Coutu DL, Cermakian N, Boivin DB. Circadian rhythms and clock genes in psychotic disorders. *Isr J Psychiatry Relat Sci* 2010; **47**: 27–35.
- 12 Paschos GK. Circadian clocks, feeding time, and metabolic homeostasis. *Front Pharmacol* 2015; **6**: 1–4.
- 13 Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M. Entrainment of the circadian clock in the liver by feeding. *Science* 2001; **291**: 490–493.
- 14 Vollmers C, Gill S, DiTacchio L, Pulivarthy SR, Le HD, Panda S. Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. *Proc Natl Acad Sci U S A* 2009; **106**: 21453–21458.
- 15 Liu Y, Mellow M, Loros JJ, Dunlap JC. How temperature changes reset a circadian oscillator. *Science* 1998; **281**: 825–829.
- 16 Diernfellner ACR, Schafmeier T, Mellow MW, Brunner M. Molecular mechanism of temperature sensing by the circadian clock of *Neurospora crassa*. *Genes Dev* 2005; **19**: 1968–1973.
- 17 McClung CR. Plant circadian rhythms. *Plant Cell* 2006; **18**: 792–803.
- 18 Konopka RJ, Benzer S. Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 1971; **68**: 2112–6.
- 19 Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD *et al.* Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* 1994; **264**: 719–25.
- 20 Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu YZ, Pray-Grant M *et al.* Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J Biol Chem* 1997; **272**: 8581–93.

- 21 Tosini G. Locomotor activity in rodents. *Methods Mol Biol* 2007; **362**: 95–101.
- 22 Edmunds LN. Physiology of circadian rhythms in micro-organisms. *Adv Microb Physiol* 1984; **25**: 61–148, 301–3.
- 23 Noordally ZB, Millar AJ. Clocks in algae. *Biochemistry* 2015; **54**: 171–83.
- 24 Lakin-Thomas PL, Bell-Pedersen D, Brody S. The genetics of circadian rhythms in *Neurospora*. *Adv Genet* 2011; **74**: 55–103.
- 25 Cha J, Zhou M, Liu Y. Mechanism of the *Neurospora* circadian clock, a FREQUENCY-centric view. *Biochemistry* 2015; **54**: 150–6.
- 26 Cirelli C. Searching for sleep mutants of *Drosophila melanogaster*. *Bioessays* 2003; **25**: 940–9.
- 27 Hall JC. Genetics and molecular biology of rhythms in *Drosophila* and other insects. *Adv Genet* 2003; **48**: 1–280.
- 28 Chiu JC, Low KH, Pike DH, Yildirim E, Edery I. Assaying locomotor activity to study circadian rhythms and sleep parameters in *Drosophila*. *J Vis Exp* 2010. doi:10.3791/2157.
- 29 King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP *et al*. Positional cloning of the mouse circadian clock gene. *Cell* 1997; **89**: 641–653.
- 30 Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP *et al*. Role of the CLOCK protein in the mammalian circadian mechanism. *Science* 1998; **280**: 1564–1569.
- 31 Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB *et al*. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 2000; **103**: 1009–1017.

-
- 32 Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X *et al.* mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 1999; **98**: 193–205.
- 33 Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S *et al.* Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* 2001; **105**: 683–694.
- 34 Albrecht U, Zheng B, Larkin D, Sun ZS, Lee CC. MPer1 and mper2 are essential for normal resetting of the circadian clock. *J Biol Rhythms* 2001; **16**: 100–104.
- 35 Yoo S-H, Ko CH, Lowrey PL, Buhr ED, Song E, Chang S *et al.* A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. *Proc Natl Acad Sci U S A* 2005; **102**: 2608–2613.
- 36 Griffin EA, Staknis D, Weitz CJ. Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. *Science* 1999; **286**: 768–771.
- 37 Sangoram AM, Saez L, Antoch MP, Gekakis N, Staknis D, Whiteley A *et al.* Mammalian circadian autoregulatory loop: A timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription. *Neuron* 1998; **21**: 1101–1113.
- 38 Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ *et al.* Feedback repression is required for mammalian circadian clock function. *Nat Genet* 2006; **38**: 312–319.
- 39 Hirota T, Lewis WG, Liu AC, Wook J, Schultz PG, Kay SA. A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3 β . *PNAS* 2008; **105**: 20746–20751.

- 40 Isojima Y, Nakajima M, Ukai H, Fujishima H, Yamada RG, Masumoto K *et al.* CKIepsilon/delta-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock. *Proc Natl Acad Sci U S A* 2009; **106**: 15744–15749.
- 41 Lamia KA, Sachdeva UM, DiTacchio L, Williams EC, Alvarez JG, Egan DF *et al.* AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* 2009; **326**: 437–440.
- 42 Hirano A, Yumimoto K, Tsunematsu R, Matsumoto M, Oyama M, Kozuka-Hata H *et al.* FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. *Cell* 2013; **152**: 1106–1118.
- 43 Shi G, Xing L, Liu Z, Qu Z, Wu X, Dong Z *et al.* Dual roles of FBXL3 in the mammalian circadian feedback loops are important for period determination and robustness of the clock. *Proc Natl Acad Sci U S A* 2013; **110**: 4750–5.
- 44 Yoo SH, Mohawk JA, Siepka SM, Shan Y, Huh SK, Hong HK *et al.* Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. *Cell* 2013; **152**: 1091–1105.
- 45 Baggs JE, Price TS, DiTacchio L, Panda S, Fitzgerald GA, Hogenesch JB. Network features of the mammalian circadian clock. *PLoS Biol* 2009; **7**: 0563–0575.
- 46 Ueda HR, Chen W, Adachi A, Wakamatsu H, Hayashi S, Takasugi T *et al.* A transcription factor response element for gene expression during circadian night. *Nature* 2002; **418**: 534–539.
- 47 Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P *et al.* A functional genomics strategy reveals rora as a component of the mammalian circadian clock. *Neuron* 2004; **43**: 527–537.

-
- 48 Crumbley C, Burris TP. Direct regulation of CLOCK expression by REV-ERB. *PLoS One* 2011; **6**. doi:10.1371/journal.pone.0017290.
- 49 Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U *et al*. The orphan nuclear receptor REV-ERB α controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 2002; **110**: 251–260.
- 50 Guillaumond F, Dardente H, Giguère V, Cermakian N. Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms* 2005; **20**: 391–403.
- 51 Cho H, Zhao X, Hatori M, Yu RT, Barish GD, Lam MT *et al*. Regulation of circadian behaviour and metabolism by REV-ERB- α and REV-ERB- β . *Nature*. 2012; **485**: 123–127.
- 52 Gachon F, Olela FF, Schaad O, Descombes P, Schibler U. The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metab* 2006; **4**: 25–36.
- 53 Zuber AM, Centeno G, Pradervand S, Nikolaeva S, Maquelin L, Cardinaux L *et al*. Molecular clock is involved in predictive circadian adjustment of renal function. *Proc Natl Acad Sci U S A* 2009; **106**: 16523–16528.
- 54 Gachon F, Fonjallaz P, Damiola F, Gos P, Kodama T, Zakany J *et al*. The loss of circadian PAR bZip transcription factors results in epilepsy. *Genes Dev* 2004; **18**: 1397–1412.
- 55 Storch K-F, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH *et al*. Extensive and divergent circadian gene expression in liver and heart. *Nature* 2002; **417**: 78–83.

-
- 56 Ukai-Tadenuma M, Yamada RG, Xu H, Ripperger JA, Liu AC, Ueda HR. Delay in feedback repression by cryptochrome 1 Is required for circadian clock function. *Cell* 2011; **144**: 268–281.
- 57 Pereira DS, Van Der Veen DR, Gonçalves BSB, Tufik S, Von Schantz M, Archer SN *et al.* The effect of different photoperiods in circadian rhythms of Per3 knockout mice. *Biomed Res Int* 2014; **2014**. doi:10.1155/2014/170795.
- 58 Van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M *et al.* Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 1999; **398**: 627–630.
- 59 Lopez-Molina L, Conquet F, Dubois-Dauphin M, Schibler U. The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J* 1997; **16**: 6762–6771.
- 60 DeBruyne JP, Weaver DR, Reppert SM. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat Neurosci* 2007; **10**: 543–545.
- 61 Menet JS, Rodriguez J, Abruzzi KC, Rosbash M. Nascent-Seq reveals novel features of mouse circadian transcriptional regulation. *Elife* 2012; **2012**. doi:10.7554/eLife.00011.
- 62 Le Martelot G, Canella D, Symul L, Migliavacca E, Gilardi F, Liechti R *et al.* Genome-Wide RNA Polymerase II Profiles and RNA Accumulation Reveal Kinetics of Transcription and Associated Epigenetic Changes During Diurnal Cycles. *PLoS Biol* 2012; **10**. doi:10.1371/journal.pbio.1001442.
- 63 Gustafson CL, Partch CL. Emerging Models for the Molecular Basis of Mammalian Circadian Timing. *Biochemistry* 2015; **54**: 134–149.

-
- 64 Ozber N, Baris I, Tatlici G, Gur I, Kilinc S, Unal EB *et al.* Identification of two amino acids in the C-terminal domain of mouse CRY2 essential for PER2 interaction. *BMC Mol Biol* 2010; **11**: 69.
- 65 Schmalen I, Reischl S, Wallach T, Klemz R, Grudziecki A, Prabu JR *et al.* Interaction of circadian clock proteins CRY1 and PER2 is modulated by zinc binding and disulfide bond formation. *Cell* 2014; **157**: 1203–1215.
- 66 Ye R, Selby CP, Ozturk N, Annayev Y, Sancar A. Biochemical analysis of the canonical model for the mammalian circadian clock. *J Biol Chem* 2011; **286**: 25891–25902.
- 67 Gallego M, Virshup DM. Post-translational modifications regulate the ticking of the circadian clock. *Nat Rev Mol Cell Biol* 2007; **8**: 139–148.
- 68 Meng QJ, Logunova L, Maywood ES, Gallego M, Lebiecki J, Brown TM *et al.* Setting Clock Speed in Mammals: The CK1 ϵ tau Mutation in Mice Accelerates Circadian Pacemakers by Selectively Destabilizing PERIOD Proteins. *Neuron* 2008; **58**: 78–88.
- 69 Kurabayashi N, Hirota T, Sakai M, Sanada K, Fukada Y. DYRK1A and glycogen synthase kinase 3 β , a dual-kinase mechanism directing proteasomal degradation of CRY2 for circadian timekeeping. *Mol Cell Biol* 2010; **30**: 1757–1768.
- 70 Lee J, Lee Y, Lee MJ, Park E, Kang SH, Chung CH *et al.* Dual modification of BMAL1 by SUMO2/3 and ubiquitin promotes circadian activation of the CLOCK/BMAL1 complex. *Mol Cell Biol* 2008; **28**: 6056–6065.
- 71 Busino L, Bassermann F, Maiolica A, Lee C, Nolan PM, Godinho SIH *et al.* SCFF β 13 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* 2007; **316**: 900–904.

-
- 72 Xing W, Busino L, Hinds TR, Marionni ST, Saifee NH, Bush MF *et al.* SCF(FBXL3) ubiquitin ligase targets cryptochromes at their cofactor pocket. *Nature* 2013; **496**: 64–8.
- 73 Etchegaray J-P, Lee C, Wade PA, Reppert SM. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature* 2003; **421**: 177–182.
- 74 Ripperger JA, Schibler U. Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat Genet* 2006; **38**: 369–374.
- 75 Katada S, Sassone-Corsi P. The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat Struct Mol Biol* 2010; **17**: 1414–1421.
- 76 DiTacchio L, Le HD, Vollmers C, Hatori M, Witcher M, Secombe J *et al.* Histone Lysine Demethylase JARID1a Activates CLOCK-BMAL1 and Influences the Circadian Clock. *Science* (80-.). 2011; **333**: 1881–1885.
- 77 Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D *et al.* The NAD⁺-Dependent Deacetylase SIRT1 Modulates CLOCK-Mediated Chromatin Remodeling and Circadian Control. *Cell* 2008; **134**: 329–340.
- 78 Nakahata Y, Sahar S, Astarita G, Kaluzova M, Sassone-Corsi P. Circadian control of the NAD⁺ salvage pathway by CLOCK-SIRT1. *Science* 2009; **324**: 654–657.
- 79 Aksoy P, Escande C, White TA, Thompson M, Soares S, Benech JC *et al.* Regulation of SIRT 1 mediated NAD dependent deacetylation: A novel role for the multifunctional enzyme CD38. *Biochem Biophys Res Commun* 2006; **349**: 353–359.

-
- 80 Doi M, Hirayama J, Sassone-Corsi P. Circadian Regulator CLOCK Is a Histone Acetyltransferase. *Cell* 2006; **125**: 497–508.
- 81 Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y *et al.* CLOCK-mediated acetylation of BMAL1 controls circadian function. *Nature* 2007; **450**: 1086–1090.
- 82 Etchegaray JP, Yang X, Debruyne JP, Peters AHFM, Weaver DR, Jenuwein T *et al.* The polycomb group protein EZH2 is required for mammalian circadian clock function. *J Biol Chem* 2006; **281**: 21209–21215.
- 83 Viré E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C *et al.* The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006; **439**: 871–874.
- 84 Bellet MM, Sassone-Corsi P. Mammalian circadian clock and metabolism - the epigenetic link. *J Cell Sci* 2010; **123**: 3837–3848.
- 85 Feng D, Lazar M a. Clocks, metabolism, and the epigenome. *Mol Cell* 2012; **47**: 158–67.
- 86 Nader N, Chrousos GP, Kino T. Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. *FASEB J* 2009; **23**: 1572–1583.
- 87 Charmandari E, Chrousos GP, Lambrou GI, Pavlaki A, Koide H, Ng SSM *et al.* Peripheral CLOCK regulates target-tissue glucocorticoid receptor transcriptional activity in a circadian fashion in man. *PLoS One* 2011; **6**. doi:10.1371/journal.pone.0025612.

-
- 88 Takahata S, Ozaki T, Mimura J, Kikuchi Y, Sogawa K, Fujii-Kuriyama Y. Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. *Genes to Cells* 2000; **5**: 739–747.
- 89 Curtis AM, Seo SB, Westgate EJ, Rudic RD, Smyth EM, Chakravarti D *et al.* Histone Acetyltransferase-dependent Chromatin Remodeling and the Vascular Clock. *J Biol Chem* 2004; **279**: 7091–7097.
- 90 Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F *et al.* SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation. *Cell* 2008; **134**: 317–328.
- 91 Duong HA, Robles MS, Knutti D, Weitz CJ. A molecular mechanism for circadian clock negative feedback. *Science* 2011; **332**: 1436–1439.
- 92 Brown SA, Ripperger J, Kadener S, Fleury-Olela F, Vilbois F, Rosbash M *et al.* PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. *Science* 2005; **308**: 693–696.
- 93 Naruse Y, Oh-hashii K, Iijima N, Naruse M, Yoshioka H, Tanaka M. Circadian and light-induced transcription of clock gene *Per1* depends on histone acetylation and deacetylation. *Mol Cell Biol* 2004; **24**: 6278–6287.
- 94 Ishizuka T, Lazar MA. The N-CoR/histone deacetylase 3 complex is required for repression by thyroid hormone receptor. *Mol Cell Biol* 2003; **23**: 5122–5131.
- 95 Yin L, Lazar MA. The orphan nuclear receptor Rev-erb α recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian *Bmal1* gene. *Mol Endocrinol* 2005; **19**: 1452–1459.

-
- 96 Feng D, Liu T, Sun Z, Bugge A, Mullican SE, Alenghat T *et al.* A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. *Science* 2011; **331**: 1315–1319.
- 97 Bugge A, Feng D, Everett LJ, Briggs ER, Mullican SE, Wang F *et al.* Rev-erba and Rev-erbβ coordinately protect the circadian clock and normal metabolic function. *Genes Dev* 2012; **26**: 657–667.
- 98 Merbitz-Zahradnik T, Wolf E. How is the inner circadian clock controlled by interactive clock proteins?: Structural analysis of clock proteins elucidates their physiological role. *FEBS Lett* 2015; **589**: 1516–1529.
- 99 Henry JT, Crosson S. Ligand-Binding PAS Domains in a Genomic, Cellular, and Structural Context. *Annu. Rev. Microbiol.* 2011; **65**: 261–286.
- 100 Möglich A, Ayers RA, Moffat K. Structure and Signaling Mechanism of Per-ARNT-Sim Domains. *Structure*. 2009; **17**: 1282–1294.
- 101 Kewley RJ, Whitelaw ML, Chapman-Smith A. The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int. J. Biochem. Cell Biol.* 2004; **36**: 189–204.
- 102 Kiyohara YB, Tagao S, Tamanini F, Morita A, Sugisawa Y, Yasuda M *et al.* The BMAL1 C terminus regulates the circadian transcription feedback loop. *Proc Natl Acad Sci U S A* 2006; **103**: 10074–10079.
- 103 Chaves I, Pokorný R, Byrdin M, Hoang N, Ritz T, Brettel K *et al.* The cryptochromes: blue light photoreceptors in plants and animals. *Annu Rev Plant Biol* 2011; **62**: 335–364.
- 104 Nangle SN, Rosensweig C, Koike N, Tei H, Takahashi JS, Green CB *et al.* Molecular assembly of the period-cryptochrome circadian transcriptional repressor complex. *Elife* 2014; : e03674.

-
- 105 Czarna A, Berndt A, Singh HR, Grudziecki A, Ladurner AG, Timinszky G *et al.* Structures of Drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function. *Cell* 2013; **153**: 1394–405.
- 106 Sancar A. Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem Rev* 2003; **103**: 2203–37.
- 107 Güler AD, Ecker JL, Lall GS, Haq S, Altimus CM, Liao H-W *et al.* Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. *Nature* 2008; **453**: 102–105.
- 108 Czeisler CA, Klerman EB. Circadian and sleep-dependent regulation of hormone release in humans. *Recent Prog Horm Res* 1999; **54**: 97–130; discussion 130–132.
- 109 Menaker M. Circadian rhythms. Circadian photoreception. *Science* 2003; **299**: 213–214.
- 110 Zordan MA, Sandrelli F. Circadian Clock Dysfunction and Psychiatric Disease: Could Fruit Flies have a Say? *Front Neurol* 2015; **6**: 80.
- 111 Wee R, Castrucci AM, Provencio I, Gan L, Van Gelder RN. Loss of photic entrainment and altered free-running circadian rhythms in *math5*^{-/-} mice. *J Neurosci* 2002; **22**: 10427–10433.
- 112 Yoshimura T, Ebihara S. Decline of circadian photosensitivity associated with retinal degeneration in CBA/J-rd/rd mice. *Brain Res* 1998; **779**: 188–193.
- 113 Provencio I, Cooper HM, Foster RG. Retinal projections in mice with inherited retinal degeneration: Implications for circadian photoentrainment. *J Comp Neurol* 1998; **395**: 417–439.

-
- 114 Berson DM, Dunn FA, Takao M. Phototransduction by retinal ganglion cells that set the circadian clock. *Science* 2002; **295**: 1070–1073.
- 115 Semo M, Peirson S, Lupi D, Lucas RJ, Jeffery G, Foster RG. Melanopsin retinal ganglion cells and the maintenance of circadian and pupillary responses to light in aged rodless/coneless (rd/rd cl) mice. *Eur J Neurosci* 2003; **17**: 1793–1801.
- 116 Davies WL, Hankins MW, Foster RG. Vertebrate ancient opsin and melanopsin: divergent irradiance detectors. *Photochem Photobiol Sci* 2010; **9**: 1444–57.
- 117 Hattar S, Liao HW, Takao M, Berson DM, Yau KW. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science* 2002; **295**: 1065–1070.
- 118 Panda S, Sato TK, Castrucci AM, Rollag MD, DeGrip WJ, Hogenesch JB *et al.* Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science* 2002; **298**: 2213–2216.
- 119 Hastings MH, Brancaccio M, Maywood ES. Circadian pacemaking in cells and circuits of the suprachiasmatic nucleus. *J Neuroendocrinol* 2014; **26**: 2–10.
- 120 Hastings M, O'Neill JS, Maywood ES. Circadian clocks: regulators of endocrine and metabolic rhythms. *J Endocrinol* 2007; **195**: 187–98.
- 121 Hastings MH, Reddy AB, Maywood ES. A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci* 2003; **4**: 649–61.
- 122 Liu C, Reppert SM. GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron* 2000; **25**: 123–128.

-
- 123 Aton SJ, Colwell CS, Harmor AJ, Waschek J, Herzog ED. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nat Neurosci* 2005; **8**: 476–483.
- 124 Harmor AJ, Marston HM, Shen S, Spratt C, West KM, Sheward WJ *et al.* The VPAC2 receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell* 2002; **109**: 497–508.
- 125 Cutler DJ, Haraura M, Reed HE, Shen S, Sheward WJ, Morrison CF *et al.* The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro. *Eur J Neurosci* 2003; **17**: 197–204.
- 126 Mrosovsky N, Edelstein K, Hastings MH, Maywood ES. Cycle of period gene expression in a diurnal mammal (*Spermophilus tridecemlineatus*): implications for nonphotic phase shifting. *J Biol Rhythms* 2001; **16**: 471–478.
- 127 Maywood ES, O'Brien JA, Hastings MH. Expression of mCLOCK and other circadian clock-relevant proteins in the mouse suprachiasmatic nuclei. *J. Neuroendocrinol.* 2003; **15**: 329–334.
- 128 Travnickova-Bendova Z, Cermakian N, Reppert SM, Sassone-Corsi P. Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. *Proc Natl Acad Sci U S A* 2002; **99**: 7728–7733.
- 129 Field MD, Maywood ES, O'Brien JA, Weaver DR, Reppert SM, Hastings MH. Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. *Neuron* 2000; **25**: 437–447.
- 130 Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG *et al.* Circadian cycling of the mouse liver transcriptome, as revealed by cDNA

- microarray, is driven by the suprachiasmatic nucleus. *Curr Biol* 2002; **12**: 540–50.
- 131 Storch KF, Paz C, Signorovitch J, Raviola E, Pawlyk B, Li T *et al*. Intrinsic Circadian Clock of the Mammalian Retina: Importance for Retinal Processing of Visual Information. *Cell* 2007; **130**: 730–741.
- 132 Kornmann B, Schaad O, Bujard H, Takahashi JS, Schibler U. System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol* 2007; **5**: 0179–0189.
- 133 Damiola F, Le Minli N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 2000; **14**: 2950–2961.
- 134 Le Minh N, Damiola F, Tronche F, Schütz G, Schibler U. Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. *EMBO J* 2002; **20**: 7128–7136.
- 135 Asher G, Reinke H, Altmeyer M, Gutierrez-Arcelus M, Hottiger MO, Schibler U. Poly(ADP-Ribose) Polymerase 1 Participates in the Phase Entrainment of Circadian Clocks to Feeding. *Cell* 2010; **142**: 943–953.
- 136 Rogers NL, Dinges DF. Shiftwork, circadian disruption, and consequences. *Econ Neurosci* 2001; **3 (9)**: 58–64.
- 137 Wang X-S, Armstrong MEG, Cairns BJ, Key TJ, Travis RC. Shift work and chronic disease: the epidemiological evidence. *Occup Med (Lond)* 2011; **61**: 78–89.
- 138 Green CB, Takahashi JS, Bass J. The Meter of Metabolism. *Cell*. 2008; **134**: 728–742.

-
- 139 Bass J. Circadian topology of metabolism. *Nature* 2012; **491**: 348–56.
- 140 O'Neill JS, van Ooijen G, Dixon LE, Troein C, Corellou F, Bouget F-Y *et al.* Circadian rhythms persist without transcription in a eukaryote. *Nature* 2011; **469**: 554–558.
- 141 O'Neill JS, Reddy AB. Circadian clocks in human red blood cells. *Nature* 2011; **469**: 498–503.
- 142 Kalsbeek A, Fliers E, Romijn JA, La Fleur SE, Wortel J, Bakker O *et al.* The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels. *Endocrinology* 2001; **142**: 2677–2685.
- 143 Dixit VD, Schaffer EM, Pyle RS, Collins GD, Sakthivel SK, Palaniappan R *et al.* Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. *J Clin Invest* 2004; **114**: 57–66.
- 144 Bodosi B, Gardi J, Hajdu I, Szentirmai E, Obal F, Krueger JM. Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. *Am J Physiol Regul Integr Comp Physiol* 2004; **287**: R1071–R1079.
- 145 Yannielli PC, Molyneux PC, Harrington ME, Golombek DA. Ghrelin effects on the circadian system of mice. *J Neurosci* 2007; **27**: 2890–2895.
- 146 Yi CX, Challet E, Pévet P, Kalsbeek A, Escobar C, Buijs RM. A circulating ghrelin mimetic attenuates light-induced phase delay of mice and light-induced Fos expression in the suprachiasmatic nucleus of rats. *Eur J Neurosci* 2008; **27**: 1965–1972.
- 147 Jha PK, Challet E, Kalsbeek A. Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals. *Mol Cell Endocrinol* 2015. doi:10.1016/j.mce.2015.01.024.

-
- 148 Kalsbeek A, Fliers E. Daily regulation of hormone profiles. *Handb Exp Pharmacol* 2013; **217**: 185–226.
- 149 La Fleur SE, Kalsbeek A, Wortel J, van der Vliet J, Buijs RM. Role for the pineal and melatonin in glucose homeostasis: pinealectomy increases night-time glucose concentrations. *J Neuroendocrinol* 2001; **13**: 1025–1032.
- 150 Kalsbeek A, Strubbe JH. Circadian control of insulin secretion is independent of the temporal distribution of feeding. *Physiol Behav* 1998; **63**: 553–558.
- 151 Doi R, Oishi K, Ishida N. CLOCK regulates circadian rhythms of hepatic glycogen synthesis through transcriptional activation of Gys2. *J Biol Chem* 2010; **285**: 22114–22121.
- 152 Okano S, Akashi M, Hayasaka K, Nakajima O. Unusual circadian locomotor activity and pathophysiology in mutant CRY1 transgenic mice. *Neurosci Lett* 2009; **451**: 246–251.
- 153 Zhang EE, Liu Y, Dentin R, Pongsawakul PY, Liu AC, Hirota T *et al*. Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. *Nat Med* 2010; **16**: 1152–1156.
- 154 Lamia KA, Papp SJ, Yu RT, Barish GD, Uhlenhaut NH, Jonker JW *et al*. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*. 2011. doi:10.1038/nature10700.
- 155 Zhu L, Zee PC. Circadian Rhythm Sleep Disorders. *Neurol. Clin.* 2012; **30**: 1167–1191.
- 156 Kanathur N, Harrington J, Lee-Chiong T. Circadian Rhythm Sleep Disorders. *Clin. Chest Med.* 2010; **31**: 319–325.

-
- 157 Reid KJ, Zee PC. Circadian rhythm disorders. *Semin Neurol* 2009; **29** (4): 393–405.
- 158 Gu X, Xing L, Shi G, Liu Z, Wang X, Qu Z *et al*. The circadian mutation PER2S662G is linked to cell cycle progression and tumorigenesis. *Cell Death Differ* 2012; **19**: 397–405.
- 159 Xu Y, Padiath QS, Shapiro RE, Jones CR, Wu SC, Saigoh N *et al*. Functional consequences of a CK1delta mutation causing familial advanced sleep phase syndrome. *Nature* 2005; **434**: 640–644.
- 160 Ando H, Kumazaki M, Motosugi Y, Ushijima K, Maekawa T, Ishikawa E *et al*. Impairment of peripheral circadian clocks precedes metabolic abnormalities in ob/ob mice. *Endocrinology* 2011; **152**: 1347–1354.
- 161 Marcheva B, Ramsey KM, Buhr ED, Kobayashi Y, Su H, Ko CH *et al*. Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* 2010; **466**: 627–631.
- 162 Maury E, Ramsey KM, Bass J. Circadian rhythms and metabolic syndrome: From experimental genetics to human disease. *Circ. Res.* 2010; **106**: 447–462.
- 163 Sahar S, Sassone-Corsi P. Regulation of metabolism: The circadian clock dictates the time. *Trends Endocrinol Metab* 2012; **23**: 1–8.
- 164 Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E *et al*. Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* 2005; **308**: 1043–1045.
- 165 Lamia KA, Storch K-F, Weitz CJ. Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci U S A* 2008; **105**: 15172–15177.

-
- 166 Sadacca LA, Lamia KA, DeLemos AS, Blum B, Weitz CJ. An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice. *Diabetologia* 2011; **54**: 120–124.
- 167 Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB *et al.* BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* 2004; **2**. doi:10.1371/journal.pbio.0020377.
- 168 Gumz ML, Stow LR, Lynch IJ, Greenlee MM, Rudin A, Cain BD *et al.* The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice. *J Clin Invest* 2009; **119**: 2423–2434.
- 169 Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, Amin RH *et al.* PER2 controls lipid metabolism by direct regulation of PPAR γ . *Cell Metab* 2010; **12**: 509–520.
- 170 Doi M, Takahashi Y, Komatsu R, Yamazaki F, Yamada H, Haraguchi S *et al.* Salt-sensitive hypertension in circadian clock-deficient Cry-null mice involves dysregulated adrenal Hsd3b6. *Nat Med* 2010; **16**: 67–74.
- 171 Raspé E, Duez H, Mansén A, Fontaine C, Fiévet C, Fruchart J-C *et al.* Identification of Rev-erb α as a physiological repressor of apoC-III gene transcription. *J Lipid Res* 2002; **43**: 2172–2179.
- 172 Steinmayr M, André E, Conquet F, Rondi-Reig L, Delhaye-Bouchaud N, Auclair N *et al.* staggerer phenotype in retinoid-related orphan receptor α -deficient mice. *Proc Natl Acad Sci U S A* 1998; **95**: 3960–3965.
- 173 Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, Zhang CY *et al.* Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1 α null mice. *Cell* 2004; **119**: 121–135.

-
- 174 Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S *et al.* PGC-1?? deficiency causes multi-system energy metabolic derangements: Muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol* 2005; **3**: 0672–0687.
- 175 Kawai M, Green CB, Lecka-Czernik B, Douris N, Gilbert MR, Kojima S *et al.* A circadian-regulated gene, Nocturnin, promotes adipogenesis by stimulating PPAR-gamma nuclear translocation. *Proc Natl Acad Sci U S A* 2010; **107**: 10508–10513.
- 176 Douris N, Kojima S, Pan X, Lerch-Gaggl AF, Duong SQ, Hussain MM *et al.* Nocturnin regulates circadian trafficking of dietary lipid in intestinal enterocytes. *Curr Biol* 2011; **21**: 1347–1355.
- 177 Gauger MA, Sancar A. Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Cancer Res* 2005; **65**: 6828–6834.
- 178 Sancar A, Lindsey-Boltz L a., Gaddameedhi S, Selby CP, Ye R, Chiou Y-Y *et al.* Circadian Clock, Cancer, and Chemotherapy. *Biochemistry* 2015; **54**: 110–123.
- 179 Hua H, Wang Y, Wan C, Liu Y, Zhu B, Yang C *et al.* Circadian gene mPer2 overexpression induces cancer cell apoptosis. *Cancer Sci* 2006; **97**: 589–596.
- 180 Fu L, Pelicano H, Liu J, Huang P, Lee CC. The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* 2002; **111**: 41–50.
- 181 Ozturk N, Lee JH, Gaddameedhi S, Sancar A. Loss of cryptochrome reduces cancer risk in p53 mutant mice. *Proc Natl Acad Sci U S A* 2009; **106**: 2841–2846.

-
- 182 Zeng Z-L, Wu M-W, Sun J, Sun Y-L, Cai Y-C, Huang Y-J *et al.* Effects of the biological clock gene Bmal1 on tumour growth and anti-cancer drug activity. *J Biochem* 2010; **148**: 319–326.
- 183 Kelleher FC, Rao A, Maguire A. Circadian molecular clocks and cancer. *Cancer Lett* 2014; **342**: 9–18.
- 184 Gery S, Komatsu N, Baldjyan L, Yu a, Koo D, Koeffler HP. The circadian gene *per1* plays an important role in cell growth and DNA damage control in human cancer cells. *MolCell* 2006; **22**: 375–382.
- 185 Dussault I, Giguère V. Differential regulation of the N-myc proto-oncogene by ROR alpha and RVR, two orphan members of the superfamily of nuclear hormone receptors. *Mol Cell Biol* 1997; **17**: 1860–1867.
- 186 Lee JH, Sancar A. Regulation of apoptosis by the circadian clock through NF- κ B signaling. 2011; **2011**. doi:10.1073/pnas.1108125108/-/DCSupplemental. www.pnas.org/cgi/doi/10.1073/pnas.1108125108.
- 187 Lee JH, Gaddameedhi S, Ozturk N, Ye R, Sancar A. DNA damage-specific control of cell death by cryptochrome in p53-mutant ras-transformed cells. *Cancer Res* 2013; **73**: 785–791.
- 188 Straif K, Baan R, Grosse Y, Secretan B, El Ghissassi F, Bouvard V *et al.* Carcinogenicity of shift-work, painting, and fire-fighting. *Lancet Oncol.* 2007; **8**: 1065–1066.
- 189 Patel D. Shift work, light at night and risk of breast cancer. *Occup. Med. (Chic. Ill).* 2006; **56**: 433.
- 190 Schernhammer ES, Kroenke CH, Laden F, Hankinson SE. Night work and risk of breast cancer. *Epidemiology* 2006; **17**: 108–111.

-
- 191 Baan R, Grosse Y, Straif K, Secretan B, El Ghissassi F, Bouvard V *et al.* A review of human carcinogens--Part F: chemical agents and related occupations. *Lancet Oncol.* 2009; **10**: 1143–1144.
- 192 Sahar S, Sassone-Corsi P. Metabolism and cancer: the circadian clock connection. *Nat Rev Cancer* 2009; **9**: 886–896.
- 193 Ahmad M, Cashmore AR. HY4 gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* 1993; **366**: 162–6.
- 194 Van der Spek PJ, Kobayashi K, Bootsma D, Takao M, Eker AP, Yasui A. Cloning, tissue expression, and mapping of a human photolyase homolog with similarity to plant blue-light receptors. *Genomics* 1996; **37**: 177–182.
- 195 Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM *et al.* Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci U S A* 1999; **96**: 12114–12119.
- 196 Anafi RC, Lee Y, Sato TK, Venkataraman A, Ramanathan C, Kavakli IH *et al.* Machine learning helps identify CHRONO as a circadian clock component. *PLoS Biol* 2014; **12**: e1001840.
- 197 Geer LY, Marchler-Bauer A, Geer RC, Han L, He J, He S *et al.* The NCBI BioSystems database. *Nucleic Acids Res* 2009; **38**. doi:10.1093/nar/gkp858.
- 198 Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T *et al.* Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 1995; **269**: 540–543.
- 199 Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platika D *et al.* Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nat Med* 1996; **2**: 585–589.

-
- 200 Chehab FF, Lim ME, Lu R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* 1996; **12**: 318–320.
- 201 Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R *et al.* Identification and expression cloning of a leptin receptor, OB-R. *Cell* 1995; **83**: 1263–1271.
- 202 Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; **395**: 763–770.
- 203 Fruhbeck G. Intracellular signalling pathways activated by leptin. *Biochem J* 2006; **393**: 7–20.
- 204 Zabeau L, Lavens D, Peelman F, Eyckerman S, Vandekerckhove J, Tavernier J. The ins and outs of leptin receptor activation. *FEBS Lett* 2003; **546**: 45–50.
- 205 Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ *et al.* Evidence that the diabetes gene encodes the leptin receptor: Identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 1996; **84**: 491–495.
- 206 Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC. Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad Sci U S A* 1996; **93**: 6231–6235.
- 207 Huang Y, Ying K, Xie Y, Zhou Z, Wang W, Tang R *et al.* Cloning and characterization of a novel human leptin receptor overlapping transcript-like 1 gene (LEPROTL1). *Biochim Biophys Acta - Gene Struct Expr* 2001; **1517**: 327–331.
- 208 Belgareh-Touzé N, Avaro S, Rouillé Y, Hoflack B, Haguenaer-Tsapis R. Yeast Vps55p, a functional homolog of human obesity receptor gene-related

- protein, is involved in late endosome to vacuole trafficking. *Mol Biol Cell* 2002; **13**: 1694–1708.
- 209 Bennett PA, Lindell K, Karlsson C, Robinson IC, Carlsson LM, Carlsson B. Differential expression and regulation of leptin receptor isoforms in the rat brain: effects of fasting and oestrogen. *Neuroendocrinology* 1998; **67**: 29–36.
- 210 Bailleul B, Akerblom I, Strosberg AD. The leptin receptor promoter controls expression of a second distinct protein. *Nucleic Acids Res* 1997; **25**: 2752–2758.
- 211 Séron K, Couturier C, Belouzard S, Bacart J, Monté D, Corset L *et al.* Endospanins regulate a postinternalization step of the leptin receptor endocytic pathway. *J Biol Chem* 2011; **286**: 17968–17981.
- 212 Touvier T, Conte-auriol F, Briand O, Cudejko C, Paumelle R, Caron S *et al.* LEPROT and LEPROTL1 cooperatively decrease hepatic growth hormone action in mice. 2009; **119**. doi:10.1172/JCI34997.3830.
- 213 LeRoith D, Yakar S. Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. *Nat Clin Pract Endocrinol Metab* 2007; **3**: 302–310.
- 214 Beauloye V, Willems B, De Coninck V, Frank SJ, Edery M, Thissen JP. Impairment of liver GH receptor signaling by fasting. *Endocrinology* 2002; **143**: 792–800.
- 215 Postel Vinay MC, Cohen Tanugi E, Charrier J. Growth hormone receptors in rat liver membranes: Effects of fasting and refeeding, and correlation with plasma somatomedin activity. *Mol Cell Endocrinol* 1982; **28**: 657–669.
- 216 Inagaki T, Lin VY, Goetz R, Mohammadi M, Mangelsdorf DJ, Kliewer SA. Inhibition of Growth Hormone Signaling by the Fasting-Induced Hormone FGF21. *Cell Metab* 2008; **8**: 77–83.

-
- 217 Wu S, Grunwald T, Kharitonov A, Dam J, Jockers R, De Luca F. Increased expression of fibroblast growth factor 21 (FGF21) during chronic undernutrition causes growth hormone insensitivity in chondrocytes by inducing leptin receptor overlapping transcript (LEPROT) and leptin receptor overlapping transcript-like 1 (LE). *J Biol Chem* 2013; **288**: 27375–83.
- 218 Pizarro A, Hayer K, Lahens NF, Hogenesch JB. CircaDB: A database of mammalian circadian gene expression profiles. *Nucleic Acids Res* 2013; **41**. doi:10.1093/nar/gks1161.
- 219 Hughes ME, DiTacchio L, Hayes KR, Vollmers C, Pulivarthi S, Baggs JE *et al*. Harmonics of circadian gene transcription in mammals. *PLoS Genet* 2009; **5**. doi:10.1371/journal.pgen.1000442.
- 220 Oishi K, Uchida D, Ishida N. Circadian expression of FGF21 is induced by PPAR α activation in the mouse liver. *FEBS Lett* 2008; **582**: 3639–3642.
- 221 Itoh E, Iida K, del Rincon JP, Kim D-S, Thorner MO. Diurnal variation in growth hormone receptor messenger ribonucleic acid in liver and skeletal muscle of lit/+ and lit/lit mice. *Endocr J* 2004; **51**: 529–535.
- 222 Huang N, Chelliah Y, Shan Y, Taylor CA, Yoo S-H, Partch C *et al*. Crystal Structure of the Heterodimeric CLOCK:BMAL1 Transcriptional Activator Complex. *Science* (80-.). 2012; **337**: 189–194.
- 223 Ptitsyn A a, Gimble JM. Analysis of circadian pattern reveals tissue-specific alternative transcription in leptin signaling pathway. *BMC Bioinformatics* 2007; **8 Suppl 7**: S15.
- 224 Zhang EE, Liu AC, Hirota T, Miraglia LJ, Welch G, Pongsawakul PY *et al*. A genome-wide RNAi screen for modifiers of the circadian clock in human cells. *Cell* 2009; **139**: 199–210.

