Identification of Novel Transcription Factors That Modulate BMAL1:CLOCK Transactivation

by

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Koç University

Graduate School of Sciences and Engineering

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ABSTRACT

Circadian clocks are self-sustained time-keeping systems that generate circadian rhythms with a period of approximately 24 hours. Circadian clocks are internal pacemakers that influence human physiology, endocrinology, xenobiotic detoxification, cell growth, and behavior. In mammals, the circadian clock mechanism involves several proteins that participate in positive and negative transcriptional feedback loops. Proteins involved in the positive feedback loop include BMAL1 and CLOCK proteins. These proteins form heterodimers and bind to E-box elements (CACGTG) in promoter of period (*Per*), cryptochrome (*Cry*) and other clock-controlled genes. PER and CRY proteins form heterodimers that interact with casein kinase I ε (CKIε) and then translocate into the nucleus where CRY acts as a negative regulator of BMAL1:CLOCK driven transcription. Genetic studies on mouse indicated that indeed there are more core clock components to regulate core clock at the molecular level. To identify components that have an effect on the BMAL1:CLOCK transactivation, high-throughput luciferase reporter assay was utilized to screen 1400 mammalian transcription factors. Initial screening showed that WW domain-containing transcription regulator protein 1 (WWTR1) is one of the top candidates that showed high repression activity for BMAL1:CLOCK driven transcription on *Per1* promoter. Herein, I demonstrate that this repression activity is achieved by physical interaction of the WWTR1 with Bmal1 protein with co-immunoprecipitation and bi-molecular fluorescence complementation assay (BiFC). To see its effect on the circadian clock, *Wwtr1* was downregulated by shRNA in NIH3T3 Per1: *dluc* and U2-OS Bmal1: *dluc* cell lines. There was damping in amplitude and advance in phase of oscillation of rhythm in both cell lines. Additionally, knockdown of *Wwtr1* affected the transcriptional regulation of the core clock genes, especially transcription of the *Bmal1* and *Cry1* genes. Furthermore, WWTR1 appears to acts as co-activator on *Cry1* and *Bmal1* promoters. ChIP analysis also demonstrates WWTR1 occupation on *Cry1* promoter. Collectively all these results suggest a potential new core clock component, WWTR1, for the regulation of circadian rhythms by repressing BMAL1:CLOCK transactivation and by regulating *Bmal1* and *Cry1* transcriptional level.

ÖZETÇE

Sirkadiyen saatler kendi devamlılığını sağlayabilen ve yaklaşık olarak 24 saatlik periyotlarla günlük salınımı meydana getiren sitemlerdir. Sirkadiyen saatler insan fizyolojisini, endokronolojisini, karaciğer detoksifikasyonunu, hücre büyümesi ve davranışını etkiler. Memelilerde, sirkadiyen saat mekanizması pozitif ve negatif transkripsiyonel geri besleme döngülerine katılan birçok protein içermektedir. Pozitif geri besleme döngüsü BMAL1 ve CLOCK proteinlerini içerir. BMAL1 ve CLOCK proteinlerinin oluşturduğu heterodimer periyot (*Per*), kriptokrom (*Cry*) ve saat kontrolündeki diğer genlerin promotörlerinde E-box (CACGTG) bölgesine bağlanır. PER ve CRY proteinlerinin oluşturdukları heterodimer kazein kinaz I ε (CKIε) ile etkileşip çekirdeğe girer ve burada CRY, BMAL1:CLOCK 'a bağlı transkripsiyonun negatif düzenleyicisi olarak davranır. Fare üzerindeki genetik çalışmalar, ana saati moleküler seviyede düzenleyen daha birçok saat bileşeninin olduğunu gösterdi. BMAL1:CLOCK transaktivasyonu üzerinde etkili olan bileşenleri tanımlamak amacıyla yüksek kapasiteli (high troughput) lusiferaz tarama yöntemi kullanılarak 1400 adet memeli transkripsiyon faktörü taranmıştır. İlk tarama, WW bölgesi içeren transkripsiyon düzenleyici protein 1 (WWTR1)'in *Per1* promotörü üzerindeki BMAL1:CLOCK transkripsiyon aktivitesini yüksek oranda baskılayan en iyi aday proteinlerden biri olduğunu gösterdi. Bu çalışmada, ko-immünopresipitasyon ve iki molekül floresan tamamlama analizini(BiFC) kullanarak WWTR1'in bu baskı faaliyetini Bmal1 ile fiziksel iletişime girerek gerçekleştirdiğini kanıtladım. WWTR1 proteininin sirkadiyen saatteki etkisini görmek için, NIH3T3 Per1: *dluc* ve U2-OS Bmal1: *dluc* hücre hatlarında Wwtr1 shRNA kullanılarak baskılandı. Her iki hücre hattında salınımın ritm genliğinde düşüş ve daha erken faz kaydedildi. Ek olarak, *Wwtr1* ifadesinin düşüşü, çekirdek saat genlerinin transkripsiyonel düzenlenişini, özellikle *Bmal1* ve *Cry1* transkripsiyonunu etkiledi. Dahası, WWTR1; *Cry1* ve *Bmal1* promotörleri üzerinde yardımcı aktivatör gibi davranmaktadır. ChIP analizi ayrıca Cry1 promotörünün WWTR1 tarafından etkilendiğini gösterdi. Bütün bu sonuçlar, WWTR1'in BMAL1:CLOCK transaktivasyonunu baskılayarak ve *Bmal1* ile *Cry1* transkripsiyonel seviyesini düzenleyerek sirkadiyen ritmi düzenlediğini ve bu sebeple potansiyel yeni bir saat bileşeni olabileceğini önermektedir.

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NOMENCLATURE

AVP, arginine vasopressin

Arntl1, Aryl hydrocarbon receptor nuclear translocator-like protein 1

AVP arginine vasopressin

ASPS advanced sleep phase disorder

bHLH basic helix loop helix domain

BiFC bi-molecular fluorescence complementation

Bmal1, mouse brain-muscle-Arnt-like protein 1(italicized, gene/mRNA)

BMAL1, human brain-muscle-Arnt-like protein 1(italicized, gene/mRNA)

BHLH E40, Basic Helix-Loop-Helix Family Member E40

CCGs, clock-controlled genes

cDNA, complementary DNA

ChIP, chromatin immunoprecipitation

Clock, circadian locomotor output cycle kaput

CK1ε, casein kinase 1 epsilon

Cry, Cryptochrome

cAMP, cyclic adenosine monophosphate

Cdk1, cyclin-dependent kinase 1

CREB cAMP response element-binding protein

CT, circadian time

Ct, cycle threshold

DBP, D site of albumin promoter (albumin D-box) binding protein

DSPS delayed sleep phase disorder

DD, constant dark conditions

Dec1/2, Differentially Expressed in Chondrocytes 1

EMT, epithelial-mesenchymal transition

E4bp4, E4 promoter-binding protein 4

GABA, Gamma-Amino Butyric acid

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GPCRs, G protein-coupled receptors

Gsk3β glycogen synthase kinase 3 beta

HAT histone acetyl transferase

HIFs, hypoxia-inducible factors

h, hour

HLF family hepatic leukemia factor

ipRGC, intrinsically photoreceptive retinal ganglion cells

LD, light-dark cycle

mRNA, messenger RNA

MLL1, myeloid/lymphoid or mixed-lineage leukemia enzyme

MOP3, Member of PAS Superfamily 3

NAD/NADH Nicotinamide adenine dinucleotide

Nfil3, Nuclear factor, interleukin 3 regulated

Per1: luc, mouse Period1: Luciferase gene construct

PACAP, pituitary adenyl cyclase activation peptide

ParbZip, proline and acidic amino acid rich basic leucine zipper

PAS, per-arnt-sim domain

ROR, RAR-related orphan receptor

Rev-erb, nuclear receptor subfamily 1, group D

RORE, RAR-related orphan receptor response element

PCR, polymerase chain reaction

Per, period (italicized, gene/mRNA)

qRT-PCR, quantitative reverse-transcription polymerase chain reaction

RHT, retinohypothalamic tract

ROR, retinoid-related orphan receptor

RT, room temperature

SCN, suprachiasmatic nucleus of the hypothalamus

SEM, standard error of the mean

shRNA, short hairpin RNA

siRNA, small interfering RNA

Sirt1, silent mating type information regulation 2 homolog aka sirtuin 1

Stra13, Stimulated By Retinoic Acid Gene 13 Protein

TAD, transactivating domain

TAZ, transcriptional coactivator with PDZ-binding motif;

TEADs, TEA domain transcription factors;

TTF1, thyroid transcription factor 1;

TTL, transcriptional-translational feedback loop

TEF, thyrotroph embryonic factor

U2-OS-B6, human osteosarcomaU2-OS cell reporter line

VPAC2, Vasoactive intestinal peptide receptor 2

VIP, vasoactive intestinal peptide

WWTR1, domain containing transcription regulator 1;

YAP, yes-associated protein

ZT, zeitgeber time

Chapter 1

INTRODUCTION

Circadian rhythms are biological processes that display an endogenous, entrainable oscillation of about 24 hours and have been widely observed in plants, animals, fungi and cyanobacteria. Circadian clocks are the endogenous biochemical timekeeping mechanisms that generate circadian rhythms to anticipate 24-hour environmental light/dark cycle and to adapt organisms to the external conditions [1].

These cell-autonomous clocks are synchronized by both endogenous and external signals to regulate their own molecular rhythm. In mammals, circadian oscillators are hierarchically organized into the central, suprachiasmatic nuclei (SCN), and the peripheral clocks. Circadian mechanisms drive the rhythmic expression of genes that are involved in physiology, behavior and biochemical events [2, 3] [4]. Dysregulation in circadian clocks is associated with the onset and development of numerous human diseases including sleep disorders, depression, metabolic and psychological disorders, cardiovascular problems and neurological disorders as well as cancer as seen in shift workers [4-7] [8-10].

Similar molecular mechanisms for the circadian clocks seem to be conserved across species with a common central mechanism. In mammals, the circadian clock is composed of an autoregulatory transcriptional network with two interlocked transcriptional-translational feedback loop (TTFL) and the oscillation in posttranslational modifications of proteins [4, 11-14]. The TTFL comprises of a positive and a negative limb interconnected and it takes approximately 24 hours to complete loop [15]. In the primary feedback loop the positive transcriptional elements include two bHLH (basic Helix-Loop-Helix) PAS-domain containing transcription factors, CLOCK (Circadian locomotor output cycles kaput) and BMAL1 (Brain and Muscle ARNT-Like-1) [16, 17]. CLOCK:BMAL1 heterodimers binds 'canonical' E-box enhancer-elements (CACGTG) in the promoter of *Period* (*Per1, Per2, Per3)* [18] and *Cryptochrome* (*Cry1, Cry2)* [19] genes that constitute the negative portion of the TTFL. The rhythmic expression of *Bmal1* is also subject to circadian clock regulation by transcriptional repressor REV-ERBα and the activator RORα [20, 21].

Recently, the canonical model has been reevaluated by finding missing component in circadian mechanism. Using high throughput luciferase assay system, 1400 transcription factors (TFs) were screened to determine their activator or repressor activity on BMAL1:CLOCK transactivation. In this thesis, five TFs that ameliorate BMAL1:CLOCK transactivation were selected and one of these, WWTR1 was undergone in more detailed analysis to define its function in circadian clock mechanism. Our results showed that WWTR1 represses BMAL1:CLOCK transactivation through interacting with BMAL1 proteins. On the contrary, WWTR1 display positive regulation on *Cry1* and *Bmal1* promoter regions indicating differential function in clock mechanism. Knockdown of *Wwtr1* influences the circadian rhythmicity in cells line; NIH 3T3 via changing circadian expression of clock proteins. Finally, we showed that WWTR1 exert its co-activation function on *Cry1* promoter using ChIP assay.

Chapter 2 gives literature review about what circadian rhythm and molecular clock mechanism and importance of circadian regulation on mammal's physiology, behavior, and biochemical events.

Chapter 3 states the rational and the aim of this study.

Chapter 4 provides detailed explanation about materials and methods used in this work.

Chapter 5 addresses detailed explanation of results of this thesis for characterization of TF, WWTR1 to demonstrate its clock related function.

In Chapter 6 and 7, obtained results are discussed and concluded with future perspectives.

Chapter 2

LITERATURE REVIEW

2.1 Biological Rhythms

Biological rhythms are periodic biological changes in an organism in response to periodic environmental changes. These changes can be position of Earth to the Sun that alters day and night time and to the Moon that alters tide level [22]. Biological rhythms are classified as circadian rhythms (in Latin “circa diem”, “approximately a day”), diurnal rhythms, ultradian rhythms, and infradian rhythms. Circadian rhythms are endogenously repeated cycles with a period around 24 hours (e.g. sleep/wakefulness cycle, body temperature, blood pressure patterns of hormone secretion, digestive secretions). Diurnal rhythms are circadian rhythms synchronized with the day/night cycle. Ultradian rhythms are biological rhythms that shows much shorter period than 24 hours (e.g. breathing, hearth rate, urination feeding cycles), while infradian rhythms are biological rhythms with a cycle of more than 24 hours (e.g. seasonal hibernation of animals, seasonal migration of animals, reproductive cycles of plants, the human menstrual cycle) [22, 23]. Of these, circadian rhythms will be introduced in more detail.

2.2 Circadian Rhythms - an Overview

Circadian rhythms are biological processes that display an endogenous, entrainable oscillation of about 24 hours. Circadian rhythms display four unique characteristics. First, circadian rhythms are endogenous time-keeping systems encoded by the genome. Second, circadian rhythms are self-sustained systems that persist in the absence of external cues with the expression of genes in a rhythmic fashion[24]. Third property is that circadian rhythms are entrainable and can be reset by exposure to external stimuli (such as light), a process called entrainment. Finally, oscillations have an ability to maintain circadian periodicity over a range of physiological temperatures known as temperature compensation that is important especially in poikilotherm organisms[25]. Circadian rhythm is driven by circadian clock or circadian oscillator, an endogenous biochemical timekeeping mechanism. Circadian oscillator

found in a diverse range of cell types in a variety of organisms from prokaryotic cyanobacteria and eukaryotic unicellular organisms to plants, insects and mammals[26]. This central mechanism generates circadian rhythms with a period of 23-25 hours depending on species to anticipate 24 hour environmental light/dark cycle and adapt organisms to the external conditions that come with the Earth's daily rotation around its axis[1]. Circadian clock protects organism against environmental changes and confer fitness during the evolution and provide an important selective advantage [27-30]. Prokaryotic cells evolved ability to get optimized benefits to survive on Earth using the most important zeitgeber, or timing cue, light. *Neurospora crassa* or cyanobacteria use their clocks to arrange metabolism and growth temporally. Photoreceptors let cyanobacteria use light for photosynthesis while protect them from ultraviolet radiation[31]. Circadian clocks convey an evolutionary advantage to higher eukaryotes to regulate behavioral, physiological and metabolic rhythms during the day. Such advantages can protect organisms from predators, and enable them to save resources and energy [32], enhancement in efficiency of metabolic processes[33], protection against mutations and cancers by regulating cell cycle and DNA damage [33] [34, 35].

2.3 Molecular Mechanisms of the Circadian Clocks

The circadian clocks are organized into three components: An input pathway, a feedback loop: a central biochemical mechanism, and an output pathway. Central biochemical oscillator can be entrained with external inputs to have a period of about 24 hours. In central biochemical mechanism, circadian mechanisms drive the rhythmic expression of genes that generate output of the circadian clock involved in physiology, behavior, cognitive processes, and biochemical events [2, 3, 36] [4]. Similar central mechanisms seem to be conserved across species especially in all eukaryotes. Central mechanism consists of a set of core clock genes of which protein products have similar roles with structural differences [37] [38]. Circadian clocks of all studied organisms are composed of a core transcription/translation feedback (auto-regulatory) loop (TTFL) with multiple interconnected loops to generate approximately a 24-hour periodicity. Fine tuning of 24-hour rhythmicity is achieved via post-transcriptional, posttranslational and epigenetic modifications[39] [40].

2.3.1 The Circadian Clock in Different Organisms

A unicellular cyanobacterium, *Synechococcus elongatus* is the simplest and the most ancient organism that possess a true circadian clock. Studies of the circadian rhythm in this organism showed that the repression is not necessary [3]. Three proteins (KaiA, KaiB, and KaiC- in Japanese "Kai", "cycle") of their central oscillator control cell division, amino acid uptake, nitrogen fixation, photosynthesis, and respiration processes. Circadian oscillation of accumulation of RNA and phosphorylation of clock proteins are observed in cyanobacteria. [41].

The circadian clock of *Neurospora crassa*, a filamentous fungus, consists of a positive element, the white collar complex (WCC) formed by heterodimerization of two nuclear transcription factors; white-collar 1 (WC-1) and white-collar 2 (WC-2), and a negative element, the frequency (FRQ)[42]. Asexual spores (conidia) formation called conidiation is controlled by the circadian clock.

Arabidopsis thaliana, the plant clock consists of several interlocked loops; a central loop with morning and evening feedback loops that are formed by the interaction of transcription factors acting as repressors. Briefly, transcription factors; late and elongated hypocotyl (LHY), circadian clock associates 1 (CCA1), function in central loop and repress time of cab expression 1 (TOC1) that acts as positive regulator of CCA1 and LHY in the morning. Pseudo response regulators (PRR); PRR7, and PRR5 are player of the morning cycle and repress transcription of CCA1, LHY. The evening loop proteins function in ubiquitination and degradation of both TOC1 and PRR5 [43-45]. The circadian clock regulates key pathways involved in biological processes such as leaf movement[46], hormone biosynthesis[47], water uptake[48], and pathogen response [49] and confers photosynthetic growth, survival, and competitive advantages to plants [49]. Plants sense light via phytochromes (red and far-red photoreceptors), cryptochromes [49] and the proteins of the Zeitlupe (ZTL) family (blue-light receptors) [50] to entrain the circadian clock.

In *Drosophila melanogaster*, circadian clock proteins; Clock (CLK) and Cycle (CYC) forms a heterodimer and binds to E-boxes (CACGTG) at target promoter of circadian genes and activate the transcription of genes; such as Period (PER) and Timeless (TIM) at midday. At dark, TIM protein binds to PER forming a dimer to protect PER from degradation because of phosphorylation by double-time kinase (DBT) and subsequent ubiquitination by

supernumerary limbs (SLIMB). PER: TIM dimers enter nucleus where they repress their own transcription, acting as negative elements of the feedback loop [20]. Beside to conical loop, secondary loops exist and regulate CLK/CYC expression via clockwork orange (CWO) and Vrille (VRI) as repressor and PAR-domain protein 1ε (PDP1ε) as activator [51, 52]. *Drosophila* senses light through different photoreceptive organs: the complex eyes, the ocelli, and the Hofbauer-Buchner eyelet, containing different opsin-based photopigments [53]. Blue-light photopigment cryptochrome (CRY) is found in the compound eyes and dorsal neuronal groups. It senses to light and functions in synchronization process. Posttranslational modifications and interaction of the CRY with TIM and PER lead to ubiquitination and proteasome dependent degradation of TIM, suppression of clock's negative feedback loop [54] [55, 56].

In vertebrates, in contrast to invertebrate clock, clock mechanism needs extra copies of key clock genes such as period and cryptochrome genes with diverse functions and expression patterns. The clock mechanism among species of vertebrates is highly conserved with minor differences [57] [58]. As a model organism, zebrafish (*Danio rerio*) is mostly studied for molecular clock during embryogenesis and the light input pathway. In zebrafish, beside to SCN, peripheral clocks are also entrained by light through one of the candidate, Cry homologs, Cry4[59]. In our study, I focused on the mammalian circadian clock that will be discussed in more detail in below part.

2.4 Overview of the Mammalian Clock System

2.4.1 Suprachiasmatic Nucleus (SCN) and Perception of Environmental Stimulus

Circadian oscillators are ubiquitous in tissues of the mammalian body and hierarchically organized into the central, SCN (suprachiasmatic nuclei) and the peripheral clocks. The SCN is located in nuclei just above the optic chiasm in the hypothalamus, as a pacemaker. The SCN is a bilaterally paired neuronal organization that consists a small cluster of neurons (~20,000) located in a ventral core region and a dorsal shell region [59, 60]. Synchronization of multiple clock cells within the SCN is one of the most important characteristic that distinguish the SCN from peripheral clocks[61]. The SCN orchestrates the other clocks in subordinate organ and tissue using direct neural signals through autonomic neural connections, neuroendocrine signals such as glucocorticoids, and indirect metabolic

signals such as circadian body temperature and food intake. SCN synchronizes physiological processes precisely within organs for a robust, accurate timekeeping [62-65].

Although circadian rhythms are endogenous (self-sustained), they are adjusted (entrained) to the local environment by external cues defined as zeitgebers, the most important of which is daylight and also feeding, emotional stress [29], exercise [68], social signals [69], temperature cycles [70], and sleep deprivation[29, 71]. The SCN is a direct target of the retinal fibers [11] that sense light as input. Thereby, the SCN entrains the circadian system to the environmental light/dark cycle [66] and acts such a transmitter between the external light/dark cycle and the internal circadian clocks to adapt organism to its environment[67]. SCN functions to coordinate organism's behavior and physiology through sleep/wakefulness, glucose homeostasis, hormonal and body temperature regulation, blood pressure. Light as prominent stimuli serves to entrain the central oscillator (SCN) to the environmental light-dark cycle. Light sensitive compartments in multicellular organism such as zebrafish and *Drosophila* can be specific nonvisual light receptors in each cell of the body. Beside to photoreceptors, flies also have CRY protein as a photoreceptor for synchronization of core and peripheral tissue clocks. In contrast, in mammals, to detect environmental light, three types of photoreceptors are located only in the outer retina of eye: Intrinsically photosensitive retinal ganglion cells (ipRGCs), the rods and cones. ipRGCs express the photopigment melanopsin(MOP), a photosensitive G-protein coupled receptor (GPCR) responding to light independently of rod/cone receptors[72-74]. Melanopsin is excited maximally by light from the blue part of the spectrum (480 nm). More recent works also proved that rods and cones also transmit light signal to the SCN with property of more sensitive to low-intensity light than melanopsin, which means the circadian system is affected by the wider range of light intensities [75, 76]. ipRGCs project photic information directly to the SCN via pathway called the retinohypothalamic tract (RHT) formed by the ipRGCs axons [77, 78]. The mechanisms include neurochemical signals such as glutamate (Glu) and pituitary adenylate cyclase-activating protein (PACAP), arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP), α-aminobutyric acid GABA, ghrelin-releasing peptide (GRP), and gap junctions. Light stimulate the release of the neurotransmitters Glu and PACAP at the terminal synapses of the RHT, and the signal is then propagated to the SCN [79] [80]. Glu activates the glutamate receptors, N-methyl-D-aspartate (NMDA) and AMPA,

which leads to membrane depolarization. Subsequently, calcium flows into the cytoplasm directly through NMDA receptors and indirectly through voltage-gated calcium channels [81]. Calcium flow activates the transcription factor cAMP-response element binding proteins that evoke chromatin remodeling and the induction of immediate early genes and clock genes in the SCN including *Per1* and *Per2* possessing cAMP response elements (CRE) in their promoters [82]. GABA is another common neurotransmitter released by nearly all the SCN neurons. The monosynaptic RHT fibers terminate in the ventrolateral part (core) of the SCN which contains VIP that is the most abundant neuropeptide transmitter within the core region of the SCN. Release of VIP from the ventral part of the SCN closes potassium channels leading to membrane depolarization of the SCN neurons [83, 84] [85] and induces *Per1* and *Per2* expression [86]. VIP and GABA neurotransmitters are responsible for phase adjustment or synchronization and response to rapid light–dark cycle change (jet-lag) in the SCN[87] [88]. Perceived retinal information is then transmits to the SCN's neuronal network; AVP containing the dorsal (shell) part of the SCN [89, 90] (Figure 1).

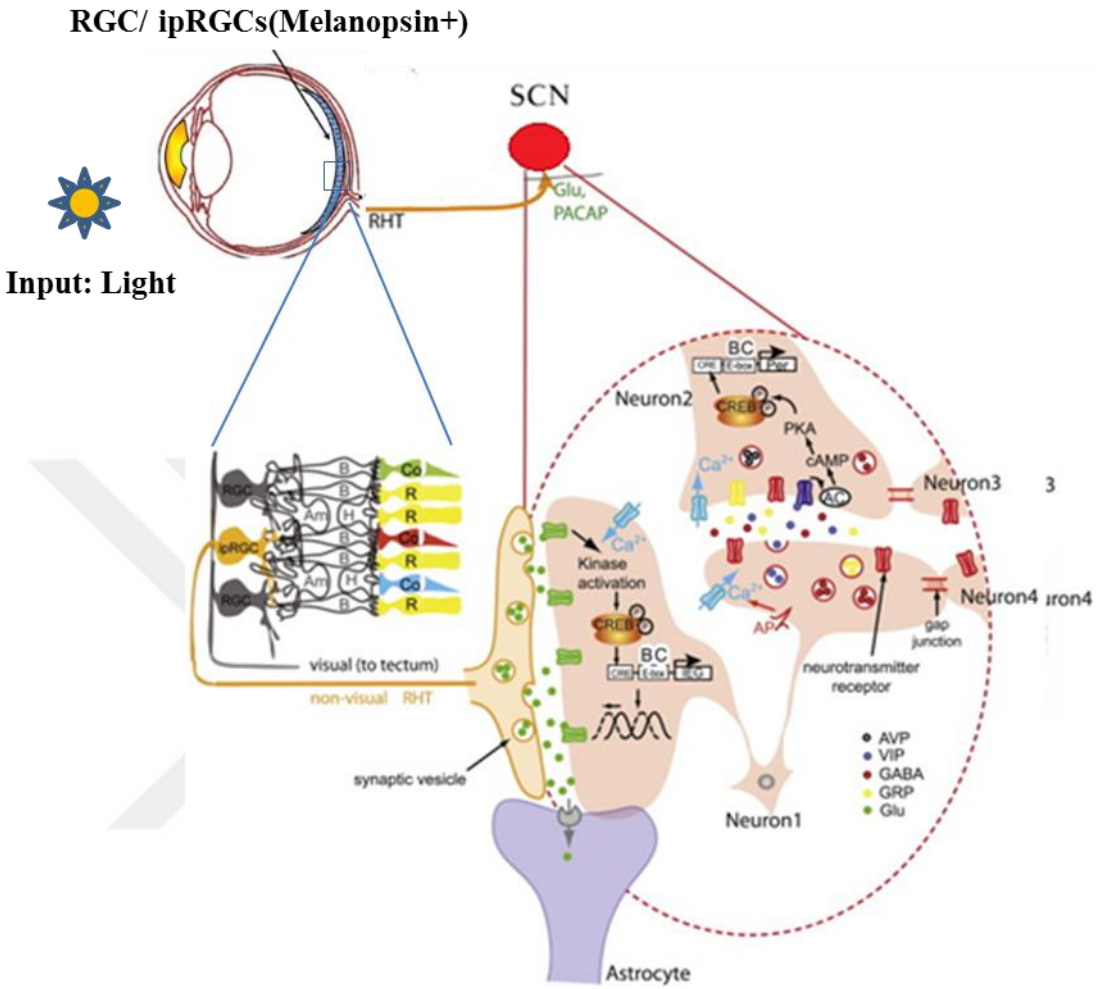


Figure 1: Representation of light input and intercellular signaling mechanisms in the SCN. Orange arrows are for photic input, and blue arrows are for nonphotic input to the SCN. Intrinsically photosensitive retinal ganglion cells (ipRGCs), retinohypothalamic tract (RHT), regular retinal ganglion cells (RGC). Adapted from [4]. Copyright 2012, with permission from Elsevier.

2.4.2 Peripheral Clocks

Peripheral clocks are dispersed throughout most organs and both neural and non-neural tissues of an animal including adrenal gland, oesophagus, lungs, liver, pancreas, spleen, thymus, skin except for the thymus and testis and also in cultured fibroblast cells such as NIH 3T3 cells[39, 91-93] [61, 94] [95]. In peripheral clocks, metabolic processes include glucose, lipid, bile acid and amino acid metabolism, fat and muscle mass regulation, oxidative and xenobiotic metabolism are coordinated with the environment light-dark cycle to ensure for instance, synchronization of digestive function and detoxication and food intake rhythmicity or diurnal cardiac function and mitochondrial energy production rhythmicity. All are achieved by circadian control of peripheral clocks by SCN to optimize these physiological processes [96, 97].

Peripheral tissues have their own endogenous self-sustained clock mechanisms but depend on SCN and tissue-specific factors for coordination or synchronization. Local mechanisms that regulate the tissue-specific gene expression patterns are food availability, hormone metabolites, temperature and TFs such as cAMP response element–binding protein (CREB). These factors can also reset the peripheral clock phases beside to SCN signals [4, 98]. By contrast to the master clock, the peripheral clocks phases can be more easily reset by the ingestion of food. Actually, food sensitivity differs among peripheral clocks, for instance, liver sensitivity to feeding/fasting cycles is higher than lung[99]. Without changing SCN clocks, in the liver, circadian clocks can be entrained by food availability or quality through nutrient sensing pathways. Circadian clock influence on metabolism such as energy metabolism is driven by AMPK (5' adenosine monophosphate (AMP)-activated protein kinase), the PPAR proteins (Peroxisome proliferator-activated receptors) and SIRT1 which sense AMP/ATP, ADP/ATP and NAD(P)+/NAD(P)/H ratios as nutrient sensors or as indicator of metabolism state modulated by feeding/fasting behavior. AMP protein kinase (AMPK) which possesses circadian expression is activated by intracellular nutrient restrictions; high ratio of AMP/ATP and ADP/ATP and low cellular energy status. Activated AMPK phosphorylates CRY1 and CK1ε causing to degradation of transcriptional repressors, CRY1 and indirectly PER2. Thereby, nutrient signals influence back circadian clock and lead to lengthen and shorten the period of the circadian oscillations[100, 101]. Interestingly, the AMPK phosphorylation site on CRY1 is absent in the organisms in which CRYs act as blue

light receptors. In these organisms CRYs degradation is induced with light that is replaced by AMPK-dependent degradation to entrain the clock in such organisms where CRYs cannot directly sense light signals anymore[102]. Another important link between circadian clock and metabolism state are the PPARs; PPARα, PPARδ, and PPARγ, having tissue-specific rhythms in mouse. PPARα which cycles in white, brown adipose tissue and liver but not in muscle is induced during fasting and so increase hepatic fatty acid oxidation and ketogenesis to adapt organism to fasting [103]. PPARγ which is selectively oscillate in white adipose tissue and liver is most abundant in adipose and activates lipid storage and lipogenesis. Conversely, rhythmic expression of PPARδ is seen in brown adipose tissue and also in liver tissues. Rhythmic influence of PPARs on clock mechanism is achieved through activation of *Nr1d1* promoter in adipose tissue of mice [104] while transcriptional activation of PPARγ activity is inhibited by PER2[105]. In addition to REV-ERBs and RORs, *Bmal1* promoter is also regulated by PPARα [106]. A key coactivator of PPAR, PGC-1 also cycles in skeletal muscle of mice besides to liver, brown adipose tissue and stimulates BMAL1 and REV-ERBα, through co-activation of the RORs [107]. In contrast to BMAL1 activators, TIeG1/KLF10/mGIF exert their repression effects by direct binding to a GC box in the *Bmal1* promoter. Feeding starved mice or adding glucose to cultured rat fibroblasts *in vitro* causes activation of TIeG1/KLF10/mGIF [108]. Another way to mediate communication between clock and nutritional state is sirtuin 1 (SIRT1) protein, a deacetylase that senses NAD+ ratio. Sirtuins need to bind its coenzyme, NAD+ for deacetylases activity. Thus, especially SIRT1 has been implicated to have important role between circadian clocks and energy metabolism via regulating glucose and lipid homeostasis, mitochondrial function and fatty-acid oxidation, oxidative stress, lifespan extension, neurodegeneration, and memory formation in mouse [109-114]. SIRT1 senses small cellular molecule NAD+ levels cycling in circadian manner and that's why, levels and activity of SIRT1 oscillate in mice. NAD+ is synthesized from nicotinamide (NAM) by an enzyme, nicotinamide phosphoribosyl transferase (NAMPT) that is under control of BMAL1/CLOCK activity. Since NAD+ generation is under control of circadian clock, all NAD+ dependent metabolic pathways also affected by circadian regulations[115, 116]. On the other hand, SIRT1 inhibits CLOCK-mediated acetylation of histone H3 and BMAL1 and leads to PER2 degradation via deacetylation [117, 118]. Thereby, activity of SIRT1 dampens the amplitude of rhythmic transcription and regulates the circadian physiology. SIRT1 is promising target for prevention of obesity and aging [119-121]. In

addition to histone proteins, Sirtuins can deacetylate other proteins, such as transcription factors. For instance, SIRT1 (Sirtuin 1) rhythmically acts in opposition to acetylation to control genes such as FOXO (Forkhead Box, subclass O) protein family PGC-1, PPAR (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1A), and the nuclear receptor LXR (Liver X Nuclear Receptor), thereby involved in many critical metabolic and physiological processes[122, 123]. NAD+ level is also under effect of poly (ADP-ribose) polymerases (PARPs) that synthesize ADP polymers from the ADP-ribose group of NAD+. PARP1 use the cellular NAD+ in response to DNA damage which means PARP1 activity also cycle with feeding through rhythmic NAD+ level[116, 124]. Moreover, PARP1 poly -ADP-ribosylates CLOCK through interaction with BMAL1/CLOCK [124]. In addition to these regulations, redox state that arises from metabolic oscillators also regulates activity of clock transcription factors. Peroxiredoxin proteins are redox-sensitive proteins that exhibit self-sustained circadian oscillation from archaebacteria, plants to other eukaryotic cells [125, 126]. It is suggested that mammals use oscillations in peroxiredoxin redox state to anticipate the generation of ROS[127].

In addition to food availability, oscillating levels of hormones including insulin and glucagon from pancreas, adiponectin and leptin from adipose, and ghrelin from stomach control many metabolic processes through control of circadian alterations[128]. Insulin produced by β-cells of the pancreas is under control of circadian system, and also studies showed that insulin acts to fire rate of clock in neurons [129, 130]. Melatonin, a lipophilic hormone, is synthesized by the pineal gland and under the control of suprachiasmatic cues and secreted at night in both diurnal and nocturnal animals [131]. Since melatonin is secreted during night-time, it adjusts energy metabolism and reproduction in photoperiodic species for seasonal changes in day length [132]. Leptin is produced in adipocytes rhythmically under control of suprachiasmatic and adipocyte clocks. This hormone acts on the mediobasal hypothalamus for food intake inhibition and influences peripheral metabolism of glucose and lipids [133, 134]. Ghrelin is synthesized in the oxyntic cells of the stomach and secreted during the resting period in rats[135]. It activates neurons of the arcuate nuclei containing neuropeptide Y and agouti-related peptide[129]. Of note, ghrelin can reset the master clock *in vitro* and can cause phase shift in fasted animal *in vivo*[136]. Glucocorticoids are mostly attracted the attention as an entraining signal for peripheral clocks. Glucocorticoids are such

as cortisol produced in the adrenal glands from cholesterol and rhythmically released at ultradian with circadian manner with highest level at dawn in diurnal species. Glucocorticoids are able to orchestrate the rhythmic expression of many metabolic and clock genes in the liver even in the presence of a lesioned SCN. The core clock genes, *Bmal1*, *Cry1*, *Per1* and *Per2* have glucocorticoid-response elements in their regulatory regions. Glucocorticoids induce the nuclear receptors including peroxisome proliferator-activated receptor (PPARα), and hepatic transcription factor HNF4α that is controlled by BMAL1:CLOCK complex through its E-boxes. These glucocorticoid receptors act as transcriptional activators of PER2.[137-139]. PPARα is a well-known regulator of the transcription of *Bmal1* [140].

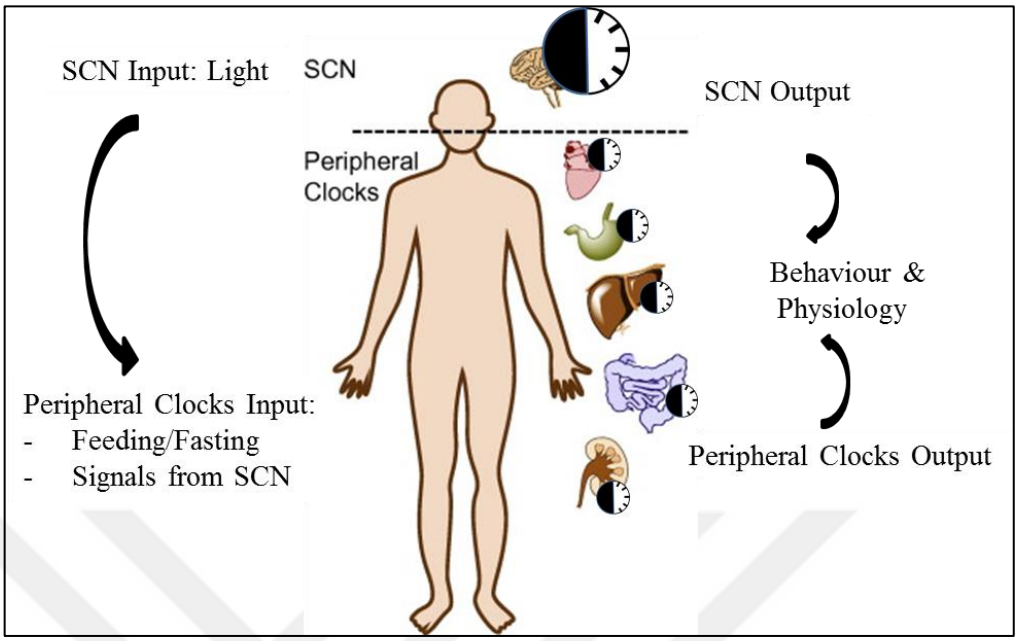


Figure 2: Circadian clocks exist both in the central nervous system and in the peripheral tissues. The figure illustrates the master clock (suprachiasmatic nucleus (SCN)) that is entrained via light while the peripheral clocks can be entrained by signals from the SCN as well as other signals such as nutrient availability. Adapted from[141], copyright 2013, with permission from Elsevier.

2.4.3 Molecular Mechanism of Circadian Clocks in Mammals

2.4.3.1 The History of Mammalian Clock Genetics

The existence of a circadian clock has been first defined in plants, in 1729, by French astronomer Jean-Jacques d'Ortous De Mairan. He recognized persisted 24 hour open and close movement of *Mimosa pudica* leaves even in the dark. After many years, in 1960s, Aschoff and Pittendrigh (the father of the biological clock) established characteristics of circadian rhythms [142]. In 1971, Konopka and Benzer opened the modern era of circadian genetics by discovering circadian clock mechanism in fruit fly (*Drosophila melanogaster)*. They defined the first component of molecular clocks, *Period* gene (*Per*) in *Drosophila* with mutagenesis studies[143]. Discovery of period gene in this organism opens the way of circadian genetics. Clock components in this fruit fly are highly conserved, which provides the discovery of corresponding clock component in zebrafish, mouse and humans. Three mouse orthologs (*Per1, Per2,* and *Per3*) of the *Drosophila Period* gene, two *Cryptochrome* orthologs (*Cry1 and Cry2*), and brain and muscle ARNT-like protein 1 (*Bmal1*), orthologue of CYC in *Drosophila,* were cloned using homology with the corresponding orthologs in other organism or paralogs in the same organism [144-146] [147] [148]. Another breakthroughs in the beginning of the mammalian clock genetics was identification of *tau* mutant, a single outbred Syrian hamster (*Mesocricetus auratus*) with endogenous free-running period of 22 hour that is less than wild type 23.5 hour periodicity [149]. After twelve years, Lowrey and his colleague reported that *tau* mutation caused by a single nucleotide change in the casein kinase I epsilon (*Ck1ε*) gene using positional synthetic cloning[150]. In 1994, using forward genetic screens, Takahashi group isolated the first single-gene mutation and identified *Clock* (circadian locomotor output cycles kaput) gene in mouse genome. Homozygous mutation affects circadian rhythms and lengthened free-running period (26–29 h) followed by a complete loss of circadian rhythmicity in constant conditions compared to wild-type mice (23.3–23.8 h) [151-153]. Clock gene encodes a novel member of the basic helix-loop-helix (bHLH)-PAS family of transcription factors. In the forebrain, the function of CLOCK is replaced by NPAS2 (Neuronal PAS domain protein-2, also called MOP4) [154] [155, 156]. bHLH-PAS proteins act in pairs to form homodimer or heterodimer with other bHLH-PAS domain containing binding partner. Hogenesch and his colleague characterized BMAL1 (MOP3) and screened against known bHLH-PAS proteins. BMAL1 acts as a heterodimeric

transcription factor with CLOCK and plays a prominent role in the generation and maintenance of circadian rhythms in animals[147] [148].

2.4.3.2 Mammalian Molecular Mechanism of the Circadian Clocks

Mammalian circadian rhythms have functional and conserved transcriptional regulatory mechanism. During the last decade, increasing numbers of so-called clock genes of transcriptional circuits for mammalian circadian rhythm are identified. The majority of these identified clock components are transcriptional activators or repressors including three basic helix-loop-helix (bHLH)-PAS (PER-ARNT-SIM) transcription factors CLOCK, NPAS2, and ARNTL1 known as BMAL1 or MOP3 [153] [155], two PERIOD (PER1 and PER2) [157], two CRYPTOCHROME proteins (CRY1 and CRY2) [19], casein kinase I epsilon and delta (CKIε and CKIδ)[158] and two retinoic acid-related orphan nuclear hormone receptors ; REV-ERBα also known as NR1D1(nuclear receptor subfamily 1, group D, member 1) and RORα (retinoid-related orphan receptor alpha)[159, 160]. A number of other transcriptional factors also thought to function in the circadian regulation but not essential for circadian rhythms generation. These proteins are four bZip-family proteins; DBP (D-box binding protein -DBP albumin D-site-binding protein), HLF (hepatic leukemia factor), and TEF (thyrotroph embryonic factor), and NFIL3 (nuclear factor, interleukin 3 regulated) also termed as E4BP4 (E4 promoter-binding protein 4)[161], three bHLH transcription factors; ARNTL2, also known as BMAL2 and DEC1 (Bhlhb2/Bhlhe40/Stra13/Sharp2) and DEC2 (Bhlhb3/Bhlhe41/Sharp1) [162], one period-related protein, PER3 (26), and three genes related to NR1D1 and RORα (NR1D2 (REV-ERBβ), RORβ, and RORγ) [21, 160]. These oscillatory genes are under the control of three types of clock-controlled elements (CCEs)—E-boxes (5’-CACGTG-3’), E’-boxes (5’-CACGTT-3’), D-boxes [5’-TTATG(C/T)AA-3’], and REV-ERBα/ROR-binding elements (RREs) [5’-(A/T)A(A/T)NT(A/G)GGTCA-3’] which are all cis-regulatory elements. Ueda et al. showed that functionally and evolutionarily conserved E/E’-boxes as morning-time elements are located on the noncoding regions of nine genes (*Per1, Per2, Cry1,Cry2, Dbp, Rorγ, Rev-Erbα/Nr1d1, Rev-Erbβ/Nr1d2, Dec1/Bhlhb2,* and *Dec2/Bhlhb3*); D-boxes are day-time elements and located on those of seven genes (*Per1, Per2, Per3, Rev-Erbα, Rev-Erbβ, Rorα,* and *Rorβ*); and RREs that are night-time elements located on those of six genes (*Bmal1/Arntl, Clock, Npas2, Cry1, E4bp4/Nfil3,* and

Rorγ) [21]. cAMP response elements (CREs) are also regulatory motifs that mediate rhythmic gene expression[163].

The circadian clock in mammals is composed of an auto-regulatory transcriptional network with two interlocked transcriptional-translational feedback loop (TTFL) and the oscillation in posttranslational modifications of proteins [4, 11-14]. The TTFL comprises of a positive and a negative interconnected limb [15]. The core transcriptional activators of the primary feedback loop (core loop) are two bHLH PAS-domain containing transcription factors, CLOCK and its binding partner BMAL1 [16, 17]. In the SCN neurons, the intracellular levels of CLOCK remain steady throughout the 24-hour period, whereas BMAL1 expression levels are high at the beginning of a subjective day and low at the beginning of a subjective night. In the positive limb of the mammalian system, the high level of transcriptional activator protein, BMAL1, promotes the formation of CLOCK: BMAL1 heterodimers through their tandem PAS domains, which also gives stability to the complex [164]. Formed heterodimers drive the transcription of central clock genes and clock control genes (CCG) such as DBP through binding to 'canonical' E-box enhancer-elements (CACGTG), within (or near) their promoter region [154, 165] and to some 'non-canonical' E-boxes (CANNTG), and some E-box like sequences (CACGTT) as well. BMAL1 and CLOCK heterodimers bind to the E-box with their amino-terminal end of the bHLH region in the basic domain. Histone acetyltransferases CBP/p300, the transcriptional coactivators of BMAL1, binds BMAL1 and activate transcription through acetylation of histone H3 at E-box sites (321-323). As CLOCK and BMAL1 activate expression of core clock genes; *Period* (*Per1, Per2, Per3*) [18] and *Cryptochrome* (*CRY1, CRY2*) [19] genes, whose transcripts and proteins slowly accumulate during the daytime, reach maximum at late circadian day and constitute the negative part of the TTFL, the primary feedback loop [166-172].As the mRNA level of *Per1, Per2, Cry1* and *Cry2* is translated in the cytoplasm, proteins of these genes accumulate in the cytosol and form a multimeric protein complex along with Casein Kinase I delta and epsilon (CKI δ/ε). This complex eventually translocate into the nucleus during the evening to inhibit their own transcription and that of other clock-controlled genes by physically interacting with the CLOCK: BMAL1 complex by recruiting the SIN3-HDAC histone deacetylase complex at histones 3 and 4 at activation phase. While tandem PAS domains of PER proteins are involved in homo- and heterodimer formation of PER proteins [173], a long,

disordered C-terminus (CBD; CRY‑binding domain) have binding sites for kinases and cryptochrome interaction, for instance, CRY proteins with CBD of PER2 protein[174, 175]. On the other hand, CRYs possess conserved photolyase homology region (PHR), an N-terminalα/β-domain, CC helix (periodic spacing of nonpolar residues common to coiled coils) and disordered C-terminal α-helical domain with flavin-binding site [176]. The C-termini contains phosphorylation sites and a nuclear localization signal. CC helix acts in competitive interaction with E3 ubiquitin ligase FBXL3 and PER through same binding sites by which degradation and stability of CRYs is controlled, respectively[177, 178].

Once in the nucleus, PER and the two CRY proteins form a large nuclear complex with over 30 polypeptide subunits which interacts with the BMAL1:CLOCK heterodimer and disrupts the CLOCK:BMAL1-associated transcriptional complex at E/E′ boxes [179] and repress BMAL1–CLOCK activity in the repression phase. CRY binding to the transcriptional activation domain (TAD) in unstructured C-terminal domain of BMAL1 with the CC helix is not indispensable for repression but cause decrease in repression activity needing additional residues to repress CLOCK:BMAL1 activity. Of note, it has not elucidated yet whether PER proteins can interact directly with the CLOCK:BMAL1 complex on DNA or not [180]. Even BMAL1:CLOCK heterodimer activity is inhibited, complex is supposed to remain bound to DNA throughout the circadian cycle, and finally be degraded after phosphorylation by various kinases. Repression on transcriptional activation of BMAL1:CLOCK completes the negative feedback loop in approximately 24 hours. The interacting positive and negative feedback loops of circadian genes ensure low levels of PER and CRY, and a high level of BMAL1 activity at the beginning of a new circadian day to start new circadian loop(Figure 3). Ripperger et al. defined the binding time of BMAL1, CLOCK, NPAS2, PER1, PER2, CRY1 and CRY2 to *Dbp* promoter during the circadian cycle. They revealed that BMAL1, CLOCK and NPAS2 as the activators bind to three locations in the promoter, introns 1 and 2 between CT0-CT12 (CT: circadian time) by recruiting and initiating RNA polymerase II (RNAPII) in a circadian manner. PER1, PER2, CRY1 and CRY2 bind the same sites to form early repressive PER: CRY complex at CT12-20, which is compassable with the canonical TTFL model as repression phase on BMAL1:CLOCK:RNAPII complex. On the other hand, CRY1 also bound to same site on *Dbp* promoter without PER proteins at CT0-CT4 and formed CRY1:BMAL1:CLOCK:RNAPII complex in a ‘poised’ state in the early morning as the late

repressive CRY1 complex. PER interaction might provide stability to CRY in early repressive complex until the late repressive phase in which CRY represses BMAL1:CLOCK complex. BMAL1:CLOCK heterodimer interacts with transcriptional activators, histone acetyltransferases CBP/p300 at CT8-CT24 but is only active at CT8-CT12 because of early repression and late repression complexes. In that study, they also found that chromatin state is modulated with daily rhythmic histone modifications; H3K4me3, H3K9ac and H3K27ac across the genome in the liver[181] (Figure 4).

Beside, PER and CRY proteins effect on function of BMAL1:CLOCK heterodimers, the rhythmic expression of *Bmal1* mRNA is subject to circadian clock regulation of nuclear receptor elements (NREs) constituting additional transcriptional circuits. This secondary (auxiliary) loop named as the stabilizing loop is crucial for precision and robustness of cellular oscillations. Feedback loop of this transcriptional circuits is induced when BMAL1:CLOCK heterodimers activate the transcription of nuclear receptors; retinoic acid-related orphan nuclear receptors, *Rev-Erbs (Rev-Erbα*, β; encoded by *Nr1d1* and *Nr1d2* genes, respectively) and retinoid-related orphan receptors, Rors (Rorα, β, and γ) [20, 21]. Rhythmically expressed transcriptional repressor REV-ERBα and the more stably expressed activator RORα subsequently compete to bind retinoic acid-related orphan receptor response elements (ROREs, RRE) present within *Bmal1*, *Clock, Npas2* and also *Cry1* promoter. While RORs activate transcription on ROREs sites, its antagonist REV-ERBs repress the same transcription process and both affect BMAL1 amplitude [182]. REVERBs recruit histone deacetylase HDAC3 that is activated by nuclear receptor co-repressor 1 (NCoR1) to inhibit BMAL1 activity[183]. In addition to ROR α/γ, genome-wide profiling in liver revealed that REV-ERBs inhibition on BMAL1 is balanced with rhythmically expressed PPARα. PPARα also affects other clock genes and many CCGs expression [106, 184, 185]. Of note, metabolic regulator PGC1α induces BMAL1 and REV-ERBα by activating RORα/γ as transcriptional co-activator[184]. As the PER and CRY protein concentration increase in nucleus, activity of CLOCK-BMAL1 heterodimer is decreased which result in less transcription of *Nr1d1* and *Nr1d2* and so derepression on *Bmal1* promoter and close in loop[186-189]. This secondary loop is also interconnected to the primary loop via PER2, which has been found to rhythmically interact with promoters of REV-ERBs to synchronize the negative and positive limbs of these TTFLs[190]. D-box elements in the promoter region have been recognized as

the third important factor that regulates circadian transcription. Time information generated by the cell clock oscillator is transmitted to clock controlled genes (CCG) in two ways. The first one is central loop of the mammalian clock to the CCGs through E-box (CACGTG) mentioned above and the second route regulates indirectly the antagonistic effects of PAR(proline and acidic amino acid-rich)-Zip transcription factors and E4 promoter-binding protein 4 (E4BP4) on the D-box elements (RTTAY GTAAY (R, purine; Y, pyrimidine)) [13]. PAR -Zip family transcription factors such as DBP, HLF, TEF, activate transcription of target clock genes by binding to the D-box elements during the day, and E4BP4 suppresses transcription of these target genes in the night. For instance *Per1* expression is modulated by DBP and E4BP4, which determine the circadian period length [190-192]. PAR proteins are themselves under the control of E-box-mediated transcription however; E4bp4 is regulated by the RRE. Thereby, they modulate CCGs indirectly in a circadian manner in several tissues, including liver, SCN and kidney [193, 194](Figure 3). Besides to clock genes, The PAR bZIP transcription factors also regulate the expression of many proteins that function in detoxification and drug metabolism[195]. In addition to these controls, repressive transcription factors, DECs (Differentially expressed in chondrocytes), have been shown to repress CLOCK/BMAL1-induced activation of the *Per1* promoter through two distinct mechanisms: competing for E-box binding and interacting with BMAL1. DEC proteins are clock-controlled proteins and belong to a new and structurally distinct class of bHLH transcription factors that interact with Class-B E-Box[196]. DEC1 modulates the phase of expression of E-Box containing genes, such as *Dec1* and *Dec2, Per1, Dbp,* and *Nr1d1* but not E'-boxes containing genes such as *Per2* and *Cry1*[197].

Circadian mechanism is conserved across the species but many specific clock control genes cycle species and tissue dependent[198]. It was believed that around 10% of active genes in mammals could be clock-controlled in a highly tissue specific manner to regulate much of cell physiology[199]. However, recently, study of Zhang et al. on circadian gene expression atlas in mammals suggested that nearly half of all protein-coding genes express in a circadian fashion [200, 201]. Rhythmicity is not only observed in gene expression, but also in protein level and post-translational modifications such as phosphorylation and ubiquitination [202, 203].

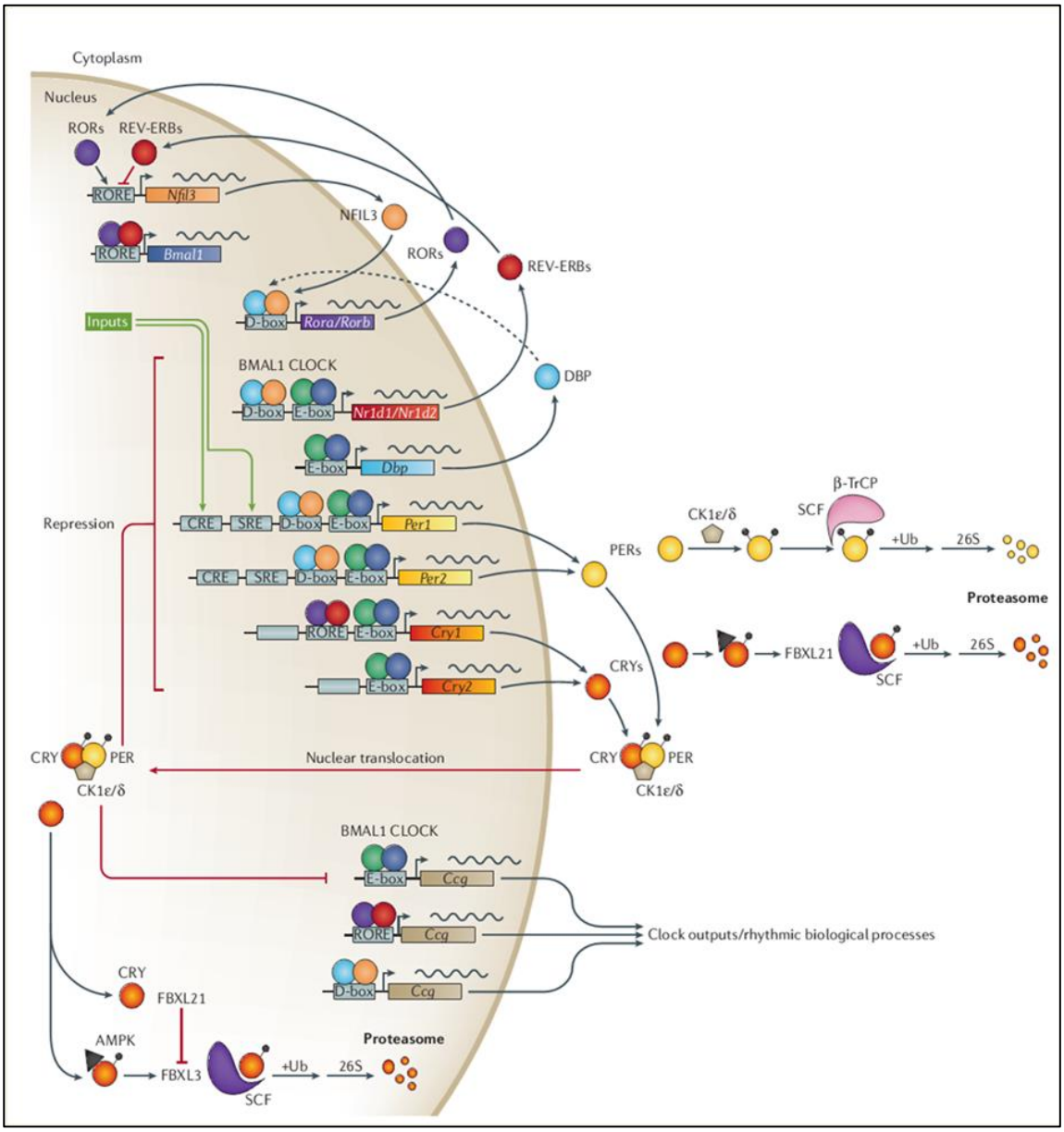


Figure 3: Model of circadian clock mechanism. It is composed of an autoregulatory transcriptional feedback network with two interlocked transcriptional-translational feedback loop (TTFL) and posttranslational modifications of clock proteins. Reprinted from [204] by permission from Nature Publishing Group, copyright 2017.

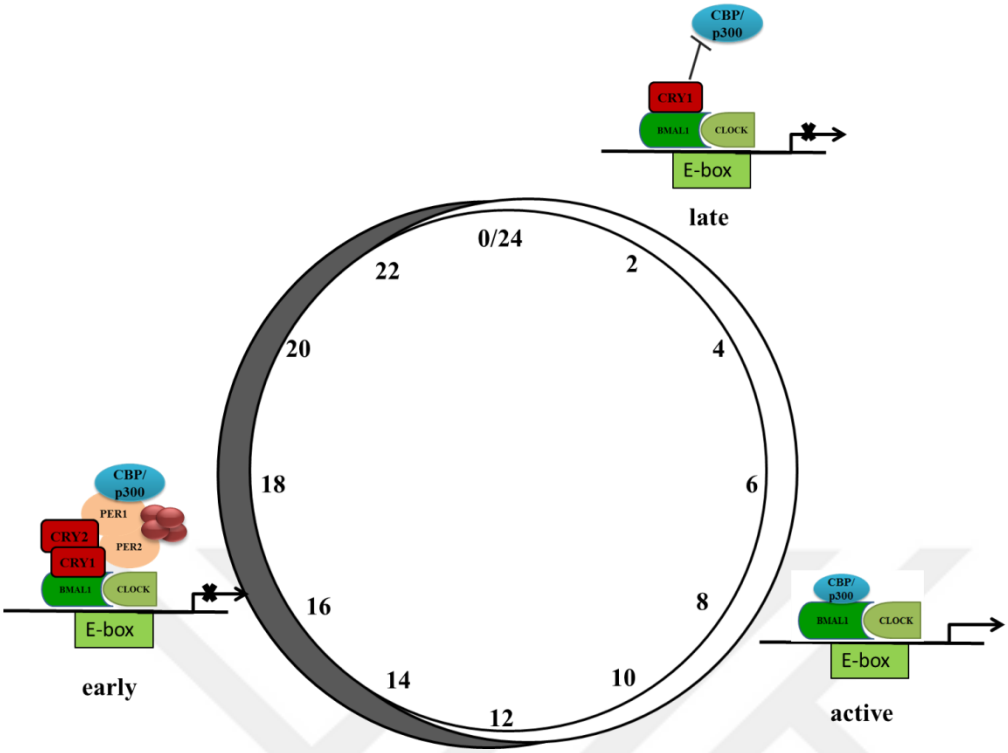


Figure 4: Rhythmic BMAL1:CLOCK binding to E-box motifs within *Dbp* promoter. Based on the ChIP-seq analysis, transcriptional regulation of Dbp promoter with the large early repressive PER:CRY complexes and the late ternary CLOCK:BMAL1:CRY complex. Adapted from [205].

2.4.3.3 Post-Transcriptional, Post-Translational and Epigenetic Regulation of Clock

As circadian pacemakers can measure time only approximately, their phase has to be adjusted every day in order to stay in synchrony with geophysical time. This is achieved via post-transcriptional, post-translational and epigenetic regulation of circadian rhythms.

Post-transcriptional regulation on circadian rhythms is obtained through modulating RNA stability via splicing, alternative polyadenylation, and poly(A) tail length regulation to alter protein levels rapidly[206-208]. On the other hand, post-translational regulation is gained via phosphorylation, ubiquitination, and SUMOylation in several of clock components. Both post-transcriptional and post-translational modifications appear to help maintain robustness and precision of ~24 hour rhythmicity, the stability and the subcellular localization of aforementioned core clock proteins.

Studies, especially RNA-seq studies on the role of post-transcriptional regulations in mammalian circadian mechanism provide identification of new CCGs and also rhythmically expressed non-protein coding transcripts: long noncoding RNAs (lncRNAs) or microRNAs (miRNAs). The link between circadian clock function and non-coding RNAs (ncRNAs) such as miRNA expression has been intensively studied. Dicer is an important enzyme in miRNA process, and dicer knock out mice has shorter circadian period due to faster translation of PER1 and PER2 proteins. Phase and amplitude of the oscillation are regulated by miR192/194 through affecting the period gene family (*Per1*, *-2*, and *-3*) mRNA stability. Unprocessed microRNAs; miR-24, miR-29a, and miR-30a that specifically target *Per1* and *Per2* affect circadian oscillation. In addition, the role of miR-132 and miR-219 involvement in modulation of the circadian period or response to light is also demonstrated. Many oscillated miRNA that control core clock genes via translational regulation have been defined in liver, retina and SCN [209-215]. miR-142-3p is reported to regulate the post-transcriptional modulation for *Bmal1* in the mouse SCN, while miR-185 oscillation controls circadian amplitude of mouse *Cry1*[214, 215]. Beside to miRNAs, studies also revealed rhythmic expression of the lncRNAs in liver and pineal gland and effect of lncRNAs, as an antisense transcript, on the transcription of core clock genes, such as *Per2* [216, 217]. Rhythmic expression of 112 long non-coding RNAs (lncRNAs) in the rat pineal gland is linked to melatonin secretion by which lncRNAs influence timing of circadian rhythms[218]. The first

reported work on the circadian post-transcriptional regulation is about *Drosophila Period* (*Per*) gene. Noncoding 3′UTR mRNA with diurnal expression determines mammalian homologue *Per1* stability besides to other cycling mRNAs, such as *Per2, Per3,* and *Cry1* [219] [220]. Post-transcriptional RNA modification such as n6-methyladenosineRNA-methylation is also thought to be involved in circadian clock speed[221].

Proteins responsible for post-translational modification of clock genes include casein kinase I family members (Casein kinase 1 delta (CK1δ) and casein kinase 1 epsilon (CK1ε)) and the F-box and leucine-rich repeat protein 3 (FBXL3) and 21(FBXL21) [222]. These proteins regulate the speed, precision, and function of the circadian clock [4, 223, 224]. Reversible phosphorylation regulates important processes such as subcellular localization, nuclear entry, formation of protein complexes, modulation of protein half-life and repressor activity of PER and CRY. PER:CRY complexes undergo proteasome-mediated degradation during the subjective night, eventually repression is relieved, which allows BMAL1/CLOCK transcription to build up again and let the initiation of a new cycle. PER1 and PER2 proteins shuttle between the cytoplasm and the nucleus and as the PER proteins accumulate in the cytoplasm; they become phosphorylated by CK1ε/δ. Phosphorylated forms are sequestered in the cytoplasm and then they become unstable after ubiquitination by specific E3 ligases, βTrCP (β-transducin repeat containing protein) and are degraded by the 26S proteasome [225, 226]. Late in the subjective day, however, CRY accumulates in the cytoplasm, promoting the formation of stable CKIε:PER:CRY complexes, which enter the nucleus at the beginning of a subjective night (265). The PER:CRY dimer is stabilized against degradation, suppresses *Cry* and *Per* genes transcription, and shuts off PER and CRY proteins synthesis. The protein phosphatase 5 (PP5) removes the phosphate group from auto-phosphorylated CKIε thereby the kinase can phosphorylate PER2 [227]. CKIε/δ-dependent phosphorylation on the PER2 protein is an important period-determining process. In the mammalian circadian clock and hamsters harboring a mutation (*tau* mutation) on CK1ε has a shortened circadian period[149, 150].

CRY1 and CRY2 post-translational modifications are achieved via AMP-activated protein kinase 1(AMPK1) and DYRK1A/GSK-3β, respectively. AMPK1 promotes CRY1 protein polyubiquitination by SKP1-CUL1-FBXL3 (SCF^{FBXL3}) ubiquitin ligase complex for proteasomal degradation [228] [229]. CRY2 is phosphorylated at Ser557 in a circadian

manner in the mouse SCN and liver by DYRK1A. This phosphorylation allows subsequent phosphorylation at Ser553 by GSK-3β [230, 231] at the two neighboring serine residues of CRY2 resulting in its proteasomal degradation. On the other hand, CRY2-FBXL3 interaction and FBXL3-mediated CRY2 degradation do not require Ser557/Ser553 phosphorylation of CRY2 [232] while FBXL21 ubiquitinates CRYs and that, surprisingly, stabilizes them [228].

BMAL1 is SUMOylated, acetylated, and phosphorylated by CK2α, CKIε and MAPK (mitogen-activated protein kinase). SUMOylation at lysine residues in the BMAL1 PAS-A domain and phosphorylation by CKIε are associated with high levels of BMAL1 transcriptional activity, while phosphorylation by CK2α and MAPK are associated with inhibition of BMAL1 transcriptional activity [233, 234] [235] [236]. CLOCK isoforms are phosphorylated *in vivo* and this phosphorylation may be a prerequisite for CLOCK's nuclear translocation. CLOCK is phosphorylated on residues Ser38, Ser42 and Ser427 which are involved in CLOCK's stability and/or transactivation capacity [237].

In addition to post-transcriptional and post-translational modifications, epigenetic factors such as DNA methylation, histone modification (e.g., methylation, acetylation, phosphorylation, and citrullination, biotinylation, ribosylation, ubiquitination, and palmitoylation), and non-coding RNAs, are another essential ways to control circadian oscillations**.** Transcription rates of the clock genes are controlled by the rhythmic acetylation/deacetylation and methylation/demethylation of the histones in the promoter regions of the clock genes [238]. Interestingly, Doi et al. reported that CLOCK protein also appears to have histone acetyltransferase (HAT) activity on histone H3 Lys-14 and H4 Lys-9 in the promoter regions of target genes for transcriptional regulation through acetylation and deacetylation [239]. Besides to histones, CLOCK has an anti-phasic dual role in which it firstly acetylates BMAL1 on Lys-537, and then acetylated BMAL1 recruits CRYs to interact with the BMAL1:CLOCK complex to repress BMAL1:CLOCK activation [240]. Furthermore, co-activator of CLOCK:BMAL1 complex, histone acetyltransferase (HAT) p300 acts with the CLOCK:BMAL1 complex to modulate *Cry* and *Per* genes expression via acetylation of histone H3 at their promoters [241]. Moreover, as mentioned above, profile of the epigenetic landscape in mouse liver showed that chromatin state is modulated with daily rhythmic histone modifications; H3K4me3, H3K9ac and H3K27ac beside to rhythmic RNA polymerase II recruitment and initiation [181].

Histone deacetylase (HDAC) works with opposite function with HAT and deacetylases histone via removing acetyl groups from ε-N-acetyl lysine residue. Deacetylation causes histone to wrap the DNA and inhibits the expression of clock genes. Multimeric protein complex along with CKI δ/ε translocate into the nucleus and recruits the SIN3 complex, containing HDAC1 and HDAC2. Additionally, HDAC1 and HDAC2 containing co-repressor complex NuRD, interacts with PER–CRY and deacetylates nearby histones. Thereby, both CRY and PER inhibit their own transcription and that of other clock-controlled genes by physically interacting with the CLOCK:BMAL1 complex. SIN3 and NuRD complexes are composed of RBAP48, CHD4 and MBD factors that recognize histone and DNA modifications [242-244]. Alenghat et al. revealed that HDAC3 represses *Bmal1* expression by recruiting nuclear receptor corepressor 1 (NCOR1) in mouse [245]. HDAC3 forms complex with REV-ERBα and NCOR1, by which several genes expressions oscillate with the fluctuation of HDAC3-related histone modification to controls metabolism such as hepatic lipid metabolism in the mouse liver[246]**.** In very recent work, Shi et al. proved that HDAC3 has a dual role in which it is a critical component in the core circadian negative feedback loop by blocking CRY1 degradation and promoting BMAL1-CRY1 association, while it also induces E-box-driven gene expression [183].

CLOCK-mediated chromatin remodeling is also influenced by another chromatin remodeling agent, Sirtuin 1(SIRT1), a member of the HDAC family. SIRT which is a NAD+ dependent class III histone/protein deacetylases, works with CLOCK to modulate the promoter of NAMPT (Nicotinamide phosphoribosyl transferase) which regulates the circadian synthesis of NAD+ [115]. SIRT1 interacts with BMAL1:CLOCK and acts opposite to CLOCK-mediated acetylation of histone H3 of BMAL1 and promotes PER2 degradation[122, 247]. Histone lysine-specific demethylase JUMONJIC and ARID domain-containing histone lysine demethylase 1a (JARID1a) has opposite function and enhances transcription by BMAL1:CLOCK through inhibiting HDAC1 function in a demethylase-independent manner[248]. Many studies have been released for the roles of DNA methylation in regulating circadian rhythms. Rhythmic occurrence of H3K4 methylation near the TSSs of clock genes is simultaneous with the acetylation of nearby lysine residues. The histone methyltransferase mixed-lineage leukemia, MLL family of methyltransferases(MLL1, MLL3) are responsible

for circadian H3K4 trimethylation and also acts as coactivators of BMAL1–CLOCK, and RORs [249, 250].

2.4.3 Effects of Circadian Clock Gene Mutations on Behavioral/ Molecular Phenotypes of Animals and Phenotypes of Cell/Tissue

In 2000s, mutations in core clock components were used to define the role of clock components at cell/tissue level and in mouse model. An unexpected result was observed for *Clock* knockout mouse model (*Clock* $^{-/-}$) that has continuous circadian rhythms with slightly shorter period in DD (dark-dark) unlike to Clock$^{\Delta 19}$ model (4 h longer period/arrhythmic) [151]. Then DeBruyne et al., reported that in SCN, NPAS2 can compensate for CLOCK [251, 252]. However, change in circadian locomotor activity and gene expression in *Npas2*$^{-/-}$ animals is also not significant, suggesting importance of Clock at molecular level and tissue dependency. Partial redundancy between CLOCK and NPAS2 was eliminated in *Clock/Npas2* double knockout animals that are arrhythmic in DD[251, 252]. On the other hand, knockout of *Bmal1* (*Arntl, Mop3*) in mice shows arrhythmic locomotor with disrupted molecular rhythms of gene expression in DD[253]. Three independent *Per1* null mutant animals; *Per1*^{ldc}, *Per1*^{Brdm1}, *Per1*$^{-/-}$, exhibit shorter free-running period compared to wild type [254-256]. Animals with two different null mutations in *Per2*, express more severe phenotypic change compared to wild types. While most animals of the *Per2*^{ldc} line show immediate arrhythmicity upon exposure to DD[254], animals with *Per2*^{Brdm1} mutation exhibit 1.5 hour shorter period for several days before experiencing arrhythmicity[257]. As expected, behavioral and molecular arrhythmicity is observed in double *Per1/Per2* knockout animals in DD condition [254] [257]. However, mice with *Per3* knockout, or mutant mice with double knockout of *Per3 /Per1* or *Per3/Per2* do not experience a change in circadian rhythmicity. Therefore PER3 is not accepted as core clock component [254] [257]. Loss of one of the cryptochrome genes exhibits opposite behavioral phenotypes on circadian behavior of mutant mice. *Cry1*$^{-/-}$ animals have a 1 hour shorter, yet *Cry2* $^{-/-}$ animals have a 1 hour longer free-running period in DD compared to wild-type animals. Loss of both cryptochrome genes in *Cry1/Cry2* double knockout animals causes complete loss of behavioral and molecular rhythmicity in DD [169, 258, 259] [260].

Mutations in auxiliary component of clock network in mammals also reveal phenotypic changes. Both *Rev-Erbα (Nr1d1)*$^{-/-}$ and *Rorα*$^{-/-}$ mutants experience 0.5 hour

shorter period and disrupted entrainment[187, 188]. In contrast, *Rorβ* $^{-/-}$ mutants show 0.5 hour longer period[261] and *Dbp*$^{-/-}$ mutant reveal 0.5 hour shorter period [262]. Mutation in casein kinase isoform such as *CK1δ (Csnk1d)* $^{+/-}$ and *CK1ε*$^{-/-}$ exhibit 0–0.5 h and 0.2–0.4 h longer circadian period compared to wild type, respectively. *CK1ε* ^{tau} mutation identified in Syrian hamster (Mesocricetus auratus) reveal 4 hour shorter circadian period [263] [148, 264, 265]. Another post translation modifier, *Fbxl3* (F-box protein) has two mutants; *Fbxl3*^{Ovtm} and *Fbxl3*^{Afh} with 2 hour and 3 hour longer period, respectively [266, 267]. Mutants with loss of *Opn4* gene required for melanopsin exhibit attenuated photic responses[268, 269].

To eliminate differences in central versus peripheral oscillators and potential intercellular interactions and also to obtain more detailed information on cellular oscillators, knockout lines of core clock components are also examined in cultured tissues and individual cells. It is important whether SCN neurons used for experiment are as in explant tissue or dissociation into single cells. Mutations in SCN neurons dissociated into single cells such as *Cry2*$^{-/-}$ show rhythmicity with a longer period and *Cry1*$^{-/-}$ or *Per1*$^{-/-}$ SCN neurons exhibit arrhythmicity in contrast to tissue explants in which mutations behave like WT. That indicates intercellular coupling among SCN neurons. While loss of *Per1, Per3, Cry1* or *Cry2* has no effect on SCN tissue circadian rhythmicity, in peripheral tissue explants (e.g., liver, lung, cornea), *Cry1*$^{-/-}$ and *Per1*$^{-/-}$ mutations cause disruption of circadian rhythmicity and *Cry2* $^{-/-}$ and *Per3*$^{-/-}$ mutant exhibit slightly longer and shorter periods, respectively, compared to WT controls. In dissociated fibroblast cells, *Per1, Per2* and *Cry1* knockout cells exhibit arrhythmicity, yet *Cry2*$^{-/-}$ cells are rhythmic with a slightly longer period [107]. Since *Bmal1*$^{-/-}$animals are arrhythmic, SCN explants from *Bmal1* $^{-/-}$ mice crossed to the Per2: *dluc* reporter line is expected to show arrhythmicity, yet, tissue explants exhibit unexpected variable rhythmicity indicated as stochastic. However, peripheral tissues from *Bmal1* $^{-/-}$ animals or single dispersed *Bmal1*$^{-/-}$ SCN neurons possess arrhythmic oscillation as expected[270, 271].

2.5 Dysregulation of Circadian Clock and Disease States

Period length of an internal clock in an organism is genetically programmed and is not exactly 24 hours. In free running rhythm, an animal with a slightly longer internal period length will exhibit drifting activity with later phase onset for each passing day. In contrast, an animal with shorter endogenous period will experience earlier phase onset. Therefore, for

fitness of organism, varied internal period length is set to 24 hour- environmental light/dark cycle by photic stimuli sensed by SCN. However, SCN adjustment of an organism clock to new schedules takes a certain number of 24-hour cycles [272][391]. In modern life, circadian clock can be disrupted with time zone changes, jet lag, space travel, psychiatric disorders, and light-emitting diodes (LED) and electronics during the evening hours which cause desynchrony between the internal and external clock[273-276].

Sleep-wakefulness and eating pattern is obvious but metabolism and the cardiovascular functions are also output of the circadian clock[277]. Hence, dysregulation of circadian clock is associated with the onset and development of numerous human diseases including sleep disorders, depression and dementia, hypertensive crises, asthma and allergy attacks, metabolic and psychological disorders, myocardial infarction, fatigue, disorientation, insomnia and distorted hormone profiles as seen in jet-lagged travelers and obesity, diabetes, cardiovascular problems, inflammatory diseases and autoimmune disorders, neurological disorders as well as cancer as seen in shift workers[4-7] [8-10, 278].

Shiftwork is the most common cause that disrupts the activity of the SCN and inhibits synchronizing of the circadian clock to environmental light. Entrainment speed of SCN and peripheral to environmental stimuli is not same, which leads to desynchronization between the SCN and the peripheral clocks. This adaptation difference, a lag, produces circadian phase shifts that results in complications and disease[279, 280]. Aforementioned proteins which sense redox AMP/ATP ratio, glucose and fatty acid level establish communication between circadian clocks and metabolism[281]. Shiftwork and sleep deprivation are implicated as detractive to this communication and cause dampen rhythms in growth hormone and melatonin secretion, reduce insulin sensitivity, and elevate circulating cortisol levels and loss of adequate blood pressure decline. These changes favor weight gain, obesity, and development of metabolic syndrome and cardiovascular organ damage [282, 283]. Of note, sleep pattern is also effect the circadian clock. Insufficient sleep or sleeping in out of phase decreases rhythmic transcripts in blood [284, 285]. Circadian disruption is also has influence on immune system in which immune cells, their gene expression and response to infections are rhythmic[286] [281].

Connections of circadian clock and the development of cancer is one of the hot topics in cancer research. Environmental factors or sleep patterns cause circadian disruption (CD)

that is related with many physiological consequences as well as tumor development. The first study about influence of environmental light and melatonin on mammary tumor induction was reported in 1960[287]. Since then, several studies have shown correlation between CD and higher cancer risk and more malignancy [288-291]. Shift work which is one of the potential causes of CD is also accepted as a potential carcinogen and cause of increase in risk of breast cancer in female night shift workers[292, 293]. Particularly, permanent shift work is not only cause for tumor development but is correlated with higher cancer incidence and more malignancy in SCN-ablated mice[294].

Beside to clock function, core clock proteins possess important roles in cell cycle progression, DNA damage responses, and genomic stability. Crosstalk between the circadian clock and cell cycle are coordinated with many layers of timing. The circadian clock regulates the cell cycle is through key checkpoints of the cell cycle, especially at the entry to S phase typically occurring at night, to ensure fidelity in DNA replication and cell division. DNA damage cause reset in the circadian phase to repair damage and/or call pro-apoptotic pathways. Thereby, disruption of circadian rhythmicity and cancer can be correlated with dysfunctional clock proteins. PER proteins, especially PER2, are implicated as tumor suppressors and as mitotic regulators [295]. Loss or unregulated PER proteins were associated with breast and endometrial cancer[296]. Many studies link circadian disruption and breast cancer on hormone melatonin and PER protein expression. PER1 and PER2 of which expressions reduce in primary breast tumors compared to normal breast tissues act as tumor suppressors in salivary gland, gastro-intestinal system, and the immune compartment [296-299]. Similarly, other studies also correlated PER protein influence on expression of breast cancer related genes, HER2, BRACA1 and estrogen receptor[300, 301]. Also, Gotoh et al. demonstrated that PER2 promotes hp53 stability via inhibiting ubiquitination of hp53 by MDM2 (Mouse double-minute 2) [302]. Because, PER2 has negative regulation on Cyclin D and Cyclin E, decrease in PER2 level is associated with acceleration tumor growth *in vivo* [303], while induction level either PER1 or PER2 leads to decrease in the growth of cancer cells and increase apoptotic rate [304].

BMAL1:CLOCK complex regulates rhythmic expression of cell cycle genes though E-box sequences in their promoters. These genes are *CCND1* (Cyclin D1 gene), *MYC* (v-Myc myelocytomatosis viral oncogene homolog avian), *MDM2*, *WEE1* kinase, *METAP2*

(Methionyl aminopeptidase), *p53, CDKN1a* (cyclin-dependent kinase 1a), and *GADD45* (Growth arrest and dna-damage-inducible, alpha). Any alteration in BMAL1:CLOCK complex function gives rise to cell proliferation dysregulation. Many studies stated that BMAL1:CLOCK complex suppression on c-MYC expression is inhibited by CRY1 [305-307] [302]. Clock controlled genes; METAP2 is an important cell cycle initiators in eukaryotes and WEE1 kinase regulates the G2/M transition timing via controlling the CYCLIN B1/CDK1 complex [306, 308]. Furthermore p20 and p21, key regulator in the G_1/S transition and expressing in a tissue-specific manner, are under clock regulation[309, 310]. Moreover REV-ERBs have modulation effect on the cell cycle inhibitor p21 WAF1/CIP1 [309]. PER with an RNA binding protein called p54nrb or NONO forms a complex that rhythmically activates the promoter of the cell cycle checkpoint gene p16-INK4A in a circadian manner, which regulates G1/S transition[311]. Circadian timing is important counter- balance between the circadian clock and cell proliferation maintenance, especially after irradiation-induced DNA-damage[312]. Cross-talk between circadian clock and DNA repair mechanism is established through interaction between PER1 and ATM (Ataxia-Telangiectasia Mutated) kinase and CHK2 (Checkpoint Kinase 2) [313]. The mammalian timeless (TIM) protein, homolog of *Drosophila* clock protein TIM, is not core clock component but mostly related to cell cycle regulators and involved in DNA damage response. TIM interacting with CRY2, CHK1)and ATR (Ataxia telangiectasia and rad3-related protein), regulates S phase in response to DNA damage [314].

Many cancer and immortalized cells show more complex circadian rhythm with aberrant expression of clock genes, most probably because of cellular heterogeneity of tumor microenvironment and dysregulation of circadian clock proteins implicated as tumor suppressors and mitotic regulators [315-317].Why some cancer cell lines are arrhythmic but some are retaining a circadian clock although they have major chromosomal disruption and metabolic abnormalities is still unsolved question.

Several studies showed that circadian alterations caused via mutation or polymorphism in different clock genes are related with abnormal sleep patterning, such as the advanced sleep phase syndrome and the delayed sleep phase syndrome, anxiety, mood disorders and addiction and metabolic phenotypes, such as type-2 diabetes or obesity[318, 319] [320-323]. As mentioned before, hamsters with a point mutation in *CK1ε*(*tau*) display a shorten circadian

period (22 h) and developed cardiomyopathy and severe renal disease when kept under 12 h light and 12 h dark because of the desynchrony between the internal and external clock[324]. *Per* mutation in mouse is associated with timing of sleep onset[325]. FASPD, (Familial advanced sleep-phase syndrome) which was the first defined human sleep disorder affects individual habits and finally cause depression. Individuals with FASPD display persistent 3-4 hour earlier sleep and awaking times, which is directly linked to core clock gene, *PER2*. Specifically, rare autosomal dominant mutation of *PER2* and missense mutation S662G, phosphorylation site of PER2 within CK1δ have been found responsible for FASPD patients. Missense mutation in *PER2* can cause increase in PER2 degradation or reduce nuclear retention of PER2. These prove the importance of post-translational regulation of PER for regulation of 24 hour - period length[326, 327]. *Cry1, Cry1/Cry2* double knockout mice and *Per2* homozygous mutant or homozygous knockout mice display tumor development after genotoxic challenge[290, 328]. However, *Cry1/Cry2* deficient double mutant mice with *p53* gene copies exhibit longer lifespan compared to only *p53*-deficient mice[329]. Change in epigenetic regulation on clock gene also may influence the cancer development. In the study conducted on breast cancer patients, hypermethylation level on the promoters of *PER1, PER2, CRY1 or BMAL1* genes is higher compared to methylation in non-cancerous tissues [330].

Genetically modified circadian mutant animals are used to study role of clock genes in metabolic processes in tissue dependent manner. Whole-body *Bmal1* KO mice experience speed up in aging processes[331], elevated glucose levels and impaired glucose tolerance and insulin secretion and disruption in triglycerides cycle [401]. Mice with liver-specific deletion of *Bmal1* experience fasting-induced hypoglycemia, while pancreas-specific deletion causes diabetes[332, 333]. Whole-body *Clock* KO mice display hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia hyperinsulinemia but also hypoinsulinaemic with age and impaired diurnal feeding cycles. In mice, *Clock* $^{\Delta 19/\Delta 19}$ causes hyperglycemia and defects in insulin release from the pancreas[333]. Double KO *Cry1/Cry2* mice have hyperinsulinemia, gluconeogenesis and accelerated respond to glucagon and glucocorticoids and gain more weight with a high-fat diet compared to WT mice[334-336]. In addition, deficiency also related with alterations in liver regeneration indicating link between circadian and cell-cycle pathways[306]. Depletion of both *Rev-Erbα* and *Rev-Erbβ* is resulted in wheel-running behavior and hyperlipidemia, hepatic steatosis and hyperglycemia [189,337, 338]. Deficiency

of BMAL1 positive regulator, *Rorα*; sg/sg (Rorα mutant) mice experience decrease in cholesterol levels and resistance to diet-induced obesity[339, 340]] that is also observed depletion of ROR co-activator, PGC1α [341]. Also, the mutation Pro385Arg in the DEC2 gene carrier individuals are considered as the natural short sleeper who can live with fewer hours of sleep per day[342].

2.6 Transcription Factors Selected for Their Repression Activity on BMAL1:CLOCK transactivation via High Throughput Luciferase Assay

High throughput luciferase assay was utilized to screen transcription factors to examine their regulation on BMAL1:CLOCK transactivation. Before proceeding with rationale and aim of this study, the current knowledge about these selected transcription factors were briefly described below

2.6.1 Nuclear factor erythroid 2-related factor 2 (NFE2L2/ NRF2)

NFE2L2 (nuclear factor erythroid 2 related factors 2) transcription factor is predominantly known as NRF2 (NF-E2-Related Factor 2). NRF2 is a basic "cap and collar" leucine zipper transcription factor, which regulates environmental stress response by activating the expression of genes for antioxidants and detoxification enzymes. The NRF2-directed environmental stress response protects cells against variety of stressors including environmental pollutants such as electrophiles and oxidizing agents, immune toxicants, and inflammation [343].

NRF2 is regulated by direct post-translational modification or its dissociation from its cysteine rich inhibitor, KEAP1 (Kelch-like erythroid-cell-derived protein with CNC homology [ECH]-associated protein), in response to different stressors. Upon exposure to stressors, the NRF2-KEAP1 complex dissociates and NRF2 translocates to the nucleus and binds to antioxidant response elements (AREs; 5'-RTGAYnnnGCR-3' where R=A or G and Y=C or T.) in the promoters of its target genes [344]. NRF2 is ubiquitously expressed, but more abundantly in metabolic and detoxification organs, such as liver, kidney and intestine, as well as organs continuously exposed to the environment, such as skin, lung and the digestive tract.

NRF2 target genes are involved in pathways for xenobiotic detoxification, antioxidants, anti-inflammatory response, DNA repair, molecular chaperones, and proteasome

systems cell growth, protein folding, cell survival, nucleotide excision repair pathway. Classic target genes including glutathione S-transferases (GSTs), heme oxygenase (HO-1) and gamma glutamylcysteine synthetase (gamma-GCS) are paramount in cellular defense against both endogenous and exogenous oxidative stressors [345]. To date, known modifications of NRF2 are phosphorylation, ubiquitination, acetylation, and SUMOylation. Phosphorylation of NRF2 occurs via the kinases PKC, PERK, MAPK, and PI3K, all of which function to activate NRF2 prior to its nuclear translocation by triggering release of binding from its repressor KEAP1. The functional consequence of NRF2 SUMOylation has yet to be fully understood. Studies have shown that NRF2 may be critically involved in various pulmonary diseases such as chronic obstructive pulmonary disease (COPD), sepsis mediated lung injury, asthma, neurodegenerative disorders and cancer [346] [347].

2.6.2 The Single-Stranded DNA–Binding Proteins; isoform 2 and 4

Ubiquitously expressed, evolutionarily conserved genes of sequence-specific single-stranded DNA-binding proteins; *SSBP2* (5q13.3), *SSBP3* (1p31.3) and *SSBP4* (19p13.1) are closely related genes. *SSBP3* is the human ortholog of a chicken gene, *CSDP* that encodes a sequence-specific single-stranded DNA-binding protein. Chromosomal localization and the putative single-stranded DNA-binding activity suggest that all three members of this family are capable of potential tumor suppressor activity by gene dosage or other epigenetic mechanisms[348]. Through a highly conserved amino terminal motif, the SSBPs have been widely studied as Ldb1(LIM-domain-binding protein 1) interaction partners, and there is considerable genetic evidence for their function in axis formation in *Xenopus*, wing development in *Drosophila*, and head morphogenesis in mice as fundamental roles in development[349, 350].

Recent studies support a role for SSBP2 as a tumor suppressor in human cancer. SSBP2 may be part of a stem cell leukemia factor, a multi-protein complex required for erythroid differentiation [351] and a JAK2 fusion partner in a patient with pre-B-cell acute lymphocytic leukemia [352]. SSBP2 is a tumor suppressor in AML by inducing cell growth arrest at G1 phase, which is accompanied by loss of c-Myc expression [353]. Huang et al. found aberrant methylation of *SSBP2* in primary ESCC(esophageal squamous cell carcinoma) and a tumor suppressive role of *SSBP2* through inhibition of Wnt signaling pathway[354]. Fleisig et al. reported that endogenous SSBP2 is recruited by E1B55K and localized to the

juxtanuclear body in transformed 293 cells[355]. The function of SSBP4 is not so clear, but analysis of sequence motifs suggests that SSBP proteins may possess a versatile array of functions.

2.6.3 WW domain-containing transcription regulator 1 (WWTR1)

WWTR1; WW domain-containing transcription regulator 1, is also known as TAZ (Transcriptional coactivator with PDZ-binding motif) is located at chromosome 3q23 and expressed highly in heart, lung, kidney and placenta. It was first identified in a cDNA screen for 14-3-3-binding proteins [356] [357, 358].

WWTR1 together with its orthologous mammalian transcriptional co-activator YAP (Yes-associated protein) acts as a downstream effector in the Hippo (SWH-Salvador/Warts/Hippo) signaling pathway. The Hippo pathway was originally characterized in *Drosophila* as tumor suppressor pathway [359] and is conserved from *Drosophila* to mammals. In mammals, Hippo signaling pathway is vital for controlling organ growth, size, intestinal stem cell and tissue regeneration [360-362] [363, 364], and as well as tumor suppression by limiting cell proliferation and promoting apoptosis [365].

WWTR1 regulation by the Hippo pathway occurs via physical interaction with upstream proteins in the Hippo pathway and phosphorylation. Cell surface regulators such as cell adhesion molecules and cell polarity complexes form the upstream components of the Hippo pathway. Kinase cascade, consisting of a serine-threonine kinase families, MST1/2 and LATS1/2 and their cofactors, SAV/WW45, regulate the downstream transducers of the Hippo cascade targets, YAP and WWTR1 by phosphorylating at serine residues (five in YAP, four in WWTR1) [366]. Upon multiple intrinsic and extracellular signals, depending to cell type, in the Hippo pathway kinase cascade, MST1/2: SAV1 complex and MAP4Ks kinase families phosphorylate LATS1/2 that is activated after binding of MOB1 (Mps one binder (Mob) 1A and 1B). Activated LATS1/2 phosphorylates and inactivates YAP/WWTR1 activity via promoting their cytoplasmic sequestration by 14-3-3, which results in degradation in cytoplasm and inhibition of their nuclear functions such as cell cycling, apoptosis, and transcription regulation [367-370]. LATS1/2 phosphorylates serine residues (S66, S89, S117, and S311) of WWTR1 at the C-terminal. Functional data indicates that S89A mutation in these serine residues leads to constitutively active WWTR1 within the nucleus, which

promotes cell proliferation, cell migration, invasion, and EMT in breast cancer cells[357, 367, 371].

Second way to regulate stability of WWTR1 by the Hippo pathway is the cell density effect. High cell density activates the Hippo pathway, which causes phosphorylation of WWTR1 at S311 by LATS1/2 and further by CK1 at S314. The phosphorylated WWTR1 creates a phosphodegron for the recognition by the F-box protein β-TrCP. As a component of E3 ligase complex, β-TrCP recruits WWTR1 to the SCF/CRL1 (β-TrCP) E3 ubiquitin ligase to carry on the polyubiquitylation and degradation [372](Figure 5) .

WWTR1 protein stability is not only under control of LATS1/2 phosphorylation but also GSK3β, CDK1 (cyclin-dependent kinase 1) or c-ABL in response to a wide range of intrinsic and extracellular signals through the Hippo signaling pathway or other pathways. Beside to LATS1/2, N-terminal of WWTR1 is also phosphorylated by GSK3β at S58 and S62, which targets WWTR1 for SCF β-TrCP-dependent polyubiquitylation and subsequent degradation in response to PTEN/PI3K/AKT pathway (phosphatidylinositol 3-kinase inhibition) regulation independently from the Hippo signaling[373]. Two independent studies also showed that during the G2/M phase of the cell cycle, CDK1 phosphorylates WWTR1 (S90, S105, T175, T285, T326, T326, and T346) and inhibits its oncogenic activity [374] [375]. Interestingly, tyrosine phosphorylation is also identified on WWTR1. Hyperosmotic stress leads c-ABL kinase to phosphorylates WWTR1 at tyrosine 316 in which form, WWTR1 binds to nuclear factor of activated T cells 5 (NFAT5) and inhibits its DNA-binding as a co-repressor in renal cells under hypertonic condition [376].

Another layer of WWTR1 regulation is through intrinsic negative YAP/WWTR1 feedback for homeostasis of YAP/WWTR1 levels within cells. Moroishi and colleagues identified that upregulated expression of YAP leads to TEADs-induced transcription of LATS1/2 and NF2 (Neurofibromin 2), which results in inhibition and degradation of WWTR1. Similarly, Moroishi et al. also showed that YAP is under negative regulation of WWTR1 depending on TEADs and LATS1/2 activation[377], whereas Finch-Edmondson and colleagues' findings stated that negative feedback loop is unidirectional [378].

Without direct DNA-binding domain, WWTR1 is a transcriptional regulator as co-activator/repressor and interacts with transcription factors via its WW domain, PDZ-binding motif, 14-3-3 binding motif, and coiled-coil domain [357] [379] [380]. WWTR1 mostly

binds to proline-proline-any amino acid-tyrosine (PPXY) motifs on transcription factor activation domain via WW motif [381, 382]. Some of the transcription factors containing PPXY motifs are c-JUN (jun proto-oncogene)[383], AP-2 (activating enhancer binding protein 2) [384], NF-E2 (nuclear factor erythroid-derived 2) [385], C/EBPα [386], EGR2 (early growth response 2) [387], MEF2 (myocyte enhancer binding factor 2)[388], PEBP2 (polyomavirus enhancer binding protein 2), RUNX2 (runt-related transcription factor 2) and PPARγ (peroxisome proliferator-activated receptor)[358, 389]. PPXY motif is mostly suggested as transcription activation domain. For instance, WWTR1 acts as co-activator of RUNX2 to induce osteoblastic differentiation through PPXY motif binding. However, WWTR1 represses PPARγ (Peroxisome proliferator activated receptor-γ) activity to attenuate adipogenic differentiation via binding to PPXY motif [389, 390].

TAZ has been also implicated to interact with multiple transcription factors including MyoD (myoblast determination protein 1) to promote myoblast differentiation[391]; TTF1(thyroid transcription factor 1) and PAX8 (paired box gene 8) to regulate thyroid development and differentiation [392, 393]; PAX3 (paired box gene 3) during embryogenesis[394]; TBX5 (T-box transcription factor) during cardiac and limb development as activator [395], CARP-1 (cell cycle and apoptosis regulatory protein 1) to attenuate apoptosis in breast cancer cells [396], SMAD2/3–4 complexes to stimulate nuclear accumulation of SMADs (the mothers against decapentaplegic homologs 2/3-4) for SMADs complex regulation via TGF-β stimulation [397, 398], and TEADs (TEA domain transcription factors)[399, 400]. The dephosphorylated WWTR1 with YAP, interacts with the most well-characterized transcription factors; TEAD family of transcription factors/transcriptional enhancer factor (TEF) in the nucleus to activate downstream set of genes regarding cell proliferation, migration, and apoptosis [400]. TEAD-1, -2, -3, and -4 proteins are redundant but active tissues specific manner during development[401]. They share highly conserved 68-amino acid TEA/ATTS DNA-binding domain for MCAT (muscle C, A and T sites) and A/T-rich sites; predominately for consensus elements CATTCC/GGAATG motifs in promoters [402]. Although WWTR1 mainly functions as a transcriptional coactivator, recent studies have elucidated that WWTR1 serves as a transcriptional repressor [403, 404]. For instance, Kim et al, demonstrated that YAP and WWTR1 acts as co-repressor of TEAD via help of the

NuRD complex to repress DDIT4 and TRAIL that are anti-proliferative and apoptotic genes [403].

Additionally, WWTR1 has been reported as a critical transcriptional modulator of mesenchymal stem cell differentiation. Hong et al. demonstrate that WWTR1 coactivates RUNX2 for differentiation of MSCs into osteoblasts and for differentiation human pulp stem cell [390, 405] while represses PPARγ that induces MSCs to differentiate into adipocytes [390, 405]. Studies mostly showed that WWTR1 acts as an oncogenic protein and is involved in cell growth-factor-independent proliferation, migration, invasion, and anchorage-independent growth, resistance to chemotherapeutics playing role in Taxol resistance and tumorigenesis and epithelial-mesenchymal transition (EMT) in cancer cells. WWTR1 expression is elevated or exhibits hyper-activation in multiple human cancers, including breast cancer, glioblastoma, lung cancer, non-small cell lung cancer (NSCLC), colorectal cancer, and oral squamous cell carcinoma [371, 406, 407] [408] [367] [356, 409] [410] [411]. WWTR1 also regulates the cancer stem cell (CSC) property of breast cancer and glioblastoma multiform (GBM) and its expression level is used as a marker in breast cancer and GBM patients [390, 405, 407]. Of note, although WWTR1 is proposed to play an important role in cell transformation, tumorigenesis and metastasis, the elusive underlying mechanisms are yet to be unraveled. WWTR1 is also involved in other pathways including BMP2 signaling pathway [32] and Wnt/β-catenin signaling pathway via interaction with disheveled (DVL) in the cytoplasm where it inhibits Wnt signaling by regulating β-catenin translocation to the nucleus [34,35].

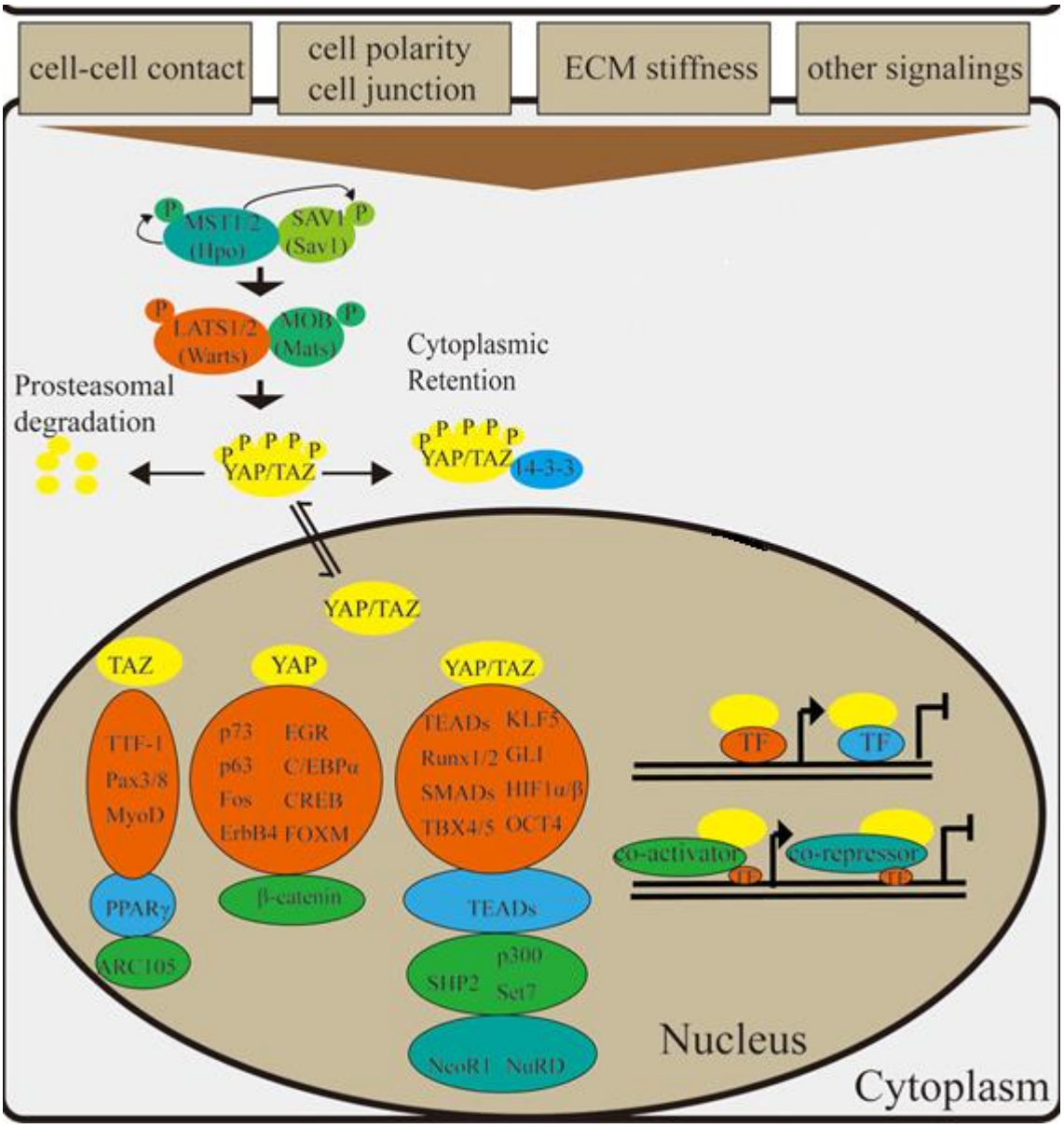


Figure 5: Schematic representation of the mammalian Hippo signaling cascade. Cell−cell contacts, cell polarity and junction proteins, ECM stiffness regulate upstream regulators such as MST1/2 with their partner SAV1 that phosphorylates LATS1/2 and MOB1. Then activated LATS1/2 kinases phosphorylate and inactivate the transcriptional coactivators/corepressors YAP/WWTR1 that are sequestered in the cytoplasm via interaction with 14-3-3 proteins or proteasome mediated degradation. In active time, YAP/WWTR1 regulates target genes transcription factors as coactivators /corepressors. Adapted with permission from [412]. Copyright 2015, American Chemical Society

Chapter 3

THESIS RATIONALE AND AIM

In the last decade, circadian clock studies have revealed great advances in many aspects. Plenty of new constituents have been discovered and proposed as candidate core clock component or as auxiliary component for regulation of the rhythmicity, the stability and precision of interlocking transcription−translation feedback loop. For instance, new functions have been proposed for NR3C1, FKBP/HSP90 complexes, CHRONO and UBE3A proteins [413-416] in the regulation of circadian rhythmicity. Also, MLL and TRAP150 proteins are indicated as a candidate clock component in the activation of BMAL1:CLOCK transactivation [249, 250]. Recently, novel function for MYC, a bHLH-containing oncoprotein, has been defined as it represses BMAL1 function by binding E-boxes [305]. However, genome-wide epistatic interaction analysis, high throughput and biochemical interaction screens revealed that abundant number of core clock components and clock regulators remain to be defined [417-419]. In the first step toward complete identification of the circadian clock core network, high throughput screens can be utilized to identify new clock components. As a target protein group, transcription factors (TFs) are good candidates that might affect BMAL1:CLOCK transactivation in the nucleus. For this purpose, nearly 1400 human and mouse transcription factor cDNAs were cloned into pBIND vector (Promega) in Hogenesch Laboratory. With the proper control, TFs were screened if they have activation or repression effect on BMAL1:CLOCK transactivation on mouse *Per1* promoter in reporter plasmid (Per1: *luc*) via luciferase reporter assay. Through the 1400 TFs, five TFs; NFE2L2/ NRF2, WWTR1, SSBP2, SSBP4 and TIRAP showed the highest repression activity on BMAL1:CLOCK transactivation. Hogenesch Laboratory also constructs a database, CircaDB (http://circadb.org) in which circadian transcriptional profiles of these TFs from mice and humans is analyzed and also using gene specific siRNA, phenotypic changes in response to these genes knockdown can be monitored [420]. According to CircaDB, all of these five TFs show a circadian transcriptional profile, which indicates they are under the

control of circadian clock. Except TIRAP, siRNA knockdown of each gene causes phenotypic change in U2-OS cell line possessing luciferase gene under control of *Bmal1* gene promoter.

According to Anafi et al., to consider a gene as a novel core clock gene, this novel component should provide five characteristics: cycling within a 24 hour period, affecting circadian behavioral rhythms in the case of mutation or knockdown in target gene, interacting with other core clock component, being expressed in most tissues and being conserved between vertebrates and flies [353]. Based on those five criteria, we started to analyze NFE2L2, SSBP2, SSBP4 and WWTR1 for their potential role in circadian mechanism. To our knowledge, except, NFE2L2, there have been no studies related to involvement of these transcription factors in circadian mechanisms. Our initial analyses were to repeat siRNA knockdown and luciferase reporter assays to confirm previous high throughput screening result. The most promising results were obtained for WWTR1; therefore this dissertation aims to characterize circadian function of WWTR1 and to define the relationship of WWTR1 and the molecular clockwork at the cellular level. To determine WWTR1 contribution in circadian mechanism and demonstrate the impact of WWTR1 on clock gene expression; luciferase assay, Co-IP, bioluminescence recording, qRT-PCR and ChIP experiments were utilized. We observed that WWTR1 has a dual role in regulation of expression of clock genes. WWTR1 interacts with BMAL1 and represses BMAL1:CLOCK transactivation, while it activates *Bmal1* and *Cry1* expression. Moreover, knockdown in *Wwtr1* affects the circadian rhythmicity in different cell lines. Based on these data, WWTR1 appears to have important function in the circadian mechanism as a transcriptional regulator. Because, WWTR1 is mostly related with cell proliferation as an oncogene and also expressed in circadian manner, elucidating WWTR1 function in circadian clock mechanism might be essential to connect clock and cell cycle and cancer fields.

Chapter 4

MATERIALS AND METHODS

4.1 Cell Lines

The human embryonic kidney cell (HEK 293T), mouse fibroblast NIH 3T3, and two reporter cell lines; human bone osteosarcoma epithelial U2-OS cell reporter line (U2-OS-B6-U2-OS *Bmal1*: *dluc*) and mouse fibroblast NIH 3T3 cell reporter line (NIH 3T3 *Per1:dluc*) were maintained in DMEM high glucose (Gibco/Thermo Fisher Scientific) supplemented with 10% FBS (PAN Biotech), 100 µg/ml streptomycin and 100 µg/ml penicillin (Multicells, Wisent Inc.) , 2 mM L-Glutamine (Gibco/ Thermo Fisher Scientific) at 37 °C in a humidified incubator with 5% CO_2. U2-OS *Bmal1*: *dluc* and NIH 3T3 *Per1*: *dluc* (a kind gift from Hogenesch Lab, University of Pennsylvania) express a destabilized firefly luciferase in pGL4.22 construct under the control of the mouse *Bmal1* promoter and *Per1* promoter, respectively. These cell lines have a functional circadian clock and are used as a model cell line for clocks studies in humans. Chemically competent *E. coli* cells; DH5α and Stbl3 (*Strep+*) are used for production of plasmids that are utilized for transfection and transduction experiments.

4.2 Determination of Effect of 1400 Transcription Factors on BMAL1:CLOCK Transactivation

Our study is based on preliminary results obtained from the two initial screens done by Dr. Kavaklı. Nearly 1400 human and mouse transcription factor cDNAs were amplified with PCR and cloned into pBIND vector (Promega) and seeded in 384 well-plates in Hogenesch Laboratory (University of Pennsylvania). All experiments were done in two groups and repeated two times. As reporter plasmid, Per*1:luc* plasmid possessing firefly luciferase gene controlled by the mouse *Per1* promoter was used [167]. In the first screen, preliminary results of transcription factors (TFs) that increase or inhibit transactivation of BMAL1:CLOCK complex were determined. Wells containing pBIND having GAL4 and pCMV-Sport6 were used to get background luminescence information for pBIND alone without marker genes.

Wells contained *Bmal1/Clock*-pCMV-Sport6 and pBIND plasmids were used to show GAL4 effect on BMAL1:CLOCK transactivation (no effect of pBIND vector is expected) and that wells containing plasmids of *Bmal1/Clock* and *Cry1* genes in pCMV-Sport6 with reporter plasmid were used as a positive control for repression of transactivation on *Per1* promoter.

In the second group**,** plasmids which contain *Bmal1/Clock*- pCMV-Sport6 and TFs genes in pBIND were constructed for transfection to mammalian cells to show whether TFs have repression or activating effect on BMAL1:CLOCK transactivity. After 16 h incubation, luciferase activity was measured with luminometer. As a control, data from wells having only marker plasmid (Per1:*luc*) indicates activity of *Per1* promoter resulted from effect of endogenous BMAL1/CLOCK and other proteins. Since some TFs have toxic effect on cell survival and cause low luciferase activity, experiments were repeated with marker plasmid and TFs only in parallel to prevent false positive results for repression activity. In this group, TFs that repress or activate BMAL1:CLOCK transactivation are determined by evaluating results with first group experiment by calculating fold of increase/decrease [$(\mathrm{pBIND} - \mathrm{TF} + \mathrm{Bmal1/Clock} + \mathrm{mPer1dLuc}) \div (\mathrm{Bmal1/Clock} + \mathrm{mPer1dLuc})$] (Table 1).

Table 1: 384-well plate content used in high throughput luciferase screening assay

Aim	**Wells content**
To measure background luciferase activity	pBind+sport6
TFs were checked whether they bind to *Per1* promoter	pBIND-TFs + Per1: *luc(*reporter plasmid)
To measure effect of GAL4 in pBIND on BMAL1:CLOCK transactivation (no effect is expected)	*Bmal1/Clock*+ G4 (pBIND)
Positive control for repression of BMAL1:CLOCK transactivation	*Bmal1/Clock* and *Cry1*
TFs repression or activation on BMAL1/CLOCK transactivation	TFs- pBIND + Per1: *luc* + *Bmal1/Clock*
As a control, for activity of Per1 promoter resulted from effect of endogenous BMAL1/CLOCK and the other proteins.	Per1:*luc*
To check toxicity effect on cell survival and to prevent false positive results for repression activity	pBIND-TFs + Per1:*luc*

4.3 Characterization of the Circadian Properties of Transcription Factors; NFE2L2, SSBP2, SSBP4, TIRAP and WWTR1

Based on initial screening, five transcription factors were selected as promising TFs that reveal repression activity on BMAL1:CLOCK transactivation. I started with TFs knockdown with siRNA in cell culture system to monitor phenotypic change in circadian rhythms of human osteosarcoma cell line; U2-OS *Bmal1*: *dluc* (U2-OS-B6) cells possessing destabilized luciferase reporter under control of *Bmal1* promoter. Then I repeated luciferase reporter assays to control repression activity of those selected TFs.

4.3.1 Real-Time Monitoring of Circadian Oscillations for Knockdown of TFs with siRNA

We aimed to see whether there would be a difference in amplitude, phase or period change in circadian oscillation in case of TFs deficiency. Therefore, knockdown effect of selected TFs on circadian phenotypes *in vitro* was determined using specific siRNA to each TF in synchronized U2-OS *Bmal1*: *dluc* cells. Cells were seeded in 35 mm dishes at density of 1.5 x 10^5 /dishes in 1.5 ml of DMEM with 10% FBS w/o pen/strep antibiotics. In following day, cells were transfected with four sets of oligomer pools designed against to each TF (Qiagen-Table 2). Solution of siRNA was prepared as mixing 10 μM stock of each siRNA solution to obtain working solution containing 2 μM of each siRNA at final. Firstly, for each dish, 3 μl of 2 μM siRNA solution (6 pmol from each) and 250 μl of Opti-MEM (GIBCO) were mixed. Meantime, 5 μl Lipofectamine RNAiMAX (Invitrogen) and 250 μl of Opti-MEM solution was prepared and incubated 5 min at RT (room temperature). Then two mixtures were combined and added onto cells dropwise. After 36-48 hour of incubation, medium of plates with >90% confluence of cells was replaced with 1.5 ml of media prepared from phenol red-free DMEM powder (sigma), 5 % FBS, 25 mM HEPES (GIBCO), 0.1 μM Luciferin(Promega), 3.5 g/l D-(+)-glucose powder(Sigma), 100 μg/ml penicillin/streptomycin, 2 mM glutamine, sodium bicarbonate (0.35 g/l), 100 nM dexamethasone (Sigma). Plates were sealed with grease and loaded in a 32-channel lumicycle (Actimetrics) in a 37 ^{o}C incubator and bioluminescence was continuously recorded as previously described [421].

4.3.2 Cloning of TFs Genes into an Expression Vector, pCDNA4/*myc*-His A and Protein Overexpression and Western Blot

Transcription factors were cloned into pCDNA4/*myc*-His A to use His/Myc tag for expression check. Coding gene sequences in pBIND vector as template were amplified by polymerase chain reaction (PCR) with gene specific primers (Table 3) having appropriate restriction sequences. The PCR conditions are applied as follows; 94^{o}C, 4 min and 94^{o}C, 30 sec; 55^{o}C, 30 sec; 72^{o}C 1.30 min for 30 cycles and then gel purification was applied to the PCR products. Purified PCR products of TFs genes were cloned into pCDNA4/*myc*-His A mammalian expression vector using determined restriction enzymes. The cloning and genetic manipulations were carried out according to Sambrook et al.[422]. The expression vector provides the sequence for 6X His and Myc tag as the fusion tag in the N-terminal of TFs. HEK293T cells were seeded in 6 well plates containing 1.5 ml media at 3x 10^6 cells/ well density. Following overnight growth at 37 °C in a humidified incubator with 5% CO_2, cells were transfected with 2 µg of the plasmid TF- pCDNA4/*myc*-His A using 3 µl TurboFect transfection reagent (Thermo Scientific) in 200 µl of serum-free DMEM. Mixture was incubated for 15-20 minutes at RT and then added dropwise to the plate of cells. After incubation at 37°C for 36 hours, cells were washed and harvested with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO4, pH 7.4) and centrifuged at 1800 x g for 5 minutes at 4 ^{o}C. Pellet was dissolved in 180 µl of distilled water, and 40 µl of 6X protein loading dye (200 mM Tris-Cl, pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol). Subsequently, cells were vortexed and heated up to 95^{0}C for 10 min. Then, they were centrifuged at 16,000 x g for 5 minutes, and cell supernatant containing total protein were loaded and separated on a 10%-15% SDS-polyacrylamide gel at 150 V for 1.5 h. After semi dry transfer to polyvinylidene difluoride membrane (Biotrace PVDF, Pall Corporation, FL, and USA) at 1 A/cm2 for 1 hour, the membrane was blocked in a solution of 5 % w/v powdered nonfat milk in tris-buffered saline with 0.1 % Tween-20(TBS-T) followed by incubation with monoclonal anti-His-HRP (ABCAM) (1:5000) for 1 hour at RT. Finally, proteins were visualized by ECL system.

4.3.3 Determination of Repression Activity of Transcription Factors; SSBP2, SSBP4, and WWTR1 cloned in pCDNA4/*myc*-His A with Luciferase Reporter Assay

After successful cloning of *Ssbp2*, *Ssbp4*, and *Wwtr1* gene into pCDNA4/*myc*-His A vector, repression activity of TFs- pCDNA4/*myc*-His A (50 ng) on BMAL1:CLOCK transactivation was repeated. Confluent HEK293T cells were seeded to a 10 cm plate 24 hour-prior to transfection to obtain 70-80% confluent cells for reverse transfection. A mixture of Per1*:luc* reporter plasmid (50 ng), *Bmal1*-pCMV-Sport6 (50 ng), *Clock*-pCMV-Sport6 (100 ng), and TFs- pCDNA or *Cry2*-pCMV-Sport6 (50 ng) is transiently transfected into HEK 293T cells using PEI transfection reagent. A mixture of Per1:*luc*, *Bmal1* and *Clock* containing plasmids was used as a positive control, while *Cry2* with Per1: *luc* was used as a negative control. The total amount of plasmid DNA was adjusted to 300 ng by adding control plasmid, pCMV-Sport6. DNA mixtures, diluted in 50 µl serum-free DMEM, were mixed with 0.9 µl transfection reagent and incubated for 20 minutes at room temperature (RT). In the meantime, cells were seeded to 96-well plate at concentration of 40 x 10^3 cells/well in 50 µl of DMEM supplemented with 20% fetal bovine serum, 4 mM L-Glutamine, 200 U/ml penicillin /streptomycin. Following to 20 min incubation, 50 µl DNA reaction mixtures were dispensed to each well. At 24 h post-transfection, 75 µl luciferase reagent (Promega) was added onto cells, mixed and after 5 min incubation, luciferase activity was measured in the Fluoroskan Ascent FL (Thermo Scientific) plate reader.

4.4 Determination of Dose-dependent Transcriptional Repression Activity of WWTR1 with Luciferase Reporter Assay

In order to have more detailed characterization on repression activity of WWTR1 on BMAL1:CLOCK transactivation, dose-dependent transcriptional repression of WWTR1 was carried out using reporter gene assay. Assay was carried out after *Wwtr1* gene was cloned into pCMV-Sport6 to have WWTR1 protein expression without any tag with primers listed in Table 3. WWTR1 repression on BMAL1:CLOCK transactivation was assessed on three different promoter regions from *Per1, Per2* and *Cry1* promoters containing E-box/boxes, that were cloned in pGL3 to obtain Per1: *luc* or Per2*: luc* or Cry1*:luc* reporter plasmids. A mixture of reporter plasmid Per1: *luc* (50 ng) or Per2*: luc* (50 ng) or Cry1*: luc* (50 ng), *Bmal1*-pCMV-Sport6 (50 ng), *Clock*-pCMV-Sport6 (100 ng), *Wwtr1*-pCMV-Sport6 (25, 50 and 100 ng

titration of plasmid) or *Cry2*-pCMV-Sport6 (1 ng) was transiently transfected into HEK 293T cells using PEI transfection reagent. Rest of procedure was same as previously described reporter gene assay in section 4.2.2.

4.5 Determination of Direct Repression activity of WWTR1 on *Per1, Per2, Cry1* and *Bmal1* promoter

To analyze direct repression of WWTR1 on *Per1, Per2, Cry1* and also *Bmal1* promoter region; HEK 293T cells were subjected to reverse transfection without Bmal1 and Clock gene co-transfection. A mixture of reporter plasmid Per1*: luc* (50 ng) or Per2: *luc* or Cry1*: luc or* Bmal1*: luc* with *Wwtr1*-pCMV-Sport6 (25, 50 and 100 ng) or *Cry2*-pCMV-Sport6 (1 ng) is transiently transfected into HEK 293T cells using PEI transfection reagent. The rest was same as previously described reporter gene assay in section 4.3.3.

4.6 Determination of Transcriptional Repression Activity of WWTR1 on BMAL1 and CLOCK interaction with Luciferase Reporter Assay

In order to determine the effect of WWTR1 on BMAL1 and CLOCK interaction, mammalian two hybrid system was used. The protocols are carried out according to manufacturer's manual [422]. In this system, the pBIND vector, the bait vector, contains the yeast GAL4 DNA-binding domain in the upstream of a multiple cloning region, and the pACT vector, the prey vector, contains the herpes simplex virus VP16 activation domain upstream of a multiple cloning region. The genes encoding the two potentially interactive proteins of interest are cloned into those bait and prey vectors. Another vector is also used as marker called pG5*luc* that contains firefly luciferase gene of which upstream is occupied by GAL4 binding site and minimal TATA box. *Bmal1* and *Clock* genes, expressed with fusion DNA-binding domain of GAL4 and activation domain of V16 reciprocally were transfected along with pG5*luc* vector and *Wwtr1*-pCMV-Sport6 into HEK 293T cells. HEK 293T cells were seeded to a 10 cm plate 24 h-prior to transfection to obtain 70-80% confluent fresh cells used for reverse transfection. A mixture of reporter plasmid PG5*luc* (50 ng) *Bmal1*-pACT (50 ng) or *Clock*-pBIND (50 ng) or vice versa, *Wwtr1*-pCMV-Sport6 (50, 100 ng) and PG5*luc* was transiently transfected into HEK 293T cells using PEI transfection reagent. PG5*luc*, *Bmal1*-pACT (-pBIND), and *Clock*-pBIND (-pACT) mix was used as a positive control for BMAL1-CLOCK interaction, while PG5*luc* alone was used as a negative control. The total

amount of plasmid DNA was adjusted to 300 ng using pCMV-Sport6. At 24 hour post-transfection, 75 µl of luciferase reagent was added to each well, and luciferase activity was measured in the Fluoroskan Ascent FL (Thermo Scientific) plate reader.

4.7 Determination of Interaction of BMAL1/CLOCK and WWTR1

4.7.1 Co-immunoprecipitation

HEK2 93T cells (1-2 x 10^6 cells /well) were seeded in 10 cm plate in DMEM (10 % FBS, 1%Pen/Strep) 16-24 hour prior to transfection. In following day, cells should be ~70%-80% confluent at time of transfection. A mixture of either 5µg of *Bmal1*-pCDNA-4-A-flag tag (lacking the His tag) and/or 5 µg of *Clock*-pCMV-Sport6-flag tag) and/or 5 µg of *Wwtr1*-pCDNA4-His/Myc-A or *Wwtr1*-pCMVsport6 was co-transfected into HEK 293T cells using the PEI reagent in 1 ml DMEM solution. Mixture was incubated for 15-20 min at RT. Then 1 ml mixture was added dropwise around the plate of cells. Cells were harvested at ~48 hour post-transfection and used for co- immunoprecipitation. Media was removed and washed with 5 ml ice-cold 1X PBS buffer and collected by centrifugation at 1800 x g for 5 minutes. PBS was aspirated and ¼ of pellet were lysed by gently resuspending in 400 µl pre-cooled lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% glycerol 1% triton 100X, and 100 µM PMSF,1% v/v protease inhibitor cocktail). After 10 min incubation, cell lysate was sonicated for 15 sec on ice at 20% output, 40% duty cycle. Cell suspension was put on the rotator in cold room for 30 min and sonicated again. Then solution was centrifuged at 16,000 x g for 10 min. The supernatants were transferred to a new 1.5 ml-centrifuge tube. Meanwhile 20 µl/tube anti-Flag M2 affinity resin (Sigma) was washed once with ice-cold TBS-200 and twice with ice-cold lysis buffer (without PIC and PMSF). %10 of supernatant was saved as input and the rest was mixed with ~ 20 µl anti-Flag M2 affinity resin/sample for overnight rotating at 4°C. Following day, tubes were spinned at 4ºC for 5 min at 5000 rpm to collect the resin. Supernatant was carefully taken and saved as flow through. Resin was washed 3X times with 1000 µl 1X TBS with 0.1 mM $CaCl_2$ and 1X times with TBS-T via centrifugation at 5000 rpm for 5 min. 20 µl ddH2O and 6 µl Laemmli loading dye mixture was added to resin and heated up to 95 ^oC for 5 min. Subsequently, the beads were collected at the bottom by centrifuging at maximum speed for 5 min. Finally, 10-15 µl of supernatant was loaded and run in 10-15 % SDS-PAGE. Following to protein transfer, membrane is blocked with 5 % milk powder, and

then incubated with anti-His-HRP conjugated antibody (Abcam, 1:5000) and anti-Flag antibody (Sigma, 1:1000) and anti-TAZ(Wwtr1) (Santa-Cruz, 1:1000) diluted in TBS-T. Visualization was achieved using ECL system.

4.7.2 Bimolecular Fluorescence Complementation Assay (BiFC)

Venus is an enhanced florescent protein separately cloned as C- and N-terminal region in BiFC-VC155 and BiFC173 vectors. In our study, *Bmal1* and *Wwtr1* genes were cloned as fused with C- and N-terminal regions using appropriate primers (Table 3) and cloning techniques. HEK293T cells (0.8-1×10^5 cells/ well) were plated in 1 mL of growth medium in 12-well plate with the 24-well chambered cover slips. Cells were grown overnight to obtain confluency around 50-60 %. Medium was removed, cells were washed with antibiotic free growth medium and then 1 ml antibiotic free growth medium was added. Next cells were co-transfected with 1 µg plasmids mixture of *Bmal1*-pBiFC-VC155 (500ng) and *Wwtr1*-pBiFC-VC173 (500 ng) or *Bmal1*-pBiFC-VC155 (500ng) with pBiFC-VC173 ((500 ng) or *Wwtr1*-pBiFC-VC173 (500ng) with pBiFC-VC155 (500 ng) using 3 µl PEI in 100 µl opti-MEM. After 4-6 hours medium was change with growth medium supplemented with antibiotics. On the following day, after 24-22 hour-incubation, cell were rinsed with PBS three times, fixed with 400 µl of 4% paraformaldehyde for 10 min and washed with PBS 3X times, permeabilized with 0.1 % triton-100 for 15 min and washed with PBS 3X times. Afterwards, cells were incubated with DAPI solution (Invitrogen) for cell nucleus staining for 10 min and washed 3X times for 5 min with PBS. Mounting medium was used to fix slice to microscope slide. After overnight incubation at 4 ^oC, cells were imaged using a confocal microscope (The Nikon Eclipse 90i upright microscope). Nuclei were stained with DAPI stain of which signal was obtained using the violet laser line (405 nm). Venus is a yellow fluorescent protein and signals were obtained by exciting via argon laser (488 nm).

4.8 Cloning shRNA Oligos into the pLKO.1 and Generation of Lentiviral shRNAs for Gene Knockdown

In order to knockdown Wwtr1 gene expression, two target oligonucleotide sequences were designed for mouse *Wwtr1* genes using The Genetic Perturbation Platform (Broad Institute) for mouse cell lines and for human *WWTR1* gene, shRNA sequence was obtained from Chan et al., and named as sh4*hWWTR1*[371] and one target oligonucleotide sequence for

Negative shRNA (Sigma SHC002 MISSION; Non-Mammalian shRNA Control) with digestion site of *PstI* enzyme (Table 4). Oligonucleotides designed for shRNA were annealed with use of a thermal cycler (Biorad). Equal volumes of both complementary oligonucleotides were mixed at equimolar concentration (100 µM concentration) and PCR was performed using the following thermal cycler program for annealing:

37 °C x 30 min for activation T4 PNK

95°C × 5 min

70°C × 10 min

70 °C x 0.1 °C /min cycle→ cool to RT for 450 cycles (cool to 4 °C for 750 cycles)

Cool to 4°C

Annealed oligonucleotide sequences were cloned into the *EcorI* and *AgeI* sites of the lentiviral pLKO.1 vector under the control of the human U6 promoter and cloning was checked by diagnostic double digest with *PstI* and *BamHI* enzymes.

In order to produce lentiviral particles, cocktail of pLKO.1-shRNA plasmid (10 µg), pCMV-ΔR8.2dvpr (9 µg) as packaging vector and pCMV-VSVG(1 µg) as envelope vector with 30 µl of PEI transfection reagent in 400 µl DMEM were transfected to 70-80% confluent HEK293T cells in 10 cm plate containing 5 ml growth medium. After 12-16 hour-incubation, 3 ml fresh growth medium was added and cells were incubated for another 24 hour. In following day medium was replaced with 8 ml fresh growth medium. Viral particles in culture medium of HEK293T cells were harvested at 72 hour and 96 hour post-transfection allowing viruses to be released into the supernatant. This supernatant was collected and filtered through the 0.45 µm filter and stored at -80 ^{o}C to be used for transduction of U2-OS Bmal1:*dluc* and NIH3T3 Per1*:dluc* cells.

4.9 shRNA mediated *Wwtr1/WWTR1* Knockdown and Real-Time Monitoring of Circadian Oscillations in NIH 3T3 Per1*:dluc* and U2-OS Bmal1:*dluc* with Lumicycle

NIH 3T3 Per1:*dluc* and U2-OS Bmal1:*dluc* cells were transduced with shRNA lentiviral particle to examine any phenotyping change in their circadian oscillation. One day before viral transduction, NIH 3T3 Per1*: dluc* ($0.5x10^5$ cells/ well) and U2-OS Bmal1:*dluc* cells ($1x10^5$ cells/ well) were seeded in a 35 mm-dish to obtain confluency about 50-70% in

the next day. Cells were treated with mixture of thawed 750 µl lentiviral particle solution, 1 µl protamine sulfate (8 mg/ml) and 250 µl DMEM after removing old medium. After 12 hours, virus-containing media was replaced with 1.5 ml fresh media. For NIH 3T3 Per1*: dluc* cells; viral transduction was repeated one more time. At 72 hour post-transfection, media of cells was replaced with 1.5 ml of DMEM (without phenol red) supplemented with FBS(10%), HEPES (25 mM), luciferin (0,1 µM), dexamethasone (0,1 mM), D-(+)-glucose powder(3,5 g/l), 1x penicillin-streptomycin, glutamine and sodium bicarbonate(0.35 g/l). Cells were incubated at 37°C for at least 7 days in lumicycle luminometer (version 2.31, Actimetrics) and bioluminescence was continuously recorded. After, first 24 hour data exclusion, baseline-subtracted raw data were fitted to a sine wave (damped) and analyzed with the LumiCycle Analysis program.

4.10 Determination of Knockdown Effect of *Wwtr1* Gene on Circadian Oscillation of Clock Gene Expression via QRT-PCR and Western Blot

At 72 hour post-transduction, NIH 3T3 Per1: *dluc* cells were harvested as 1st samples for western blot and RNA isolation as time 0 (CT0; CT, circadian time 0). For western blot, cells were collected with scraper and stored at -20 ^{o}C until to be use. Another replica of plate was lysed with 1 ml PEQLAB reagent with pipetting several times followed by 3 min incubation at RT and stored at -80 ^{o}C until total RNA isolation. Rest of the cells were synchronized with dexamethasone in serum free DMEM containing 10 mM HEPES (pH 7.5) for 2 hours. Then the cells were harvested at every 4 hour over the course of 24 hours and stored at -80 ^{o}C. Total RNA was extracted from stored cells according to manufacturer's manual (PEQLAB). Briefly, cells were thawed on ice and 0.2 ml chloroform per 1 ml of reagent was mixed with sample by shaking vigorously for 15 sec. Following 2 min incubation at RT, samples were centrifuged at 12,000 x g for 15 minutes at 4 °C. RNA containing colorless upper aqueous phase was separated and mixed with 0.5 ml isopropanol by inverting 15 times. After 10 min incubation on ice, tubes were centrifuged at 12,000 x g for 10 min at 4 °C. Pellets were washed with 1 ml of 75 % ethanol and then centrifuged at 12,000xg for 5 minutes at 4 °C. RNA pellet were dried for 20-30 min at RT. Dried pellets were dissolved in 40-50 µl of RNAse/DNAse free water and incubated at 55–60 °C for 10–15 min to facilitate dissolution. RNA concentration was measured with NanoDrop (Thermo Scientific, USA) at 260 nm and integrity of total RNA was checked with 0.4-0.5 % agarose gel prepared with

DEPC water. Around 3-4 µg of RNA was treated with 1 µl of DNAse enzyme (Thermo Scientific) for 30 min at 37 °C to get rid of DNA. Then EDTA was added and incubated for 10 min at 65 ^{o}C to stop DNAse activity. Ethanol precipitation was carried out by mixing samples with 0.1 V RNAse/DNAse free sodium acetate and 2.5 V cold EtOH. Samples were allowed to incubate for 2 hours at -80 ^{o}C followed by incubation for 30 min on ice. After centrifugation at maximum for 40 min, samples were washed with 75 % EtOH and air dried for 20-30 min. Next, samples were checked for concentration and integrity. 1-1.5 µg of cleared RNA was converted to cDNA in 16 µl reactions solution containing 1 µl Oligo (dT) primers, 2 µl of 10 mM dNTP mix and RNAse/DNAse free water. Mixture was incubated for 4 min at 70 ^{o}C. After addition of 2 µl 10X reaction buffer, 1 µl RNAse inhibitor and 1 µl M-MLUV reverse transcriptase(BioLabs), reaction was incubated for 1 hour at 42 °C and for 10 min at 90 °C. cDNA solution volume was completed to 100 µl with ddH_2O. Real time PCR reactions were carried out on thermal cycler (CFX96^{TM} real Time systems, Bio-RAD). Real time PCR solution contained 2 µl cDNA solution, 8 µl SYBR Green qRT-PCR reagent, and 3 µl of 5 pmol reverse and forward primer mixture and 7 µl ddH_2O. cDNA denaturation was carried out for 10 min at 95 ºC followed by forty cycles of denaturation at 95 ºC for 15 sec and annealing at 60 ºC for 30 sec and extension at 72 ºC for 30 sec. Specific primer sequences for *Wwtr1*, clock genes were designed using NCBI/ Primer-BLAST (Table 5). Relative gene expression values were determined by delta–delta CT method and normalized with respect to cells transduced with shNeg at CT0. *Gapdh* was used as housekeeping gene to normalize the gene expression levels of each sample.

Protein level was analyzed via western blot technique. Pellet was dissolved in 120 µl of SDS-lysis buffer (50 mM Tris-Cl, pH 6.8, 2 mM EDTA, 120 mM DTT, 1% SDS, 8% glycerol, PIC and PMSF). Subsequently, cells were sonicated for 2 x10 sec and boiled at 95^{0}C for 10 min, and then put on ice. Then, they were centrifuged at 16,000 x g for 5 min, and supernatant was collected to a fresh tube. The total protein concentration was determined by Bradford protein assay (Biorad). Equal total protein were loaded and separated on a 10% SDS-polyacrylamide gel. After wet blotting to polyvinylidene difluoride for 50 min, the membrane was blocked in a solution of 4 % w/v powdered nonfat milk in tris-buffered saline/Tween-20 followed by incubation with anti-Bmal1 antibody (1:1000, Santacruz biotechnology, Inc.), anti-Cry1 antibody (1:5000, Bethyl Laboratories, Inc.), anti-Dbp

antibody (1:2000, Abcam, Inc.), and anti-TAZ (1:1000, Santacruz biotechnology, Inc.) for overnight at 4 ^{o}C. Finally, proteins were visualized by ECL system via ChemiDoc™ Touch (BioRad) and analyzed with Image Lab 5.2(BioRad).

4.11 Chromatin Immunoprecipitation

For co-immunoprecipitation experiment Wwtr1-PLL3.7 construct was used to transfect NIH 3T3 fibroblast cells. *Wwtr1* was firstly cloned into pEGFP-C1 vector using primers with *HindIII* and *EcorI* sites(Table 3) to obtained *Wwtr1* construct with EGFP at the N termini. Then Wwtr1-EGFP was subcloned into PLL3.7 lentiviral vector using *NheI* and *EcorI* restriction enzymes. Two plates of 0.7-1x10^{7} fibroblast cells in 10 cm dishes were transfected with Wwtr1-PLL3.7 or PLL3.7 plasmid (10 µg) with PEI reagent. At 48 hour after transfection, firstly, 37 % formaldehyde was added drop-wise directly to adherent cell cultures in a chemical fume hood to obtain a final concentration of 1% (v/v). Plates were rotated gently at RT for 8-10 min. To stop crosslinking reaction, 2.5 M Glycine was added to get final concentration of 0.1 M-0.125 M and incubated with shaking for another 5-8 min at RT. Then cells were washed (6 ml X2) with cold PBS. Next, 5 ml ice-cold PBS added onto cells and cells were scraped and transferred into 15 mL tube. Another 3 mL of PBS was added to dishes; remaining cells were scraped again and transferred to the same tube. The cells were centrifuged at 2000-3000 x g for 5 min at 4 ^{o}C and supernatant was removed and pellet was washed with PBS one more time. Cell pellets can be stored at -80 °C, or lysed directly to proceed to experiment. Pellets were lysed in 1ml ice-cold ChIP lysis buffer I (1x10^{6} cells/100 µl sample; 50 mM Tris-HCl pH 8.0, 85 mM KCl, 0.5% NP40, proteinase inhibitor cocktail and PMSF) and incubated for 10 min on ice. Nuclei were recovered by centrifugation in a microfuge at 2000 x g for 10 min at 4 ^{o}C. Supernatant was aspirated off carefully and pellet was resuspended in 700 µl ChIP lysis buffer II (2X10^{6} cells/100 µl sample; 50mM Tris-HCl pH7.5, 100mM NaCl, 0.4% SDS, 0.4% deoxycholate, 0.2% Nonidet P 40, and 10mM EDTA with proteinase inhibitor cocktail and PMSF). 300 µl of lysed cells was put into special tubes and sonicated in Bioruptor with 4^{o}C water bath for 50 min with settings of power level: high; duty cycle: 30 sec on 30 sec off. Samples were vortexed and spinned for every 10 min. The insoluble material was removed by centrifugation at maximum speed for 15 min at 4 ^{o}C. After removing 50 µl of supernatant to check DNA concentration and fragment size, remaining supernatant which contained fragmented chromatin was stored at -80 ^{o}C until use. Following

reverse crosslinking process, if DNA fragment size was concentrated around 100-500 bp, stored samples were diluted 1:4 using ice-cold ChIP dilution buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1.1 % Triton X-100, with protease inhibitor cocktail and PMSF). Diluted chromatin samples were first precleared by incubating with 20 µl Protein A/tube for 30-45 min at 4 ^{o}C. After centrifuge, supernatant was recovered and 10% of the chromatin was set aside at -20 ^{o}C for use as input. The remaining chromatin was incubated with the anti-Wwtr1 (Novus Bio: 1:400) and rabbit IgG (3-4 µg)(Santa Cruz, Inc.) as negative control at 4°C for overnight with rotating. On the next day, 20 µl of Protein agarose A (sigma) for each reaction was washed with IP buffer (25 mM Tris-HCl pH 7.2, 150 mM NaCl) spinning at 2500 x g for 3 min at 4 ^{o}C. Chromatin samples with Protein agarose A were rocked in cold room for 1-2 hours. Beads were pelleted by spinning at 2500 x g for 3 min in cold room. Supernatant part was discarded without disturbing the beads. Beads were washed sequentially with buffers (Low Salt Wash Buffer: 0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1,150 mM NaCl; High Salt Wash Buffer : 0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl; LiCl Wash Buffer: 0.25 M LiCl, 1%NP40, 1% sodium deoxycholate, 1 mM EDTA, 10mM Tris pH8.1; 1X TE : 1 mM EDTA, 10 mM Tris-HCl pH 8.1) by rocking for 5 min and spinned down 2500 x g for 3 min in cold room. Elution was performed with fresh prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). 150 µl of elution buffer was added to beads and mixture rocked at RT for 15 min followed by spinning down at 2500 x g for 3 min. Supernatant was transferred to fresh tube without disturbing the beads. This step was repeated again, and eluents were combined. Both inputs and eluents were applied to reverse crosslinking process. Firstly, RNAse was added to sample at final concentration of 50 µg/ml and mixtures were incubated at 37 ^{o}C for 30 min. Then, proteinase K was added at a final concentration of 0.4 mg /ml and mixture was incubated at 50 °C for 1 hour. Crosslinks were reversed by gentle shaking at least 4 hours to overnight at 65 °C in elution buffer containing 150 mM NaCl. Samples can be stored at –20 ^{o}C until later use. DNA was purified using PCR purification kit (Qaigen) and can be eluted up to 100µl H_2O. Input DNA was first diluted 100-200 folds and then 3 µl ChIP DNA and 3 µl of diluted input DNA are subjected to real time PCR using promoter targeted primers (Table 6).

Chapter 5

RESULTS

5.1 A High Throughput Screen Assay to Determine the Effect of 1400 Transcription Factors on BMAL1:CLOCK Transactivation

In order to determine the effect of 1400 transcription factors on BMAL1:CLOCK driven transactivation, a high throughput screen assay was utilized in which all experiments were done in two groups and repeated two times. In the first group, TFs, alone in pBIND vector, were screened for whether they affect the luciferase expression under the control of *Per1* promoter in reporter plasmid (mPer1: *luc*). Beside, wells containing pBIND (GAL4) and pCMV-Sport6 were used for background luminescence information without marker plasmid. Wells containing BMAL1/CLOCKand pBIND plasmids were also examined to show GAL4 effect on BMAL1:CLOCK transactivation (no effect of pBIND vector is expected). In this group analysis were done to eliminate background and false positive results for better assessment. In the second group**,** plasmids which contain *Bmal1/Clock* in pCMV-Sport6 and TFs genes in pBIND were co-transfected to mammalian cells to monitor activation and repression influence of TFs on BMAL1:CLOCK transactivation on *Per1* promoter. Wells containing plasmids of *Bmal1/Clock* and *Cry1* genes were used as a positive control for repression on the transactivation. After 16 h incubation, luciferase activity was measured with a luminometer. Data from wells having only marker plasmid (Per1:*luc*) with the activity of *Per1* promoter resulted from endogenous Bmal1/Clock and other proteins' binding was used as a control for normalization. Since some TFs might have a toxic effect on cell survival and cause low luciferase activity, these experiments were repeated with marker plasmid and TFs only to eliminate false positive results for repression activity. In this group, TFs that repress or activate BMAL1:CLOCK transactivation were determined by comparing results with the first group experiment by calculating fold of increase/decrease [$(\mathrm{pBIND} - \mathrm{TF} + \mathrm{Bmal1/Clock} + \mathrm{mPer}luc) \div (\mathrm{Bmal1/Clock} + \mathrm{mPer1}luc)$]. After the analysis, six TFs that exert the highest repression activity on BMAL1:CLOCK transactivation were selected for further analysis.

Transcription factors showing repression effect on BMAL1:CLOCK transactivation are Nuclear factor erythroid 2-related factor 2 (NFE2L2/ NRF2), WW domain-containing transcription regulator protein 1 (WWTR1), Tata box-binding protein-like protein 1 (TBPL1), Single-stranded dna–binding proteins 2 and 4 isoforms (SSBP2, 4) and Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP). To our knowledge, except, NFE2L2, there have been no studies related to involvement of these transcription factors in circadian mechanisms. There are several studies about how clock regulates Nfe2l2 function in oxidative stress regulation [423] [424] but NFE2L2 function in clock mechanism has not been shown yet. Our goal was to characterize circadian function of these transcription factors that repress the BMAL1:CLOCK transactivation at the cellular level.

5.2 Characterization of the Circadian Properties of Transcription Factors; NFE2L2, SSBP2, SSBP4, TBPL1, TIRAP and WWTR1

In this study, we designed our experiments according to four criteria that target transcription factors have to possess to be considered as a core clock component. These criteria are having 24-hour transcriptional cycle, interacting with other core clock proteins, affecting circadian behavioral rhythms in mutation or knockdown case and being phylogenically conserved between vertebrates and flies [4]. In following section, firstly I examined the TFs knockdown effect on clock oscillation and then repeated luciferase gene assay to verify repression activity of TFs. Based on these data, which is shown in below, I decided to go forward with WWTR1 and characterize its clock function in more detail both at the molecular and cellular levels.

5.2.1 Real-Time Monitoring of Circadian Oscillations with LumiCycle for Knockdown of TFs

I would like to explore the effect of 5 genes on circadian clock by siRNA on U2-OS Bmal1-*luc* cells. U2-OS Bmal1: *dluc* cell line which stably expresses luciferase gene under control of the promoter has been used in a model cell line in the field [421, 425]

Cells were transfected with siRNA (purchased from Qiagen containing four sets of oligomers) against *SSBP2, SSBP4, WWTR1, NFE2L2* and *TIRAP* genes. Additionally, to determine the efficiency of the knockdown for each gene, cells treated with siRNA and control siRNA were collected and subjected for the qPCR analysis. qPCR of siRNA treated

cells verified that each gene expression reduced to the 20-30% (data not shown). As seen in Figure 5a, knockdown of the *SSBP2* attenuates the amplitude of oscillation while knockdown of the *SSBP4* increases amplitude of oscillation compared to negative siRNA treated cells**.** Decrease in amplitude level for *SSBP2* knockdown is around 10 %, while increase in amplitude level for *SSBP4* knockdown is nearly 3-fold. A reduction in expression of *SSBP2* gene causes almost 1 hour advance in the phase of oscillation while no significant change was observed in period length for both gene knockdown (Figure 5b).

Knockdown of the *WWTR1* gene by siRNA in U2-OS cell line has great impact on amplitude in which 70 % damping in amplitude and approximately 1 hour advanced phase of oscillation without any change in period time are observed (Figure 6a-b). Similar studies were also carried out with U2-OS cell line treated with *NFE2L2* and *TIRAP* siRNA. Analysis of the lumicycle results indicated *NFE2L2* knockdown decreases the amplitude to the 50 % and delays the phase (Figure 7) while *TIRAP* depletion reveals no change compared to non-transfected U2-OS cells (Figure 8). Collectively all these result indicated that TFs, being studied in this study, have impact on circadian clock by altering the phase and the amplitude of circadian oscillation with exception of the *TIRAP*. To see their effect is whether through BMAL1:CLOCK driven transcription, I, then, decided to perform experiments described in next section.

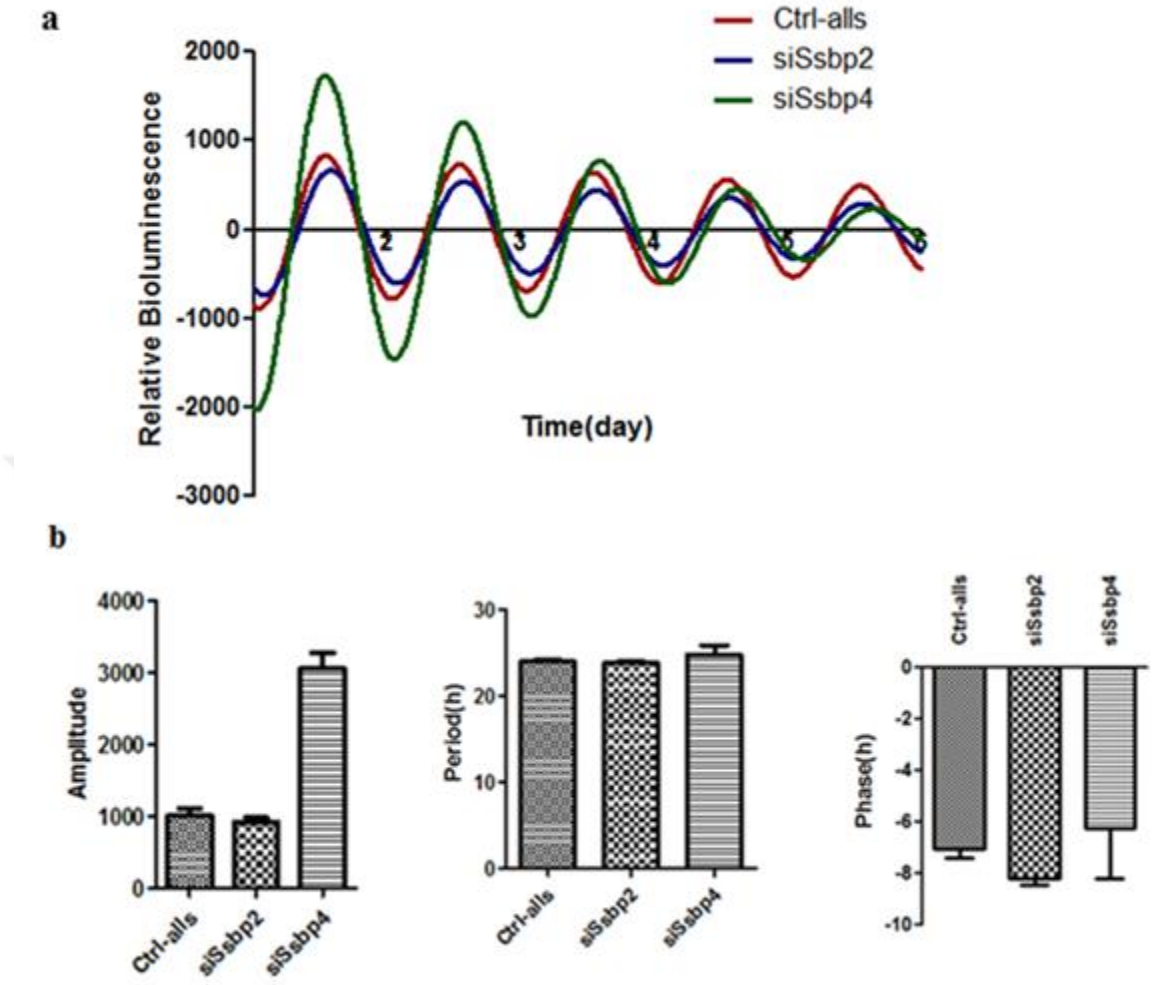


Figure 6: (a) Subtracted bioluminescence records of SSBP2 and SSPB4 knockdown and ctrl siRNA treated U2-OS Bmal1: dluc cells (b) Mean amplitude, period and phase change of circadian oscillation in cells with transfected siRNA targeting SSBP2 and SSBP4 and control siRNA. Error bars represent standard error of mean from 2 experimental repeats.

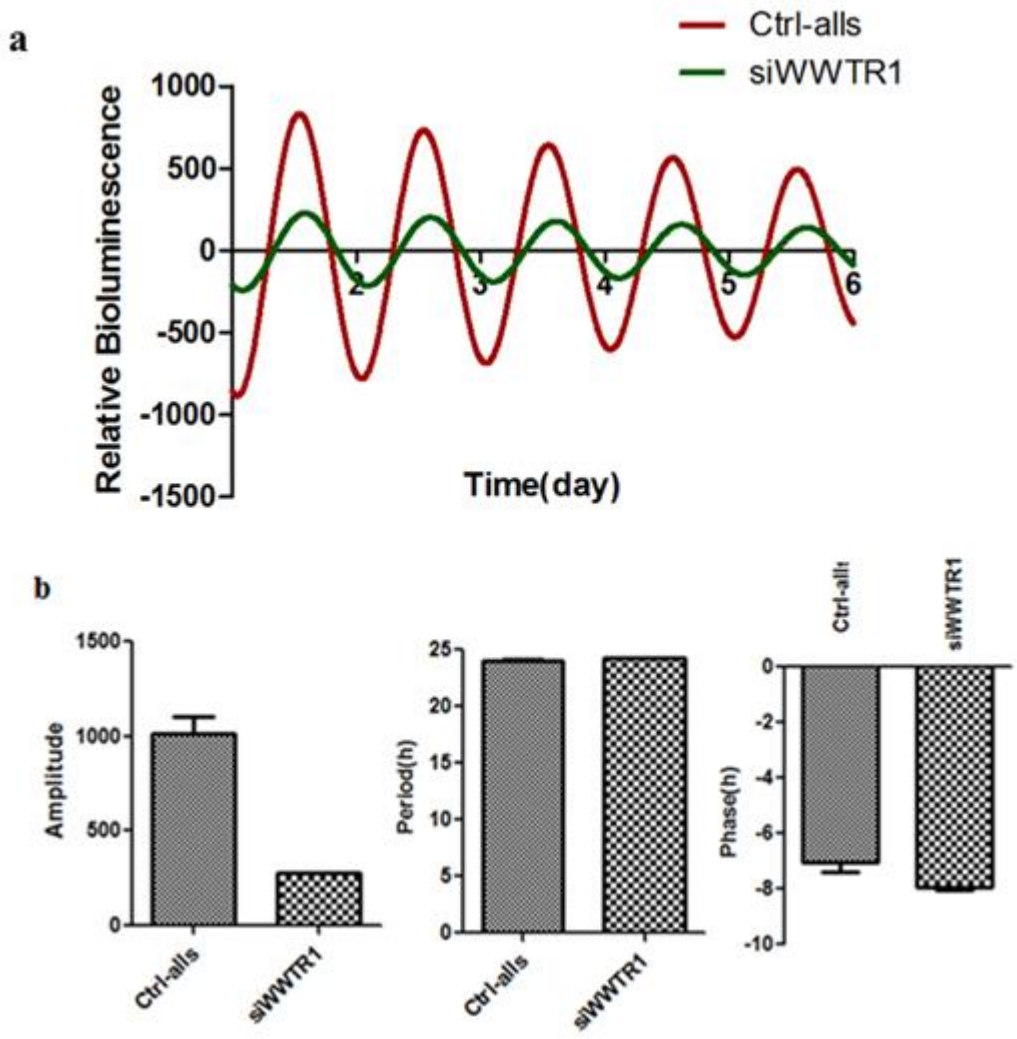


Figure 7: (a) Subtracted bioluminescence records of WWTR1 knockdown and ctrl siRNA treated U2-OS Bmal1: dluc cells (b) Mean amplitude, period and phase change of circadian oscillation in cells transfected with siRNA targeting WWTR1 and control siRNA. Error bars represent standard error of mean from 2 experimental repeats.

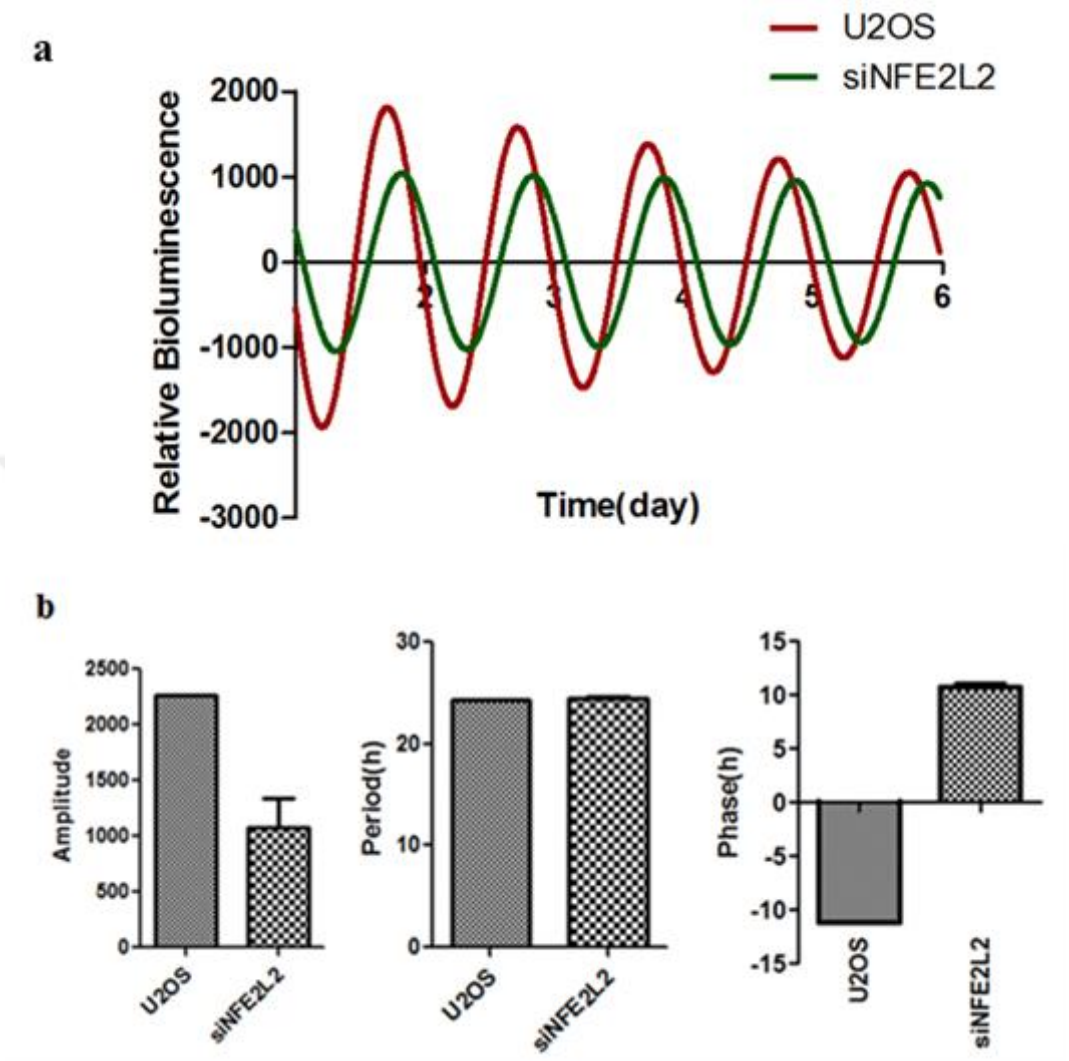


Figure 8: (a) Subtracted bioluminescence records of NFE2L2 knockdown and wild type U2-OS Bmal1: dluc cells (b) Mean amplitude, period and phase change of circadian oscillation in cells transfected with siRNA targeting NFE2L2 and U2-OS Bmal1: *dluc* without any treatment. Error bars represent standard error of mean from 2 experimental repeats.

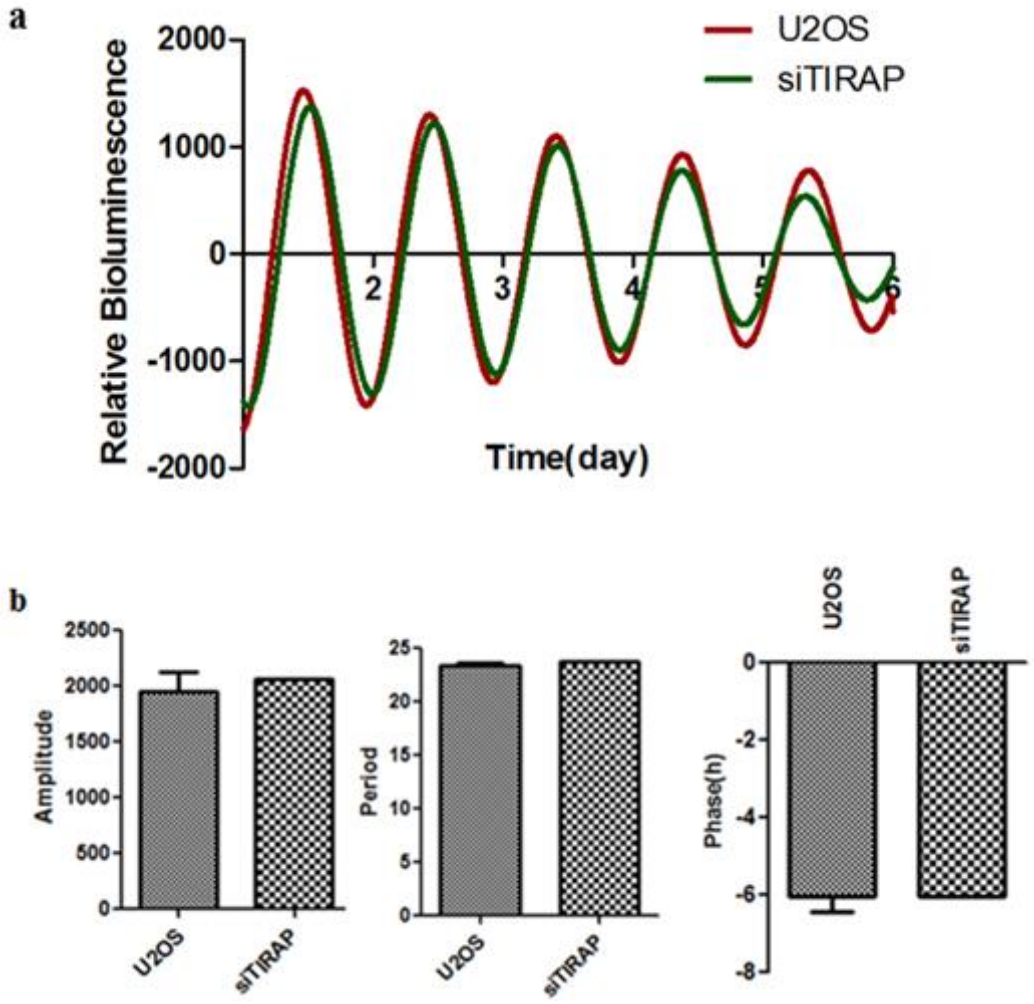


Figure 9: (a) Subtracted bioluminescence records of TIRAP knockdown and wild type U2-OS Bmal1: dluc cells (b) Mean amplitude, period and phase change of circadian oscillation in cells transfected with siRNA targeting TIRAP and U2-OS Bmal1: *dluc* without any treatment. Error bars represent standard error of mean from 2 experimental repeats.

5.2.2 Repression Activity of TFs; SSBP2, SSBP4 and WWTR1, on BMAL1:CLOCK Transactivation

In order to compare with and validate initial screen, we repeated luciferase reporter assay. Since siRNA knockdown of TIRAP gene did not exhibit any circadian phenotypic change and TBPL1 repression activity was low compared to other selected TFs in initial screens, I decided to not further characterize these two genes in detail. To understand how these genes affect circadian rhythm at cellular level, their repression activity on BMAL1:CLOCK driven transcription was assed via Per-*luc* assay commonly used in the field[167]. Therefore, I first amplify the cDNAs of the *Wwtr1*, *Ssbp2*, *Ssbp4*, and *Nfe2l2* by PCR (Figure 9a), and then cloned into mammalian expression plasmid pCDNA4-His/Myc-A (pCDNA). After sub cloning of these genes, their expression levels were assessed by anti-HIS, which is His/Myc tag was fused at the C-terminus of the each gene (Figure 9a) by Western blot. As expected the WWTR1, SSBP2, and SSBP4 proteins were expressed with expected molecular mass with the exception of NFE2L2 protein with apparent molecular mass 130 kDa that is higher than expected (Figure 9b). Next, WWTR1, SSBP2 and SSBP4 repression activities on the BMAL1:CLOCK driven transcription were measure by luciferase reporter assay. Analysis of the results revealed that WWTR1 displayed repression activity on BMAL1:CLOCK while SSBP2 and SSBP4 had no effect on BMAL1:CLOCK driven transcription (Figure 10).

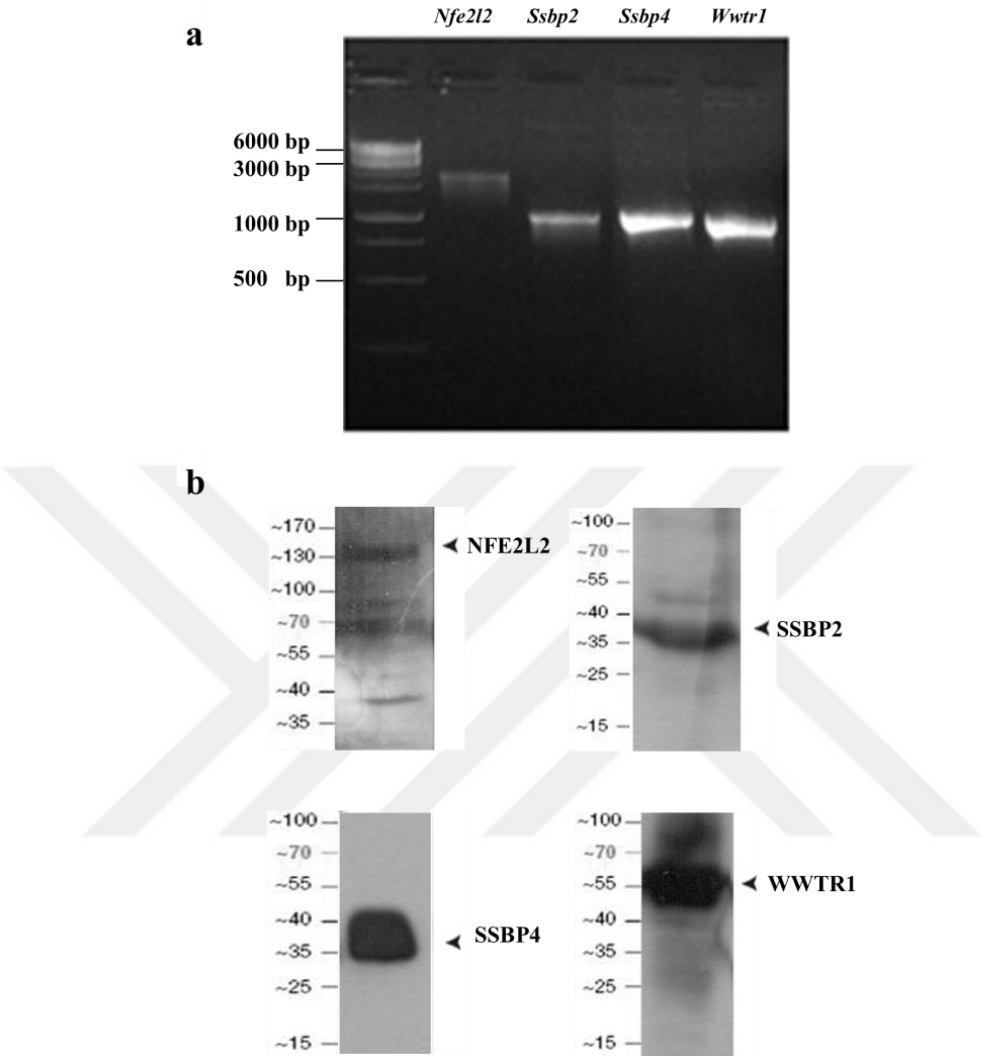


Figure 10: (a) PCR amplification of TF; Nfe2l2, Ssbp2, Ssbp4, Wwtr1(b) Protein expression of TFs in pCDNA4-His/Myc-A mammalian expression vector obtained from transiently transfected HEK 293T cells.

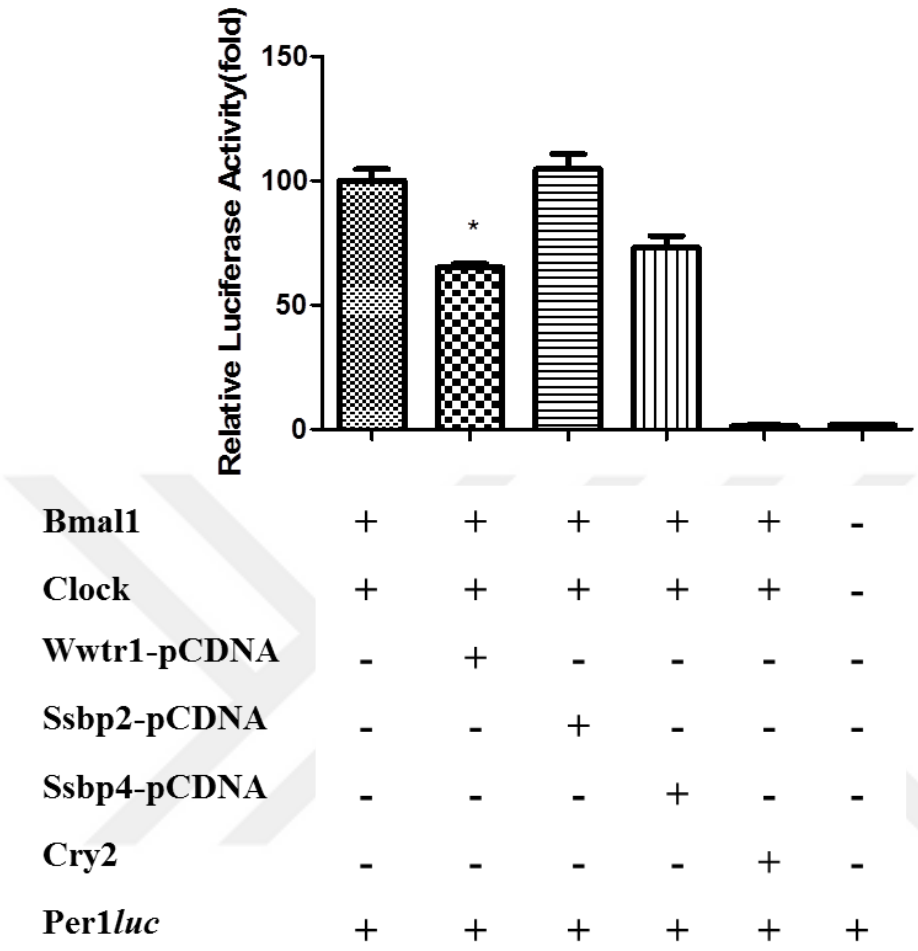


Figure 11: Transcriptional repression activity of WWTR1, SSBP2 and SSBP4 on BMAL1:CLOCK transactivation was analyzed by Reporter gene assay. Luciferase activity was normalized with respect to co-transfection of Clock, Bmal1 and mPer1*luc* plasmids. Experiment was performed in triplicate and repeated one time. Mean ± SEM (n = 3).Student's t test, *p(0,0216) < 0.05.

5.3 Characterization of the Circadian Properties and Role of WWTR1 in the Circadian Clock Mechanism

5.3.1 Activity of WWTR1 on BMAL1:CLOCK Transactivation

In order to eliminate possible tag effect on Wwtr1 protein activity in luciferase reporter assays, I decided to clone *Wwtr1* gene into pCMV-Sport6 and checked its expression with western blot using anti-Wwtr1 antibody (Figure 11a). Then repression activity of the Wwtr1 on BMAL1:CLOCK was investigated on Per1: *luc,* in which mouse *Per1* promoter fused with reporter gene luciferase. As seen in Figure 11b, WWTR1 represses the BMAL1:CLOCK activity on mice *Per1* promoter in a dose dependent manner. To see whether this repression activity of the WWTR1 is promoter specific or not, similar experiments were performed using Per2: *luc* and Cry1: *luc* reporter plasmid, containing mouse *Per2* and *Cry1* promoter regions, in a dose depended manner. Similar to Per1: *luc* results, WWTR1 also had repression activity on the mice *Per2* promoter in a dose dependent manner (Figure 11c). As the concentration of *Wwtr1* gene dosage was increased from 25 ng to 100 ng, the repression activity was increased from 55% to 90% on *Per1* promoter (Figure 1b) and from 57% to 77 % on *Per2* promoter, respectively (Figure 11c). These data demonstrate that WWTR1 has dose dependent repression activity on BMAL1:CLOCK transactivation on cloned *Per1* and *Per2* promoters, which consist of three E-box and two E-box-like elements, respectively. However, WWTR1 exhibits an antagonistic activity on BMAL1:CLOCK driven transcription on the mice Cry1: *luc,* in which *Cry1* promoter (-1.5 to +300) has one E-box site. WWTR1 elevates the *Cry1* promoter driven luciferase transcription in a dose dependent manner (Figure 11d). Taken all results together WWTR1 had repression activity for BMAL1:CLOCK activity on both *Per1* and *Per2* promoter while it enhances *Cry1* promoter activity event with co-expression of BMAL1/CLOCK, which implies its dual role on different clock genes promoters.

After observing these antagonistic results a similar experiments were performed without co-expressing BMAL1 and CLOCK on *Per1, Per1, Cry1* and also *Bmal1* promoter to see whether WWTR1 activity occurs through repression of BMAL1:CLOCK and/or specific to E-box. Results obtained from luciferase reporter assay show that Wwtr1 has subtle repression activity on *Per1* and *Per2* promoter and this possibly due to the interaction of the

WWTR1 with endogenously expressed BMAL1 and CLOCK (Figure 12 a-b). However repression level is not as high as with co-overexpression of BMAL1 and CLOCK genes indicating that the repression effect of WWTR1 is through BMAL1:CLOCK transactivation (Figure 12 a-b).

On the other hand, WWTR1 enhance luciferase activity on both *Bmal1* and *Cry1* promoters, indicating WWTR1 binds certain motifs within the promoter regions of these genes via interacting with some other factors and elevates transcription of the genes (Figure 12 c-d). Positive influence of WWTR1 on Bmal1 promoter provides us two important conclusions. First, repression activity of WWTR1 is specific to *Period* genes promoters, and conceivably through inhibition of BMAL1:CLOCK transactivation. Second, WWTR1 has positive regulation on *Bmal1* promoter that means WWTR1 regulate clock mechanism through core clock component, BMAL1. Moreover, WWTR1 also has positive regulation on *Cry1* promoter even that has E- box site with or without BMAL1/CLOCK overexpression. This result suggests that for *Cry1* promoter, WWTR1 co-activates another transcription factor that is prevalent compared to WWTR1 repression effect on BMAL1:CLOCK complex.

These results clearly showed that WWTR1 exerts its effect by interacting with core clock proteins of either BMAL1 or CLOCK on the E-box to regulate their activity and also behaves as an enhancer for *Bmal1* and *Cry1* in positive part of feedback loop.

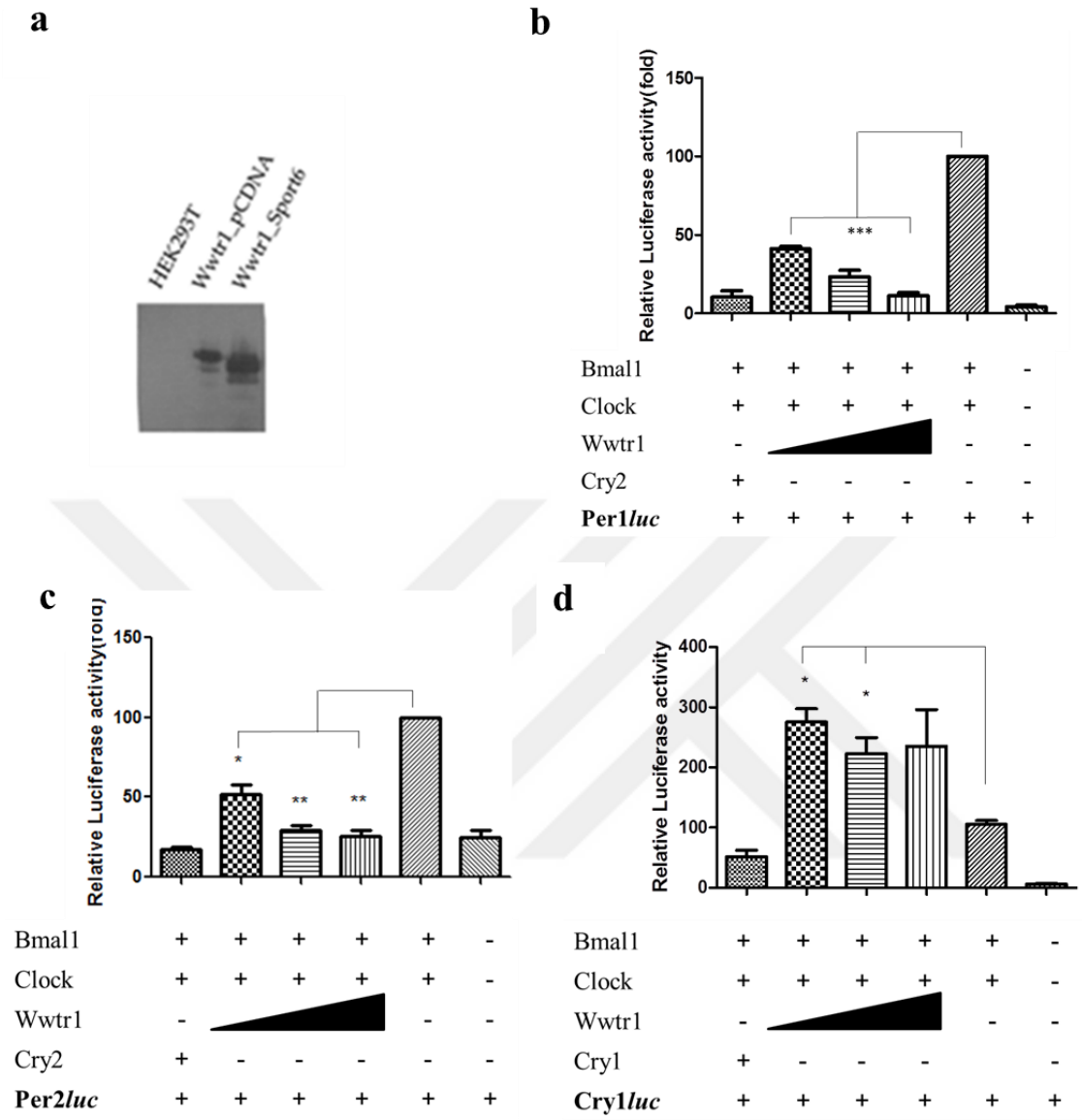


Figure 12: (a) Expression *Wwtr1*-pCMV-Sport1 was confirmed by western blot. Dose-dependent transcriptional repression activity of WWTR1 on BMAL1:CLOCK transactivation was analyzed by luciferase assay. Luciferase activity of CLOCK/BMAL1 on (b) Per1*luc* (c) Per2*luc* (d) Cry1*luc* promoter is compared to co-transfection with different dose of *Wwtr1* (100 ng, 50 ng, 25 ng). Luciferase data normalized with respective co-transfection of *Clock*, *Bmal1* and Per1*luc*/ Per2*luc*/ Cry1l*uc* reporter plasmid which is set to 100 % in each experiment. Error bars represent standard error of mean from 3 experimental repeats. Student's t test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

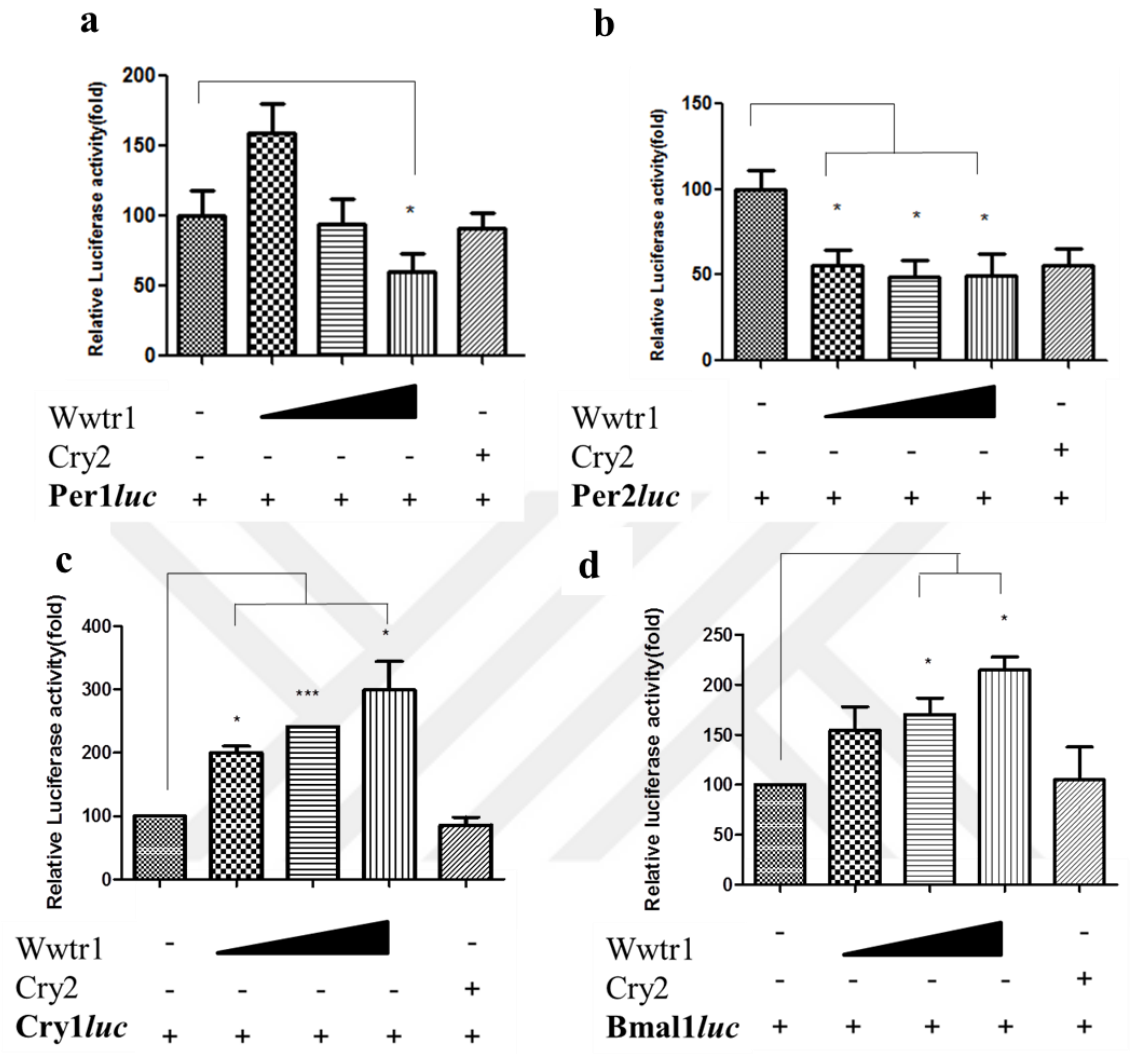


Figure 13: Direct repression activity of WWTR1 on luciferase activity under control of (a) *Per1*, (b) *Per2*, (c) *Cry1*, (d) *Bmal1* promoter was analyzed by luciferase assay. Luciferase data normalized with respective to transfection of reporter plasmids set to 100 % in each experiment. Error bars represent standard error of mean from 3 experimental repeats. Student's t test, *$p < 0.05$, **$p < 0.01$.

5.3.2 Determination of Transcriptional Repression Activity of WWTR1 on BMAL1 and CLOCK Interaction

To confirm the repression activity of the WWTR1 is through the BMAL1:CLOCK complex, mammalian two hybrid system was used. We had pBIND and pACT vectors where *Clock* and *Bmal1* in both are expressed with fusion DNA-binding domain of GAL4 and activation domain of V16, reciprocally. Then all plasmid with appropriate combination were transfected along with pG5*luc* reporter plasmid and *Wwtr1*-pCMV-Sport1 into HEK293T cells. All luciferase activities were normalized to the activity of cells transfected with *Clock /Bmal1* and pGL5*luc* reporter plasmid. In cells, co-transfected with Wwtr1, luciferase activity is significantly decreased to 10-25 % in a dose dependent manner compared to cells transfected with only *Clock* and *Bmal1* (Figure 13). This result supports the repression effect of WWTR1 on CLOCK: BMAL1 transactivation through the physical interaction with protein.

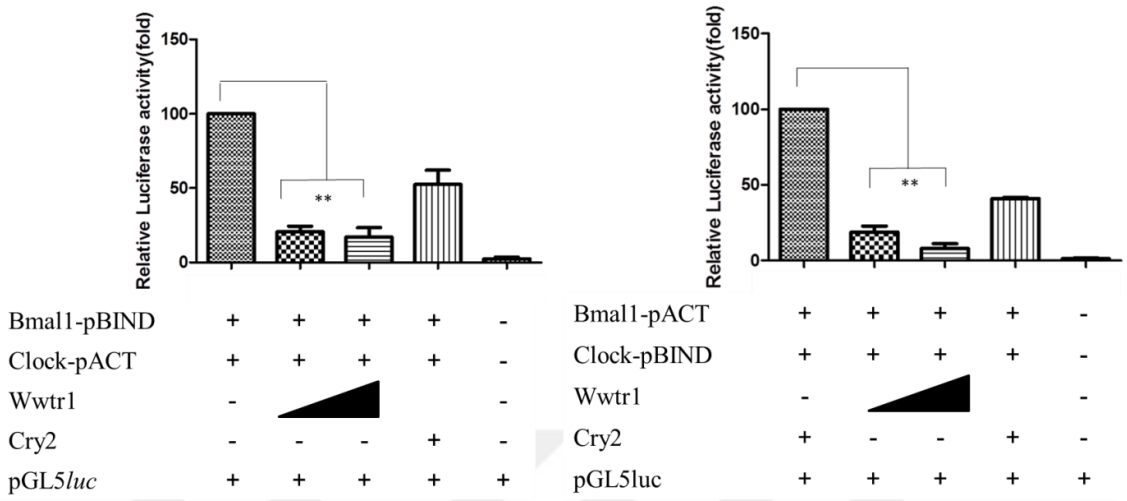


Figure 14: Transcriptional repression activity of WWTR1 on BMAL1 and CLOCK interaction was determined using mammalian two-hybrid system. Luciferase values were normalized to cells transfected with *Clock/Bmal1* and pGL5luc reporter plasmid set to 100 % in each experiment. Error bars represent standard error of mean from 3 experimental repeats. Student's t test, **$p < 0.01$.

5.3.3 Determination of Physical Interaction of BMAL1 and WWTR1 with Co-immunoprecipitation

Repression activity of WWTR1 on BMAL1:CLOCK complex directed us to examine the importance of the physical interaction between WWTR1 and BMAL1 or CLOCK protein. WWTR1 does not have DNA binding site but functions as a co-activator or co-repressor for transcription factors [451] in the Hippo pathway. Therefore, repression activity of WWTR1 might be through interaction with BMAL1:CLOCK complex via acting as a co-repressor for BMAL1 or CLOCK transcription factors. Therefore, to see interacting partner of the WWTR1, I performed co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation assay (BiFC). Co-IP was carried out from HEK293T cells transfected with plasmids encoding *Bmal1*-Flag and *Wwtr1*-His or *Clock*-Flag and *Wwtr1* genes. Co-IP result shows physical interaction of WWTR1 with BMAL1 but not with CLOCK protein. These results suggested that WWTR1 may exhibit its co-repression activity through physical interaction with BMAL1 (Figure 14a-b).

5.3.4 Bimolecular fluorescence complementation assay (BiFC) and co-localization of Bmal1 and Wwtr1 proteins in nucleus.

In order to confirm in vivo interaction between BMAL1 and WWTR1, I used BiFC system. *Bmal1* and *Wwtr1* genes were cloned as fused with the C and N terminal of Venus proteins in BiFC vectors. In the case of interaction of BMAL1 and WWTR1, C- and N-terminal of Venus get close proximity, which results in fluorescence signal. Since, in BiFC system nonspecific signals can be observed with the empty vectors, I also transfected cells with *Bmal1*-pBiFC-VC155 or *Wwtr1*-pBiFC-VC173 with the empty pBiFC- VC173 and pBiFC-VC155 as control, respectively to compare with cells co-transfected with Bmal1 and *Wwtr1*. *Bmal1*-pBiFC-VC155 and pBiFC-VC173 combination shows fluorescent signals in nucleus (Figure 15a) while in *Wwtr1*-pBiFC-VC173 and pBiFC-VC155 combination signals come from both nucleus and cytoplasm (Figure 15b). When Bmal1-pBiFC-VC155 and Wwtr1-pBiFC-VC173 were overexpressed, morphology and localization of signal differed from negative controls. Signals were condensed in nuclei specific to BMAL1 and WWTR1 interaction. Physical interaction between Wwtr1 and BMAL1 is confirmed with BiFC

system, which supports repression activity of WWTR1 on BMAL1:CLOCK complex through interaction with BMAL1 (Figure 15c-d).

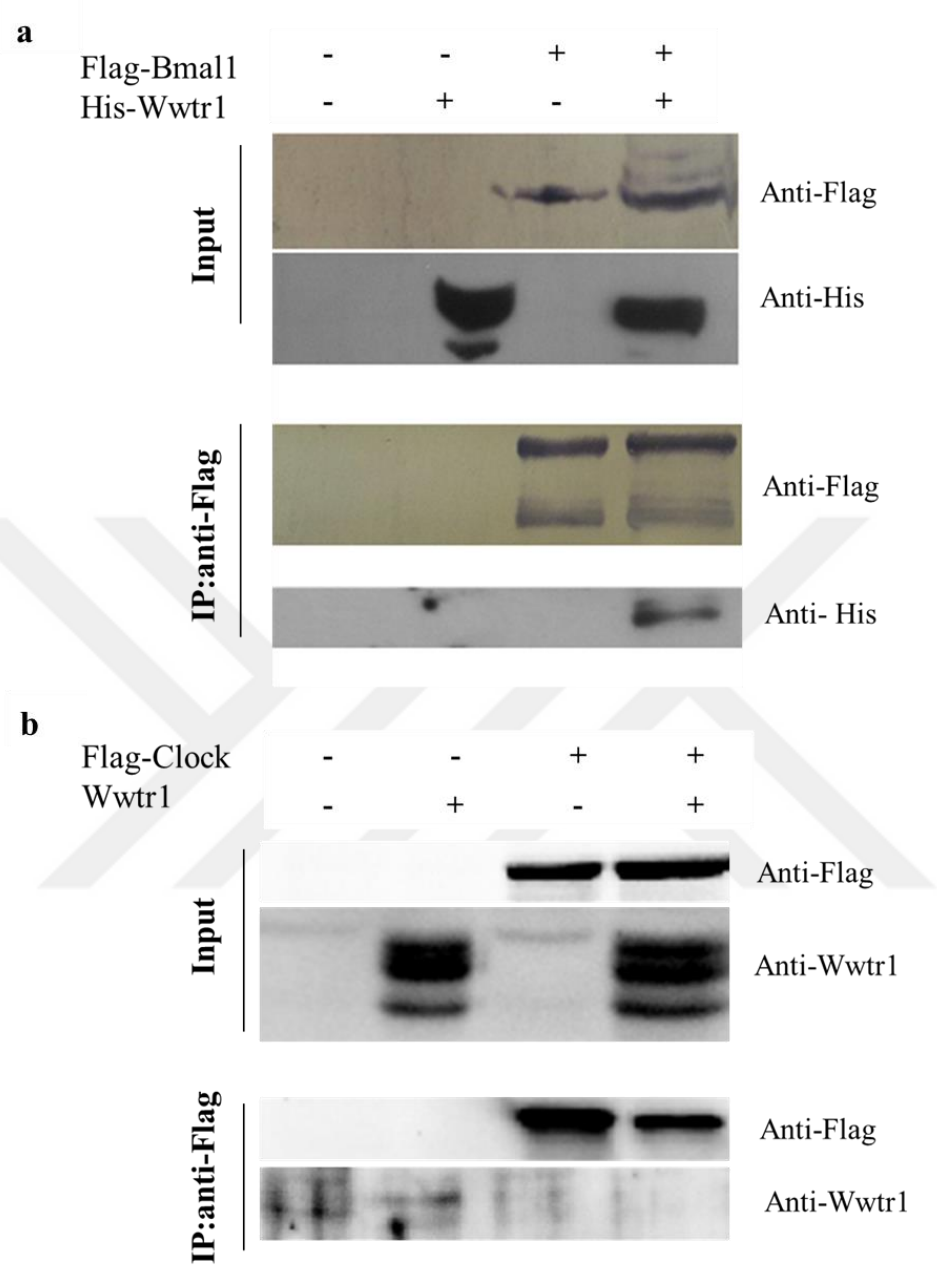


Figure 15: Co-immunoprecipitation of WWTR1 with BMAL1 and CLOCK from HEK293T cells transfected with (a) *Bmal1*-Flag-pCDNA and *Wwrt1*-pCDNAHis/myc-4A and (b) *Clock*-Flag-pCMV-sport6 and *Wwrt1*-pCMV-sport6

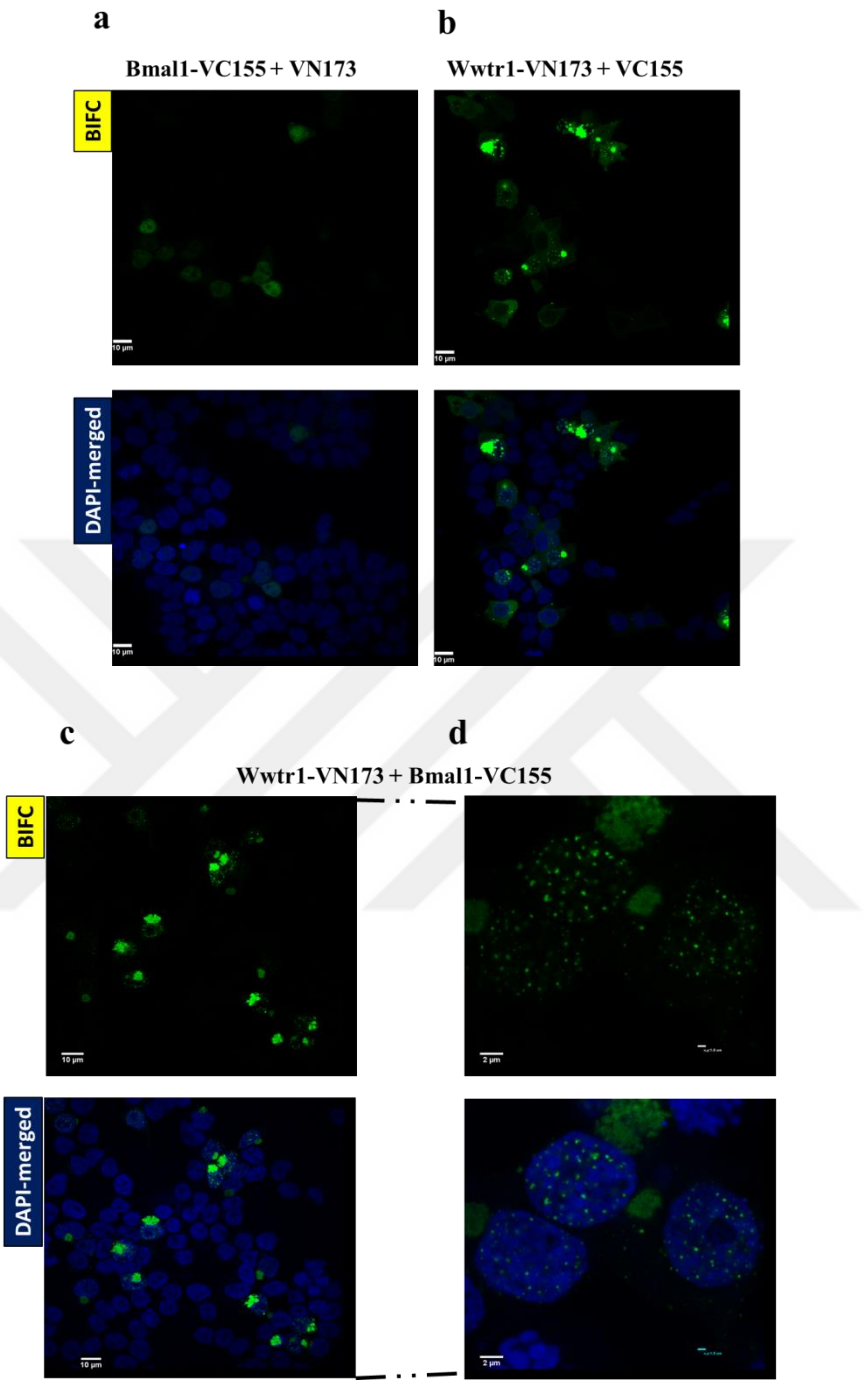


Figure 16: BMAL1 and WWTR1 interaction and co-localization were confirmed with BiFC system in HEK 293T cells. Cells co-transfected with (a) *Bmal1*-pBiFC-VC155 and empty vector (b) *Wwtr1*-pBiFC-VC173 and empty vectors (c) *Bmal1*-pBiFC-VC155 and *Wwtr1*-pBiFC-VN173 and (d) more detailed image of the *Bmal1* and *Wwtr1* co-transfected cells. DAPI was used to stain the nuclei. Images were taken at 20X (a-c) with scale bar: 10µm and 100X (d) magnification with scale bar: 2 µm.

5.3.5 Circadian Oscillation of *Wwtr1* in NIH 3T3 and U2-OS Cell Lines

To get an insight about the circadian transcriptional profiles of *Wwtr1*, we seek for expression data of *Wwtr1* from CircaDB (http://circadb.org) in which researchers have identified more than 3000 genes oscillating under circadian control. *Wwtr1* displays circadian transcriptional profile from time course expression experiments from mice tissue such as hypothalamus and liver [420]. In this study, we used mouse fibroblast NIH 3T3 and U2-OS cell lines and in order to determine expression profile of *Wwtr1* in these cell lines, cultures were synchronized at 48 hour post-seeding and collected at every 4 hour for 24 hours. Real time analysis showed that *Wwtr1* has circadian dependent expression in both cell lines (Figure 17). Our results beside to data obtained from CircaDB demonstrate that *Wwtr1* expression is under tight control of circadian clock in different tissues. Interestingly, in U2-OS cell culture, *Wwtr1* expression peaks around CT04, whereas in NIH 3T3 its expression peaks to around CT16. Circadian-dependent transcription peak at different time course implies that *Wwtr1* expression profile is tissue specific and maybe its circadian function is also tissue specific.

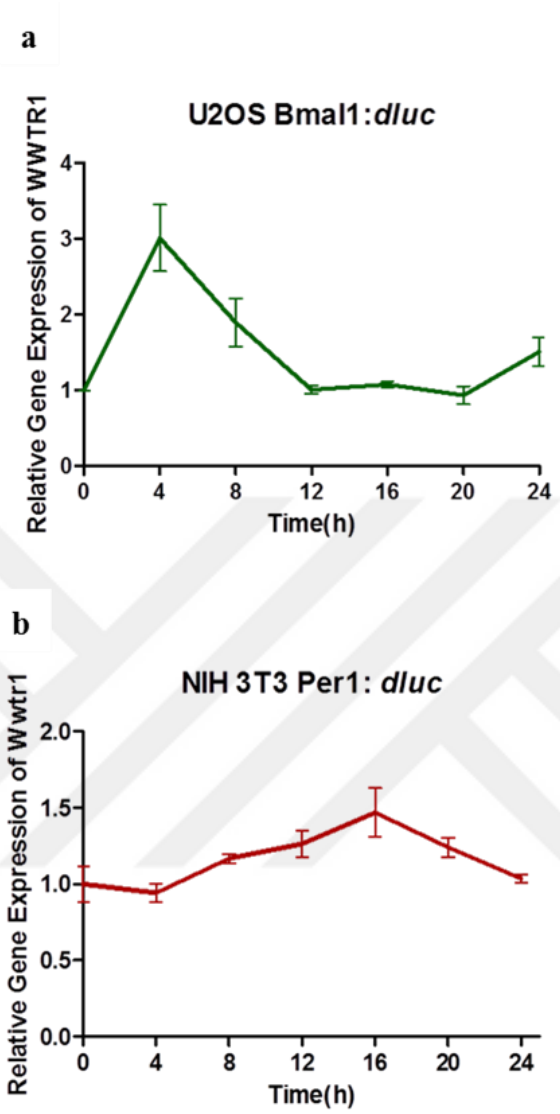


Figure 17: Circadian oscillation of *Wwtr1* in (a) U2-OS Bmal1: *dluc* and (b) NIH 3T3 Per1: *dluc*. Samples were collected at every 4 h and *Wwtr1* expression was drawn relative to CT0. *Gapdh* gene is used for normalization of gene expression.

5.3.6 Real-Time Monitoring of Circadian Oscillations with Lumicycle for Knockdown and Overexpression of *Wwtr1*

Another characteristic needs to be shown to consider WWTR1 as a clock component is that their absences result in change of circadian oscillation. Therefore, *WWTR1* was downregulated by shRNA designed for *Wwtr1* gene. Target oligonucleotide sequences of shRNA for *hWWTR1/mWwtr1* along with negative control shRNA (shNeg) were cloned into pLKO.1 vector. The presences of the products were confirmed by the diagnostic cut with *BamHI a*nd *PstI* restriction enzyme and expected products with 600 bp- fragments were observed by the DNA agarose gel electrophoresis. For mouse *Wwtr1*, two shRNAs constructs were named as sh1*mWwtr1* and sh2*mWwtr2* (Figure 17) and for human *WWTR1* gene, shRNA sequence was obtained from Chan et al., and named as sh4*hWWTR1*[371] were used. Each of shRNA construct was confirmed via sequencing shRNA-pLKO.1 plasmid with U6 primer (Macrogen, the Netherlands). Next, second generation lentiviral system were generated in HEK293T cell line using plasmids mixture containing pCMV-ΔR8.2dvpr and pCMV-VSVG. After packing these constructs into lentil virus, they were used in transduction experiment to achieve *Wwtr1/WWTR1* knockdown in both NIH3T3 and U2-OS cell lines.

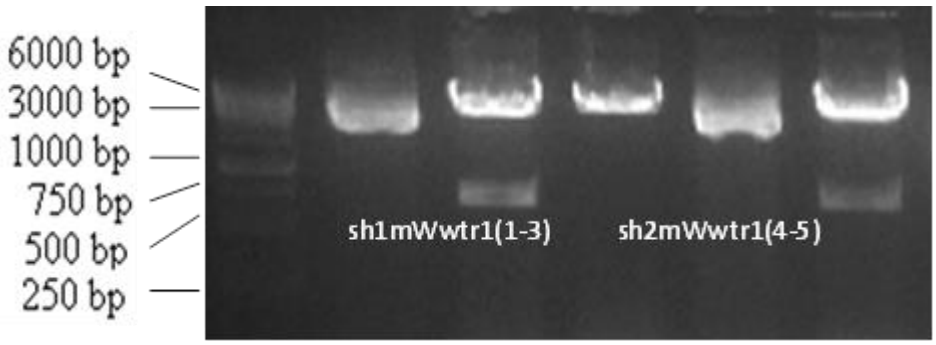


Figure 18: Diagnostic cut with *BamHI* and *PstI* enzymes to control cloning of shRNA oligonucleotides in pLKO.1 to obtain about 600 bp - fragment.

To assess the effect of knocking down *Wwtr1/WWTR1* in mouse NIH 3T3 Per1: *dluc* and human U2-OS Bmal1: *dluc* cell lines on circadian clock, circadian oscillation of both cell lines were monitored via a 32-channel lumicycle (Actimetrics).

In NIH 3T3 Per1: *dluc*, *Wwtr1* knockdown caused significant change in amplitude and phase of oscillation. Cells exhibits 30% damp in amplitude and nearly 1.3 hour-advanced phase of oscillation compared to shNeg treated cells. The circadian period remains same as 22.16 and 22.2 hours for shNeg and sh*Wwtr1* transduced cells, respectively (Figure 19a-b). To assess the degree of the knockdown of *Wwtr1* in NIH 3T3 Per1: *dluc* compared to cells transfected with negative shRNA, mRNA and protein levels were measured with qPCR and western blot, respectively. As shown in Figure 19c, mRNA level drops to 16% and intensity of protein band decreases compared to negative control. Similarly, *Wwtr1* downregulation, in U2-OS Bmal1: *dluc* cells, shows nearly same phenotype in the amplitude, period and phase. ShRNA targeting *WWTR1* decreases amplitude and advances phase significantly compared to negative shRNA. Dampened level of amplitude is around 70% and phase is nearly 2.5 h advanced for cells treated with shNeg (Figure 20a-b). The efficiency of the knocking down in this cell line was assed at mRNA and protein levels by qPCR and western blot with. Cells treated with shRNA has reduced level of *Wwtr1* to 80 % mRNA along with decreased level of the protein (Figure 20c). Raw data obtained from lumicycle analysis are represented in appendix B. All these results suggest that WWTR1 required maintaining amplitude in both cell lines. Since WWTR1 acts as enhancer for BMAL1 and CRY1, depletion of WWTR1 causes impairment in persistence of clock robustness. On the other hand, via repressing BMAL1:CLOCK activity on *Period* genes promoters, it also has regulatory effect on negative arm of feedback loop.

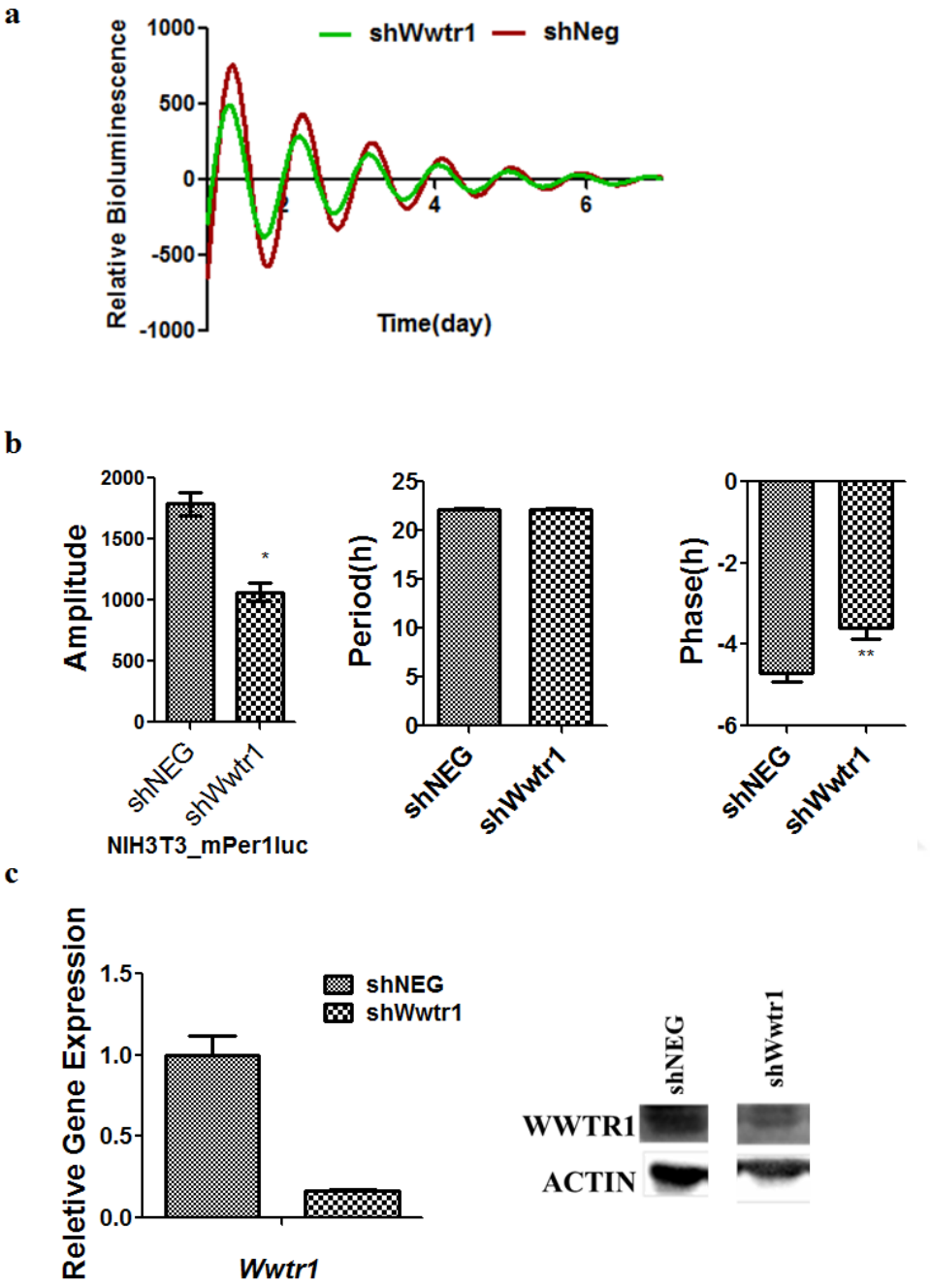


Figure 19: (a) Subtracted bioluminescence records (b) amplitude, period and phase change of NIH3T3 Per1: *dluc* transfected with shRNAs targeting *Wwtr1* (sh1m*Wwtr1*+ sh2m*Wwtr1*) and shNeg. (c) *Wwtr1* downregulation is confirmed with qPCR and western blot analysis. Error bars represent standard error of mean from 3 experimental repeats. Student's t test, *$p < 0.05$, **$p < 0.0$

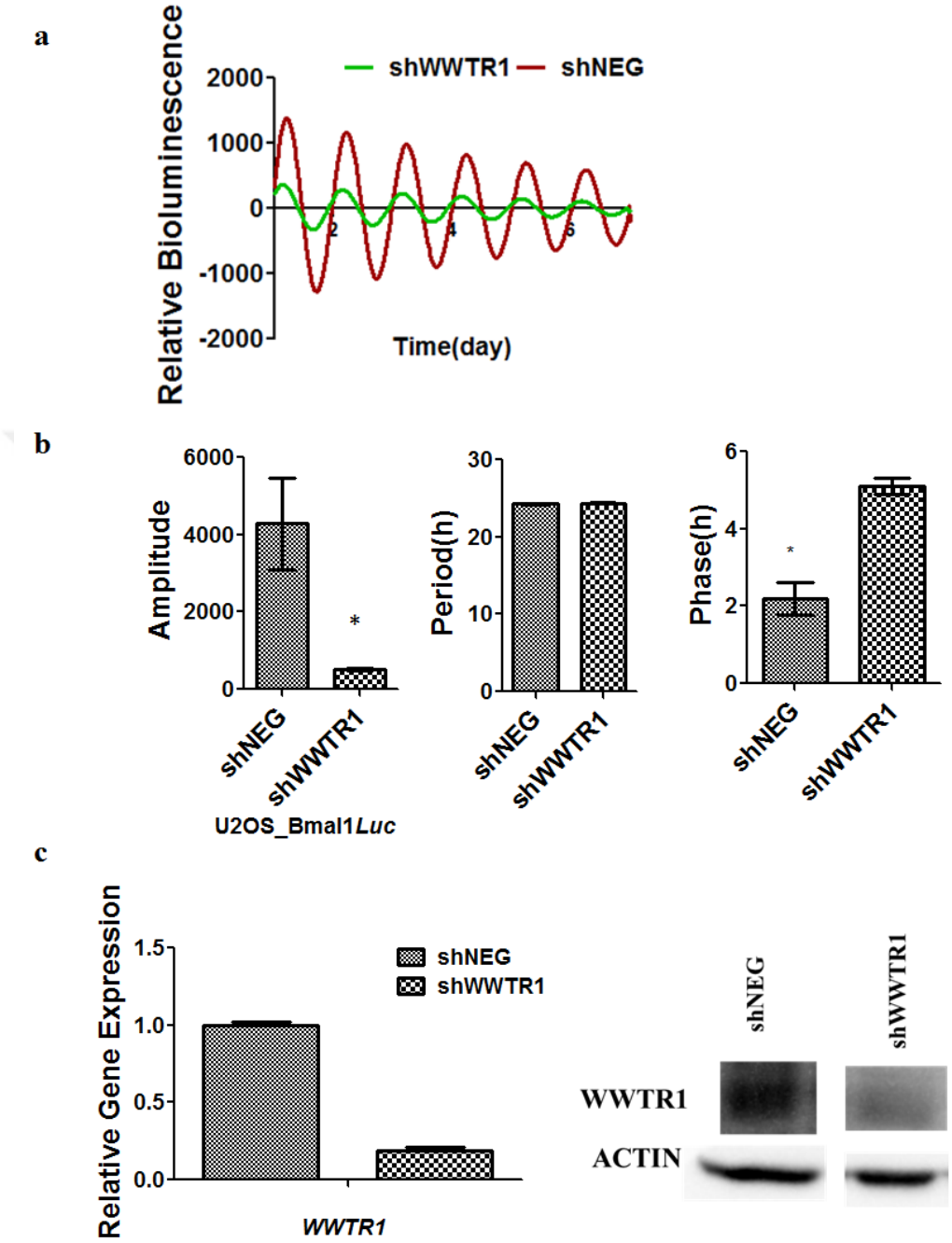


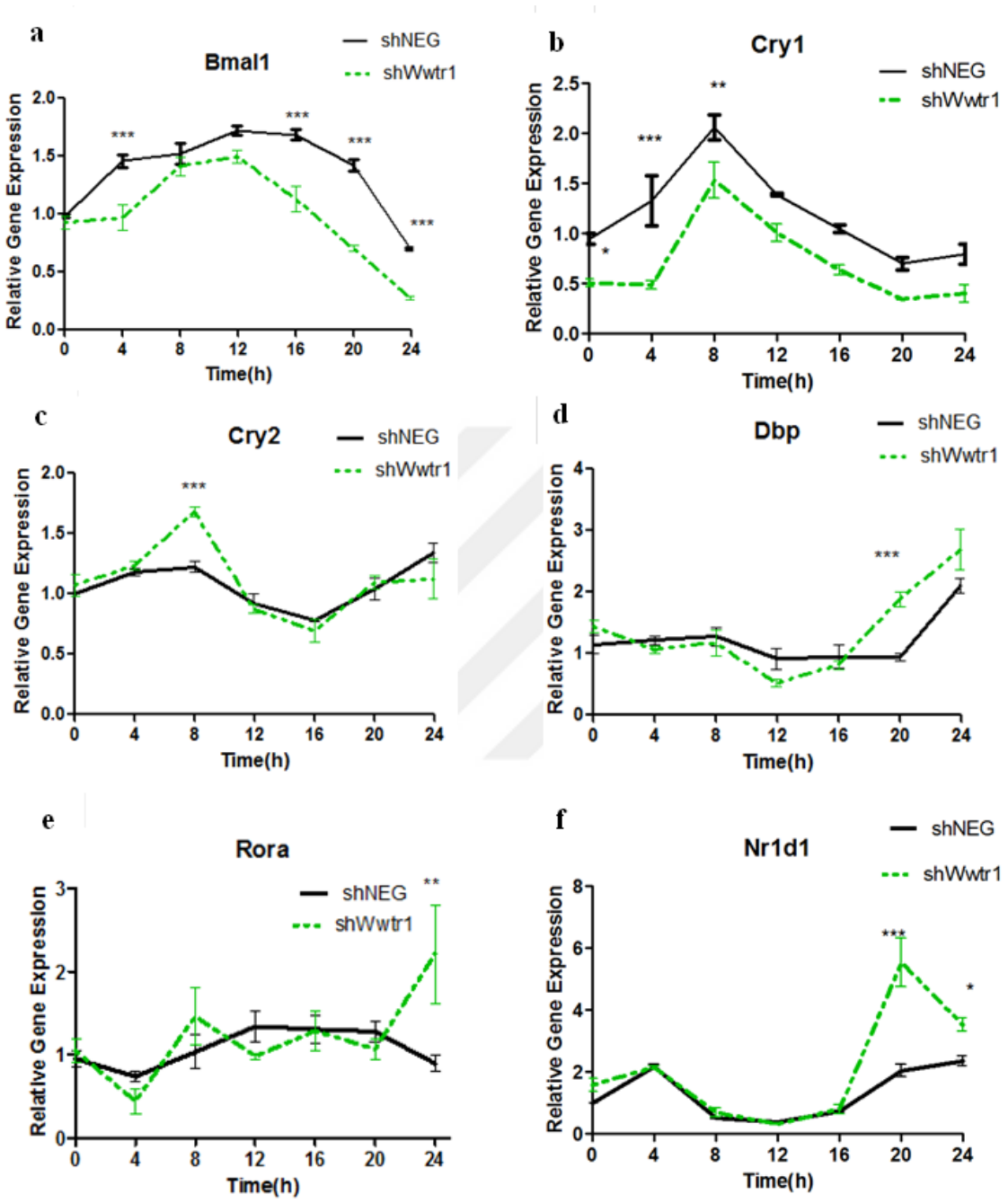
Figure 20: (a) Subtracted bioluminescence records (b) amplitude, period and phase change of U2-OS Bmal1: dluc transfected with shRNAs targeting *WWTR1* (sh4h*WWTR1*) and shNeg. (c) *WWTR1* downregulation is confirmed with qPCR and western blot analysis. Error bars represent standard error of mean from 3 experimental repeats. Student's t test, *p < 0.05, **p < 0.01

5.3.7 Determination of Knockdown Effect of *Wwtr1* Gene on Circadian Oscillation of Clock Gene Expression

The effect *Wwtr1* knockdown on clock genes oscillation in NIH 3T3 Per1: *dluc* was analyzed by qPCR and western blot. Cells were synchronized with dexamethasone and collected at every 4 hour for 24 hours. All qPCR results were normalized to cell sample transduced with shNeg and collected at CT0 (CT, circadian time).. Analysis of the results revealed that for the reduced level of *Wwtr1* expression*;* transcriptional levels of both *Bmal1* and *Cry1* gene expression were dramatically affected, in which the transcriptional level of both genes were reduced in almost all time points (Figure 21a, b). These results are consistent with luciferase reporter assay (Figure 12 c, d) in which *Wwtr1* overexpression induces luciferase expression under control of *Bmal1* and *Cry1* promoter regions. On the other hand, E-box driven genes, *Cry2, Per1* and *Per2,* express anti-phasic to *Bmal1* as expected. *Per1* and *Cry2* possess a significant increase in expression at CT08. Most probably at that time, repression effect of WWTR1 on BMAL1:CLOCK complex releases because of downregulation of *Wwtr1*. Since *Nr1d1, Rora* and *Dbp* gene accepted as direct target of BMAL1, we expected to observe decrease in their expression. Interestingly, between CT0-20, the mRNA level of *Rev-Erbα, Rorα and Dbp* in *Wwtr1* deficiency are conserved with the exception of expression increase around CT20-24 time points. These data implicates that *Wwtr1* deficiency causes dysregulation in canonical clock mechanism not only influencing *Bmal1* and *Cry1* expression but also clock control genes. I also confirmed *Wwtr1* knockdown for all time points for one circadian cycle (Figure 21i).

In addition to mRNA level, I also examined protein level of clock genes; *Bmal1*, *Cry1*, *Dbp,* and *Wwtr1* to find out whether decrease in mRNA level results in also decrease protein level or any post-translational modification counterbalances this effect. We analyzed BMAL1, CRY1, DBP, and WWTR1 protein levels in *Wwtr1* knockdown NIH 3T3 Per1: *dluc* cells for 24 hour with western blot (Figure 22). Since luciferase reporter assay demonstrate that WWTR1 positively regulate *Bmal1* expression, knockdown of *Wwtr1* causes damp in *Bmal1* and *Dbp* expression level at CT16-20 time range. On the other hand, CRY1 protein level peaks at CT12-16 range and protein level was damped at time CT12-20 in *Wwrt1* deficient samples. Decrease in CRY1 and BMAL1 protein level is expected because, via luciferase

assay and real time assay, we demonstrated that WWTR1 acts as activator on *Cry1* and *Bmal1* expression. Of note, we also demonstrated that WWTR1 protein level is showed circadian oscillation with peak around CT16-20 in NIH 3T3 Per1: *dluc* and shRNA transduction decreased protein level for all time points (Figure 22 d).


a
Bmal1
shNEG
shWwtr1
Relative Gene Expression
Time(h)
b
Cry1
c
Cry2
d
Dbp
e
Rora
f
Nr1d1

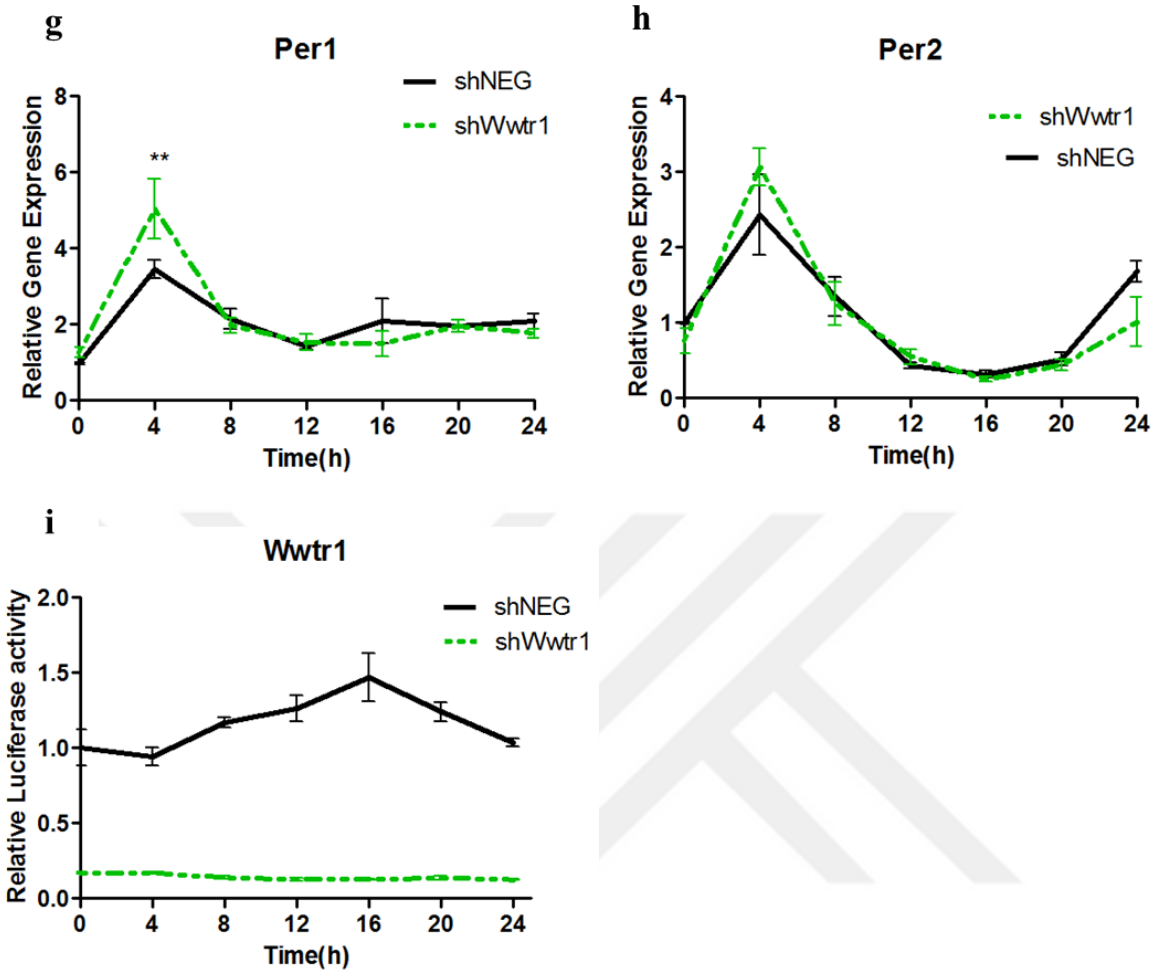


Figure 21: Quantitative RT-PCR analysis for expression of clock and clock controlled genes; (a) *Bmal1*, (b) *Cry1*, (c) *Cry2*, (d) *Dbp*, (e) *Rora*, (f) *Nr1d1*, (g) *Per1*, (h) *Per2* and (i) *Wwtr1*, for *Wwtr1* knockdown in NIH3T3 Per1: *dluc* cell line over 24 h time course at every 4 h. *Gapdh* was used as housekeeping gene to normalize the gene expression level. Error bars represent standard error of mean from 3 experimental repeats. Student's t test, *p < 0.05, **p < 0.01, ***p < 0.001.

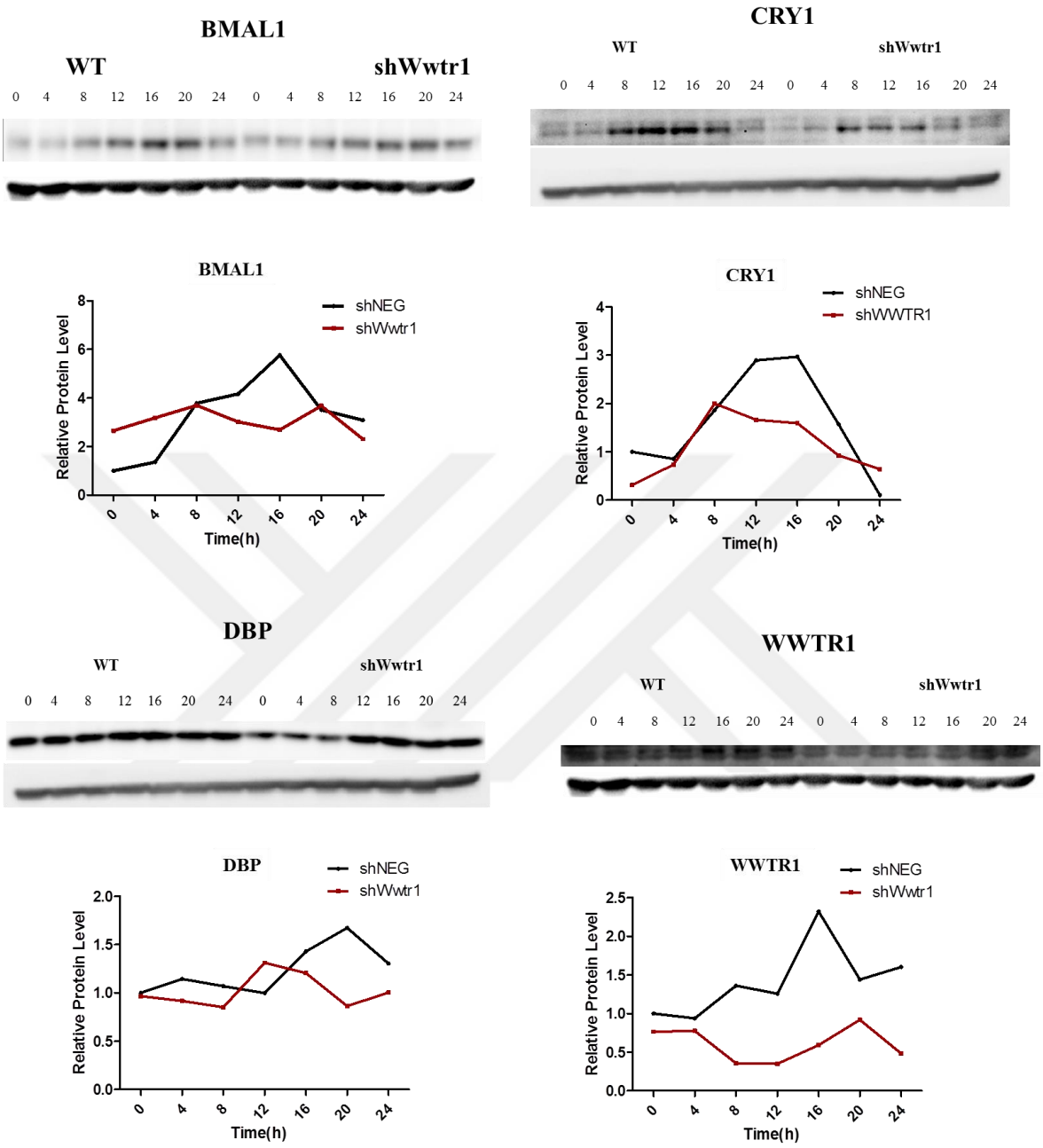


Figure 22: Western blot analysis for protein expression of clock and clock controlled genes (a)BMAL1, (b) CRY1, (c) DBP and (d) WWTR1) for *Wwtr1* knockdown in NIH3T3 Per1: *dluc* cell line over 24 h time course at every 4 h. ACTIN was used as housekeeping gene to normalize the protein level.

5.3.8 Chromatin Immunoprecipitation

In order to determine, WWTR1 occupation on clock gene promoter regions as co-activator or co-repressor, we performed ChIP experiment. We used primers amplifying to E-box containing promoter regions of mouse *Per2*, *Cry1* and *Dbp* genes, and RRE site containing promoter region of *Bmal1* gene. WWTR1 chromatin interaction is expressed as percentage of input and normalized to rabbit IgG as a negative control. In this work, the most promising result is obtained for *Cry1* promoter region in which the chromatin enrichment is around 2 fold. The designed sequence is from TSS of *Cry1* that contains E-box. However that does not mean, WWTR1 and activated TF directly bind to E-box but we can speculate that this region is important for regulation of *Cry1* via WWTR1. Data obtained from other primer amplifications show high variability between two experiments. Repeating experiment with higher level of Wwtr1 protein expression may facilitate WWTR1 and DNA co-precipitation efficiency with target transcription factors (Figure 23).

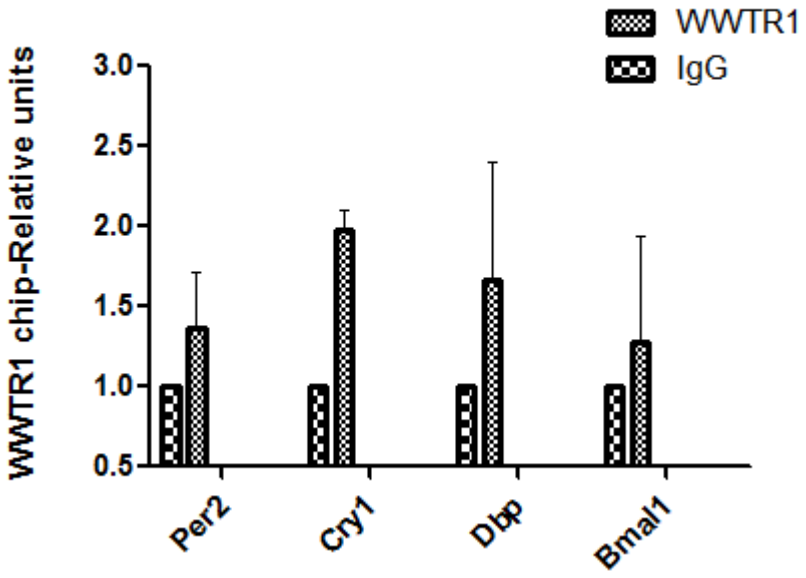


Figure 23: Determination of WWTR1 chromatin binding sites by ChIP assay and analysis by quantitative PCR. Primers obtained from *Per2*, *Cry1* and *Dbp* promoter containing E-box, and *Bmal1* promoter possessing RRE sequence. Data are calculated as percentage of input and normalized to negative control IgG. Error bars represent standard error of mean from 2 experimental repeats.

Chapter 6

DISCUSSION

The circadian clock in mammals is composed of an auto-regulatory transcriptional network with two interlocked transcriptional-translational feedback loop [4, 11-14]. The TTFL comprises of a positive and a negative interconnected limb [15]. Genome-wide epistatic interaction analysis proposed that there are a number of additional factors and regulators are present for the stability of core clock at the molecular level [417-419]. Beside, mechanism behind the core feedback loop remains to be explored. Although similar mechanisms for the core clock loops seem to be conserved across species especially in all eukaryotes, while in *Drosophila,* PER is the main negative regulator, in mammalian clock mechanism, PER paralogs; PER1, PER2, PER3 each have independent roles in SCN circadian clock[254]. For instance, PER3 function is important for peripheral circadian cycle but negligible for the brain[426]. Studies showed that CRY can repress CLOCK: BMAL1 complex without PERs in reporter assays and so CRYs are thought to be the main repressors. Moreover, PER abundance draws away CRY from CLOCK: BMAL1: CRY complex and also possess phase dependent positive role on circadian gene expression as showing buffering effect[170] [427] [428, 429]. Therefore, the PER function and also regulation in feedback loop are still need more clarifications.

In last decades, many candidate genes have been proposed to be core clock genes to elucidate the genetics of the mammalian circadian clock. In order to answer the questions related to the transcriptional/translational feedback loop, canonical model must be reevaluated. Finding regulation mechanism on BMAL1:CLOCK complex as core player in clock is a rational approach. Therefore, firstly we used high throughput luciferase assay to screen transcription factors whether any of them has any regulation on BMAL1:CLOCK transactivation. Herein, based on the initial screening result, our main focus was to seek function of one of the TFs, WWTR1, in clock mechanism. In order to state that WWTR1 is a novel core clock gene, we purposed to demonstrate that WWTR1 assures some characteristics

such as cycling with a 24-hour period, affecting circadian behavioral rhythms in the case of mutation or knockdown, interacting with other core clock component and expressing in most tissues [415].

Firstly, we started with determination of knockdown effect of selected TFs on circadian rhythm of U2-OS Bmal1: *dluc*. *SSBP2, SSBP4, WWTR1, NFE2L2* and *TIRAP* gene expression in U2-OS Bmal1: *dluc* cells were downregulated using specific siRNA pool and circadian rhythm was recorded and analyzed. Except TIRAP knockdown, all other selected TFs affect the amplitude and phase of clock rhythm. Interestingly, as orthologs genes, knockdown in *SSBP2* and *SSBP4* shows reverse phenotypes for amplitude level, which deserves to be analyzed in detail in the future. The circadian effect on NFE2L2 has been already shown[423] [424], but NFE2L2 function in clock mechanism has not been elucidated yet. We observed the most severe amplitude change for Wwtr1 gene knockdown. In fact, change in amplitude implicates change in robustness of clock mechanism that needs further characterization. For this purpose, we repeated luciferase reporter assay for repression activity of these TFs. Although SSBP2, SSBP4, WWTR1 and NFE2L2 proteins showed significant repression in initial screen, after cloning into pCDNA their repression activity decreases substantially. However, the most promising target, WWTR1 was cloned in pCMV-Sport6 without any tag. Thereby based on data obtained from bioluminescence and luciferase reporter assays we focused on characterization of WWTR1 function in circadian clock.

Herein, I started with analyzing the detailed repression act of *Wwtr1* gene on BMAL1:CLOCK activation for mouse *Per1*, *Per2*, and *Cry1* promoter. I used increasing concentrations of *Wwtr1* gene with co-transfection of *Bmal1* and *Clock* gene to prove the concentration dependence of inhibition. Interestingly, WWTR1 has dual influence on *Period* and *Cry1* promoter. We expected to observe repression activity on all these promoters that harbor E-box but dose dependent repression was only seen for *Per1* and *Per2* promoters. The number of E- box sites is higher in *Period* promoters compared to cloned sequence of mouse *Cry1* promoter that has only one. In contrast to *Period* promoters, WWTR1 has a positive effect on *Cry1* promoter region. Even we co-transfect *Wwtr1* with Bmal1/Clock, its activation on Cry1 promoter is dominant to its repression on BMAL1:CLOCK transactivation seen on *Period* promoter sites. Since WWTR1 acts as co-repressor and co-activator, and exerts its

function though binding to transcription factor, it might activate another unknown TF that binds to *Cry1* promoter and induces its expression.

Direct repression activity of WWTR1 on mouse *Per1* and *Per2* promoters was also analyzed to determine the repression level of WWTR1 on these promoters with endogenously expressed BMAL1 and CLOCK proteins. Beside, to elucidate E-box specific repression of WWTR1, I also examined WWTR1 influence on mouse *Bmal1* and *Cry1* promoters that contains defined RRE and E-Box transcription binding sites, respectively. For *Per1* and *Per2* promoter region, result is competable with co-transfection of *Bmal1* and *Clock* genes. WWTR1 shows repression activity but not as effective as with co-overexpression of BMAL1 and CLOCK proteins. Both data support the repression activity of WWTR1 on BMAL1:CLOCK transactivation on promoters of *Period* genes is through BMAL1:CLOCK complex. As a co-activator/repressor, WWTR1 does not have DNA binding site, and may act as a co-repressor for endogenous BMAL1:CLOCK complex or through another transcription factors in a complex form. Therefore I used mammalian two hybrid assay to confirm direct WWTR1 repressions activity on BMAL1:CLOCK complex. Expression level of WWTR1 negatively correlates with activation of BMAL1 and CLOCK on GAL4 binding site. We cannot state that WWTR1 influences the BMAL1 and CLOCK interaction but, it is obvious their transactivation is negatively affected via WWTR1. WWTR1 may repress the BMAL1:CLOCK complex by binding directly to the BMAL1:CLOCK complex or recruit other transcription factors to disrupt BMAL1:CLOCK complex function. Indeed, physical interaction of WWTR1 with clock component, direct or indirect in a complex, would help us to clarify repression action. Hence, co-immunoprecipitation assay for WWTR1 protein with BMAL1 or CLOCK would be useful to conclude this statement. Co-immunoprecipitation assay was applied to BMAL1 or CLOCK with WWTR1. While we observed interaction between BMAL1 and WWTR1, WWTR1 did not interact with CLOCK protein. As a future work, truncated or mutated version of WWTR1 can be used to prove interaction site with BMAL1 and also effect on repression. We also applied BiFC system to support BMAL1-WWTR1 interaction. *Bmal1* and *Wwtr1* genes cloned as fused with the C and N terminal of Venus proteins display fluorescent signal in nucleus, which indicates their interaction and also co-localization site. Compared to *Bmal1* or *Wwtr1* co-transfection with empty vector which gives primarily nonspecific signal in nucleus or nucleus and cytoplasm, respectively, signals

of BMAL1 and WWTR1 co-transfection come from only nucleus, mostly as nuclear bodies with interaction specific morphology. Together, these results demonstrate that WWTR1 associate with BMAL1, thought which it may exert its repression activity on BMAL1:CLOCK transactivation to close the negative feedback loop in repression phase.

On the other hand, WWTR1 behaves as an activator on *Bmal1* and *Cry 1* promoters. For *Cry1* promoter, we obtained consistent result with the co-overexpression of *Wwtr1* with *Bmal1* and *Clock* genes. Despite of having one E-box site in *Cry1* promoter, we again observed increase in luciferase activity. We can speculate that WWTR1 has specific repression through endongenously expressed Bmal1 and Clock on E-box, but *Cry1* promoter has another specific binding sites on which WWTR1 has more dominant co-activation effect compared to inhibitory effect of WWTR1 on BMAL1:CLOCK complex. Interestingly, for *Cry1* promoter region, as I increased the *Wwtr1* concentration to 100 ng, I observed that WWTR1 enhancer effect decreases. This result suggests that WWTR1 dosage is also crucial for clock regulation that brings us to phase dependent function of WWTR1. Most probably, WWTR1 exert its co-activator role on mouse *Cry1* and *Bmal1* promoter with unknown TF. Unknown actors as binding partners of WWTR1 have positive effect on *Bmal1* and *Cry1* promoter need to be further investigated in future work. WWTR1 functions as a coactivator or co-repressor for transcription factors such as TEAD and SMAD family with or without Yap protein[430]. In the study of Lin et al., they showed SMAD2 binds *Bmal1* promoter and enhances its activation [424]. Moreover SMADs nuclear localization is induced via *Wwtr1* activation [397], hence, positive regulation of BMAL1 via WWTR1 can be through SMAD2 transcription factor.

We also revealed that WWTR1 exhibits daily oscillations in two types of culture; U2-OS and NIH 3T3 cells, signifying WWTR1 is subject to clock control. Actually WWTR1 has two non-canonical E-box sites in 2.5 kb promoter region. Therefore, clock regulation on WWTR1 and so on Hippo pathway is another interesting topic that should be uncover for the future work.

In the initial screen, I used siRNA for *Wwtr1* to determine knockdown effect on phenotype of U2-OS Bmal1: *dluc* cell line, I observed decreased in amplitude and phase change. It is important to show knockdown effect in different tissues, therefore I also used

mouse cell line, NIH3T3 Per1: *dluc* cell line. In order to analyze knockdown effect on circadian oscillation of NIH 3T3 and also clock genes expression, I designed shRNAs specific for *Wwtr1*. In this cell line, *Wwtr1* knockdown caused damped amplitude and advance phase of oscillation. In the luciferase reporter assay, WWTR1 showed a dual role, acting as repressor on *Period* but activator on *Cry1* and *Bmal1* promoter regions. Even we expected an increase in amplitude of oscillation for NIH 3T3 Per1: *dluc* cell line expressing luciferase under control of *Per1* promoter, because WWTR1 has positive influence on *Bmal1* and *Cry1* gene expression, depletion in WWTR1 expression attenuates robustness of clock mechanism resulting decrease in amplitude of oscillation. We monitored same phenotype in the amplitude, and phase for the *WWTR1* depletion in U2-OS Bmal1: *dluc* cells in initial screen. siRNA targeting WWTR1 treatment decreased amplitude and advanced phase compared to control siRNA, that is expected because, depletion of WWTR1 leads to downregulation in *Bmal1* and so damp in amplitude level.

To determine knock down effect of WWTR1 protein on circadian oscillation of clock genes expression, I used qPCR and western blot to analyze the change in mRNA and protein level of clock genes and Wwtr1, respectively. The most significant influence of deficit in *Wwtr1* gene is observed on *Bmal1* and *Cry1* mRNA level without eliminating their rhythmicity. As expected, in depletion of *Wwtr1*, *Bmal1* gene expression is reduced, which leads to attenuation in robustness of cycle and in turn affecting the phase and amplitude of the oscillation. While mRNA level of *Nr1d1, Rorα* or *Dbp* increased around CT20; *Cry2*, *Per1* and *Per2* that have E-box containing promoter and anti-phasic oscillation with *Bmal1* exhibit increase in expression at CT08. Increase in *Nr1d1* expression causes decrease in Bmal1 and so amplitude of oscillation. Therefore it would be interesting to further examination on the dependence of *Nr1d1* gene to *Wwtr1* gene. Moreover, between CT0-20, the mRNA level *Rev-Erbα* and *Rorα* in *Wwtr1* deficiency is conserved therefore change in *Bmal1* and *Cry1* is cannot be resulted from *Rev-Erbα* repression or Rorα activation but due to WWTR1 deficiency. *Dbp* gene which is under direct control of BMAL1 showed nearly conserved gene expression for the most time points but higher expression only at one point of cycle. In NIH 3T3 Per1: *dluc* the protein level of WWTR1 peaks at around CT16 and knockdown in *Wwtr1* tightly reduces both BMAL1 and CRY1 protein around this time point. DBP protein level was displayed anti-phasic expression compared to negative control. As a clock control gene, *Dbp*

expression phase might be altered due to decrease in both *Bmal1* and *Cry1* gene expression. Collectively, *Wwtr1* knockdown decreases mRNA level of *Bmal1* and also *Cry1*, while overexpression of WWTR1 facilitates the luciferase activity under control of *Cry1* and *Bmal1* promoter. These data indicate that WWTR1 may act as a co-activator in circadian regulation by activating the promoter of *Bmal1* and *Cry1* promoter. Thus, luciferase reporter assay, qPCR and western blot results let us to state that main transcription activators of the molecular clock, BMAL1, and major repression actor CRY1 are under regulation of WWTR1.

In NIH 3T3, results for mRNA and protein level of BMAL1 and CRY1 are consistent with luciferase assay data. For this cell line, we can say that WWTR1 have dual role, maybe phase dependent role, on clock component. In activation phase, WWTR1 positively regulates *Bmal1* and *Cry1* whereas in repression phase, it negatively modulates the expression of *Per1, Cry2, Per2* and *Nr1d1* that have E-box in their promoter through inhibiting BMAL1:CLOCK transactivation. These results drive us to perform chip experiment to confirm WWTR1 recruitment onto clock gene promoters such as the Cry1 promoter. I used primers designed to promoter region of mouse *Per2, Cry1* and *Dbp* containing E-box, and also *Bmal1* possessing RRE sequence. Results obtained from qPCR after performing ChIP are expresses as percentage of input. However these enrichments are not as high as any transcription factor that binds DNA directly, therefore co-immunoprecipitation step with transcription factor partners that directly binds to DNA is crucial. Primers for RRE sequence of *Bmal1* promoter does not enrich the chromatin implicating Wwtr1 reguation is not through Rev-Erbα or Rorα nuclear receptors. Also Per2 E-box specific primer did not show a significant amplification. *Bmal1, Per2* and *Dbp* promoter specific enrichments show high deviation between two experiments. This variability could be due to a number of factors such as weak interaction between WWTR1 and TFs such as BMAL1, or insufficient overexpression of WWTR1 with PEI reagent. We could try other sets of primers for remaining E-boxes or improve protein-protein crosslink between WWTR1 and BMAL1 or better overexpression system to perform more efficient ChIP experiment. In addition, at 16-20 h post- synchronization, WWTR1 protein level peaks, and applying ChIP at that time will give better results

On the other hand, chromatin enrichment data obtained from *Cry1* promoter is around 2-fold. This data obtained with *Cry1* E-box primer set that amplifies 150 bp (-60 b) containing

one E-box sites. When I analyzed sequence of mouse *Cry1* promoter, there is one TEAD binding site AGGTATGC (+314 bp to TSS) and CATTCA (+391 bp to TSS). In fact, canonical sequence is (A/T)GGAAT(G/T)(T/C) or CATTCC(A/T) but a single nucleotide change within last three bases in canonical sequence is recognized by TEADs {Kim, 2015 #260} [431]. One canonical TEAD binding site is also found onto *Bmal1* promoter (-718 bp to TSS). WWTR1 is known as TEAD co-activator, which can explain its activation on *Cry1* and *Bmal1* promoter. In a comprehensive study of Zanconato et al., they conducted genome-wide screen for YAP/WWTR1/TEAD binding site in MDA-MB-231 cells. In their data, they obtained *CRY1* promoter and *ARNTL, ARNTL2, NR1D1* and *DBP* enhancer sites as YAP/WWTR1/TEAD common binding sites. Moreover, they indicated that CRY1 and ARNTL2 are associated to at least one YAP/WWTR1/TEAD binding site and down-regulated in YAP/WWTR1-depletion[432]. Interestingly, recent work showed that YAP and WWTR1 also function as co-repressor of TEAD to inhibit apoptotic gene expression and promote cell growth and survival. This repression is mediated by mi-2/nucleosome remodeling and deacetylase (NuRD) complex in which histone deacetylase (HDAC) and chromatin remodeling subunit (CHD4), metastasis associated subunits (MTA1) determine the effect of NuRD complex on YAP repression activity. In this work, they speculated that there must be another transactivator which converts YAP/WWTR1-TEAD into a repressor [403]. NuRD complex also involves in circadian clock feedback limb as corepressor[244]. Interestingly CHD4 subunit of NuRD complex promotes BMAL1-CLOCK transcriptional activity, while remaining complementary NuRD subunits (MTA2, MBD2, GATAD2a, HDAC1, and RbAp48) are recruited by the PER complex to reconstitute the repression on DNA-bound BMAL1-CLOCK. Totally, based on these studies, WWTR1 may act as a repressor for *Period* genes through BMAL1:CLOCK complex maybe with a complex but as an activator for *Bmal1* and *Cry1* via interacting with different partners. Therefore, transcription factors as binding partner of WWTR1 on these promoters should be also elucidated to clarify WWTR1 mechanism of action in clock system. In addition, WWTR1 might act phase dependent manner such as PER2 and HDAC2 that behaves activator and repressor at different phase of cycle. Therefore determination of WWTR1 binding times on clock related promoters is very important for future work.

The findings from these studies suggest a novel role for WWTR1 in regulating the expression of clock genes and demonstrate WWTR1 is a critical component for the core molecular clock machinery. Dual role of WWTR1 on clock gene expression may be result of co-activator and co-repressor activity of WWTR1 in activation and repression phase. Two opposite roles: CRY1 and BMAL1 activation and repression on BMAL1:CLOCK transactivation; ensure robustness of circadian mechanism and so maintenance of high-amplitude circadian gene expression (Figure 24).

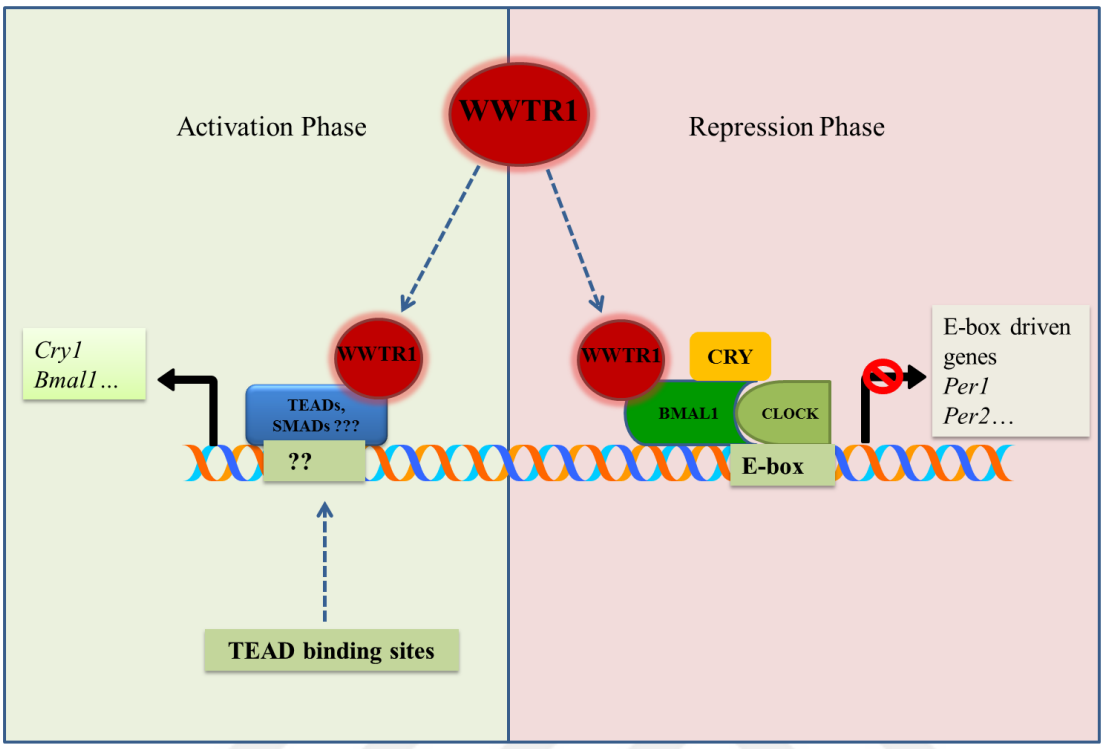


Figure 24: The proposed model for WWTR1 distinct role in circadian clock mechanism. WWTR1 binds to BMAL1 and inhibits BMAL1:CLOCK transactivation in repression phase and also stimulates transcription of *Cry1* and *Bmal1* expression via recruiting unknown transcription factor in activation phase.

Chapter 7

CONCLUSION AND FUTURE DIRECTION

In present study, our major goal was to elucidate the complexity in the mammalian circadian clock mechanism and connections with other by identifying a novel clock component. Herein, we screened transcription factors and focused on WWTR1 as target that regulates BMAL1:CLOCK transactivation and also clock gene expression.

WWTR1 is mostly related to cell proliferation as an oncogene, but I found a novel function as a regulator in clock mechanism. Based on the data in this study, the following conclusions can be drawn: I demonstrated that *Wwtr1* gene expression is regulated by circadian clock in NIH 3T3 and U2-OS cell lines. Then, I determined its dual role in clock mechanism. While it acts as repressor for BMAL1:CLOCK transactivation onto *Per1* and *Per2* promoter region, it functions as activator for *Cry1* and *Bmal1* promoters. Next, I define its interaction with BMAL1 via co-immunoprecipitation and BiFC system. The phenotypic change of genetic deficiency of *Wwtr1* is demonstrated in NIH 3T3 Per1*: dluc*. It is a co-activator/co-repressor of many transcription factors that have different tissue specific functions in mammals [390]. Finally, I test whether WWTR1 localized on clock gene promoters via any TFs. ChIP assay and also luciferase reporter assay support that WWTR1 enhances *Cry1* expression. In summary, our study demonstrates that WWTR1 expression is critical for regulation of the BMAL1:CLOCK transactivation and also core circadian transcription factors, CRY1 and BMAL1, which totally influences the robustness of oscillation. Up to know all oncogenic proteins have been found as negative regulator for clock, specifically through *Bmal1* and *Period* genes. In contrast to previous works, this research suggests WWTR1 exhibits different features. Thus, it has roles in cell proliferation and cancer development, the level and function of WWTR1 would be important for crosstalk between clock and cell cycle and also cancer process. In the near future, due to its bi-directional modulation, it will be necessity to further understand WWTR1 role in chronobiology to anticipate and develop therapies for human clock disorders related to damped amplitude and impairment of clock with cancer development. Thereby, it can be used to determine time of application of chemotherapeutic agents.

In order to uncover WWTR1 function in clock network, some future works should be done. As future works, primers that detect TEADs or SMADs binding sites should be used in ChIP experiment. Also it would be important to test mutation version of promoter cloned for luciferase reporter assay to show site specific WWTR1 activation on *Cry1* and *Bmal1* promoter regions. Moreover, to examine phase dependent association of WWTR1 with the promoter of clock genes, ChIP assays can be performed using samples obtained from livers of wild-type mice or MDM3 hepatocytes across a circadian cycle. Furthermore, the main question remaining to be answered is binding partner of WWTR1 for *Cry1* and *Bmal1* activation. For this purpose, screening known TFs partners of WWTR1 via endogenous Co-IP could be useful. In addition, this finding needs to be tested with an experiment that reveals WWTR1 action on clock as an output. Oncogenic proteins also affects metabolism in cells such as glucose metabolism or glutaminolysis [305]. In order to show WWTR1 influence on clock system, it will be helpful to analyze cellular metabolism in case of overexpression and deficiency of *Wwtr1* gene, for instance in MDM3 hepatocytes or in U2-OS osteosarcoma cells lines. Also beside of NIH 3T3 cell line, it would be useful to monitor circadian oscillation in MDM3 hepatocytes cell line following knockdown and overexpression of WWTR1.

Even we have not have whole picture for Wwtr1 mechanism of action, completion of this project will be crucial to uncover the interlocked relationship between clock and cell cycle pathways and contribute understanding of molecular mechanism behind clock and cancer development.

VITA

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APPENDIX A

Table 2: List of siRNAs from Qiagen

Target Gene	**Qiagen siRNAs**
NFE2L2	SI03246950 (FlexiTube siRNA) SI03246614 (FlexiTube siRNA) SI04320904 (FlexiTube siRNA) SI04223009 (FlexiTube siRNA)-GS4780
SSBP2	SI04323452 (FlexiTube siRNA) SI04240985 (FlexiTube siRNA) SI04154864 (FlexiTube siRNA) SI04141242 (FlexiTube siRNA -GS23635
SSBP4	SI04366432 (FlexiTube siRNA) SI04269489 (FlexiTube siRNA) SI00733712 (FlexiTube siRNA) SI00733691 (FlexiTube siRNA)-GS170463
WWTR1	SI00111237 (FlexiTube siRNA) SI00111230 (FlexiTube siRNA) SI00111223 (FlexiTube siRNA) SI00111216 (FlexiTube siRNA)- GS25937
TIRAP	SI03108308 (FlexiTube siRNA) SI03075135 (FlexiTube siRNA) SI03037062 (FlexiTube siRNA) SI00147042 (FlexiTube siRNA)- GS114609
AllStars Negative Control	SI03650318

Table 3: List of the primer sequences for cloning

Primers	**Sequence**
mNfe2l2-p*CDNA-4-His/Myc-A*	Nfe2l2-pCDNA-KpnI-F:TGGTACCGAATGATGGACTTGGAGTTG
	Nfe2l2-pCDNA-NotI-F:GAGCGGCCGCCAGTTTTTCTTTGTATCTGG
mSsbp2-p*CDNA-4-His/Myc-A*	Ssbp2-pCDNA-EcorI-F:AGATATCCAATGTACGGCAAAGGCAAG
	Ssbp2-pCDNA-NotI-R:GAGCGGCCGCCACACGCTCATGGTCATGCT
mSsbp4-p*CDNA-4-His/Myc-A*	Ssbp4-pCDNA-EcorI-F:AGATATCCAATGTACGGCAAGGCGAGC
	Ssbp4-pCDNA-NotI-R:GAGCGGCCGCCACACGCTCATGGTCATGCC
mWwtr1-p*CDNA-4-His/Myc-A*	Wwtr1-pCDNA-EcorI-F:AGATATCCAATGAATCCGTCCTCGGTG
	Wwtr1-pCDNA-NotI-R:GAGCGGCCGCCACAGCCAGGTTAGAAAGGG
Bmal1- p*CDNA-4-His/Myc-A*	Bmal1-pCDNA-EcorV-F: TGCAGATATCGCCACCATGGCGGACCAGAGAATGGAC
	Bmal1-pCDNA-Flag-NotI-R: CTCGAGCGGCCGCCTACTTATCGTCGTCATCCTTGTAATCCAGCGGCCATGGCAAGTC
Wwtr1-pCMV-sport6	Wwtr1-sport6-EcorI-F: CCGGAATTCCGATGAATCCGTCCTCGGTG
	Wwtr1-sport6-HindIII-R:CGTAAGCTTTTACAGCCAGGTTAGAAAG
mBmal1-EcoRI- pBiFC-VN155	Bmal1-EcoRI- pBiFC-VN155-F: CCCGAATTCGGATGGCGGACCAGAGAAT
	Bmal1-XhoI- pBiFC-VN155-R: TACCTCGAGACAGCGGCCATGGCAAGT

Wwtr1-pBiFC-VN173	Wwtr1-HindIII-pBiFC-VN173-F: GACAAGCTTATGAATCCGTCCTCGGTGC
	Wwtr1-EcorI-pBiFC-VN173-R: GATGAATTCGCCAGCCAGGTTAGAAAG
Wwtr1-eGFP	W- pEGFP-C1-HindIII-FW: CTCAAGCTTCGATGAATCCGTCCTCGGT
	W- pEGFP-C1-EcorI-Rev: GCAGAATTCTTACAGCCAGGTTAGAAAG

Table 4: List of shRNA sequences

Oligo Name	**Oligo Sequence**	**REF**
hWwrt1_sh4_CDS_Chan2008_F	CCG GAG AGG TAC TTC CTC AAT CAC ACT GCA GTG TGA TTG AGG AAG TAC CTC TTT TTT G	[371]
hWwrt1_sh4_CDS_Chan2008_R	AAT TCA AAA AAG AGG TAC TTC CTC AAT CACACT GCA GTG TGA TTG AGG AAG TAC CTC T	[371]
mWwrt1_sh1_CDS_TRCN0000095951_F	CCG GCA GCC GAA TCT CGC AAT GAA TCT GCA GAT TCA TTG CGA GAT TCG GCT GTT TTT G	This study
mWwrt1_sh1_CDS_TRCN0000095951_R	AAT TCA AAA ACA GCC GAA TCT CGC AAT GAATCT GCA GAT TCA TTG CGA GAT TCG GCT G	This study
mWwrt1_sh2_3UTR_TRCN0000095949_F	CCG GCC TGC ATT TCT GTG GCA GAT ACT GCA GTA TCT GCC ACA GAA ATG CAG GTT TTT G	This study
mWwrt1_sh2_3UTR_TRCN0000095949_R	AAT TCA AAA ACC TGC ATT TCT GTG GCA GAT ACT GCA GTA TCT GCC ACA GAA ATG CAG G	This study
shRNA_Negative_SHC002_F	CCG GCA ACA AGA TGA AGA GCA CCA ACT GCA GTT GGT GCT CTT CAT CTT GTT GTT TTT G	Sigma SHC002 MISSION; Non-Mammalian shRNA Control
shRNA_Negative_SHC002_R	AAT TCA AAA ACA ACA AGA TGA AGA GCA CCAACT GCA GTT GGT GCT CTT CAT CTT GTT G	Sigma SHC002 MISSION; Non-Mammalian shRNA Control

Table 5: List of RT-PCR primer sequences

Gene	**Forward**	**Reverse**
hGAPDH	TGCACCACCAACTGCTTAGC	ACAGTCTTCTGGGTGGCAGTG
hWWTR1	GCTCAGATCCTTTCCTCAATGG	CGTTTTCTCCTGTATCCATCTCAT
hBMAL1	GCCCATTGAACATCACGAGTAC	CCTGAGCCTGGCCTGATAGTAG
hCLOCK	GGCACCACCCATAATAGGGTA	TGTTGCCCCTTAGTCAGGAAC
hCRY1	ACAGGTGGCGATTTTTGCTTC	TCCAAAGGGCTCAGAATCATACT
hCRY2	CTACCGGGGACTCTGTCTACT	ACTGGGTAGTGGTCTTGGGC
hPER1	CCTCTTCCAGGATGTGGATGA A	CAACTGCAGAATCTTCTTGTGGA
hDBP	GAGGAACTTAAGCCCCAGCC	CCTCGTTGTTCTTGTACC
hRORα	ACTACACACCAGCATCAGGC	GCAGGTTTCCAGATGCGATT
hREV-ERBα	AGACATGACGACCCTGGACT	ATGGTGGGAAGTAGGTGGGA
mGapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
mWwtr1	CCGTCCATCACTTCCACCTC	TGTGGGTTGGTTCTGAGTCG
mBmal1	GACTACCAAGAAAGTATGGACACA	TTTGTCCCGACGCCTCTTTT
mClock	AGAGCGCGAAGGAAATCTGG	ACTGACCATCAGAACTCTTGCT
mCry1	CAACGTGGGCATCAACAGG	GATGTTCCATTCCTTGAAAAGCC
mCry2	TCGTCTGTGGGCATCAACC	CCCATTCCTTGAACAGCCTT
mPer1	GAAACGGCAAGCGGATGGAG	GGAGGACGAAACAGGGAAGG
mPer2	GAGCGCCACCAAGTGACGG	GGTGGGACTTGGGGAGAAGT
mDbp	GGGCCACTCTGGGAGCC	GCGGGATCAGGTTCAAAGGT

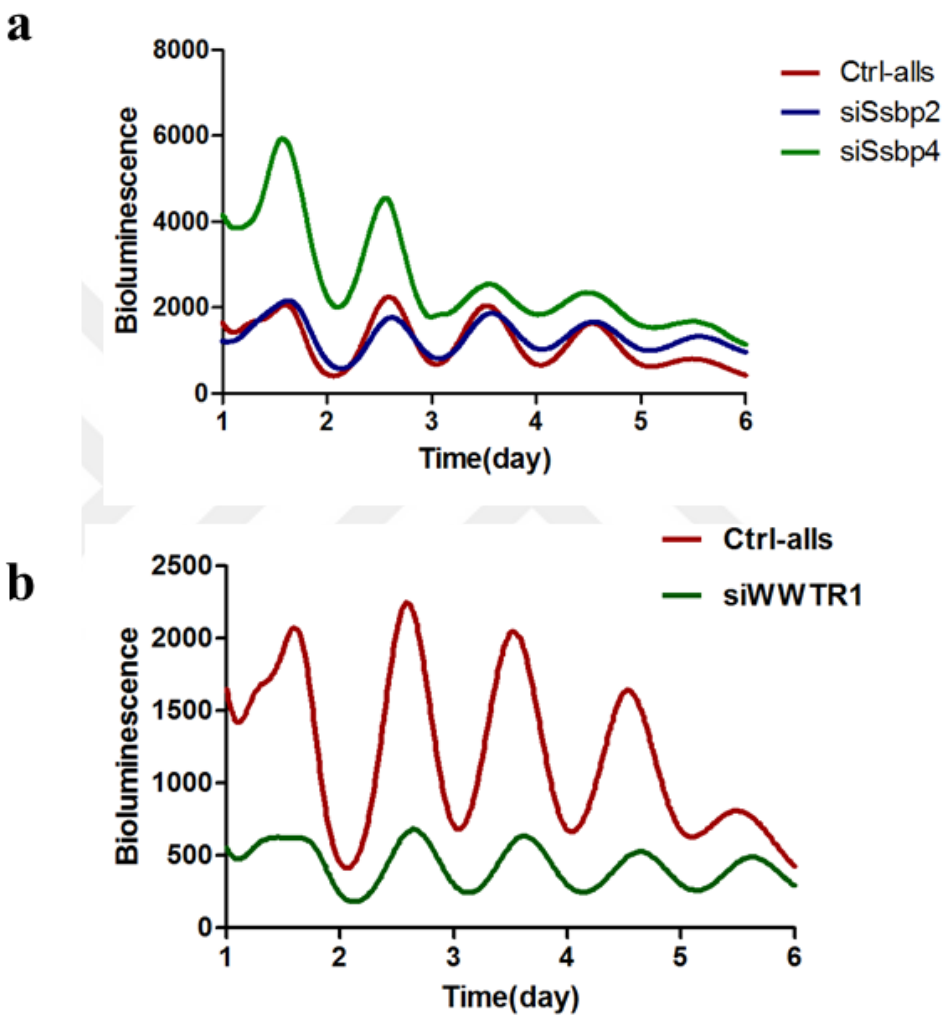
mRorα	ATTAGGATGTGCCGTGCCTT	GCGATTTCGTCTTCGGTCAG
mRev-Erbα	GGCCATCACAACCTCCAGTT	GGAGCCACTAGAGCCAATGT

Table 6: List of primers for ChIP assay

Name	**Sequence**	**Reference**
mPer2E(E-box)1-F	GTGGGTGCAGAGAGCCTCTGCTG	**[433]**
mPer2E1-R	CTCCCTGCTTCTAACTTGCTCTAGAC	
mCry1-Ebox-F	GCACGCGGGGGTCTGAGCCA	**[434]**
mCry1-Ebox-R	CCGGTCCCGAGGCTGCCCG	
mDbp3-F	GCCTGGAATGTATGAGCTAGCA	**[180]**
mDbp3-R	GGCACCGGAGTAGGCAAGA	
mBmal1-RRE-F	GGATTGGTCGGAAAGTAGGTT	**[434]**
mBmal1-RRE-R	CGGGTAAACAGGCACCTC	
mCry1-RRE-F(1st intron)	TCATTGTGATGGGAGTATGC	**[434]**
mCry1-RRE-R	TCCAAAAGATGATTTCAACA	

APPENDIX B

Raw Data of Bioluminescence Records



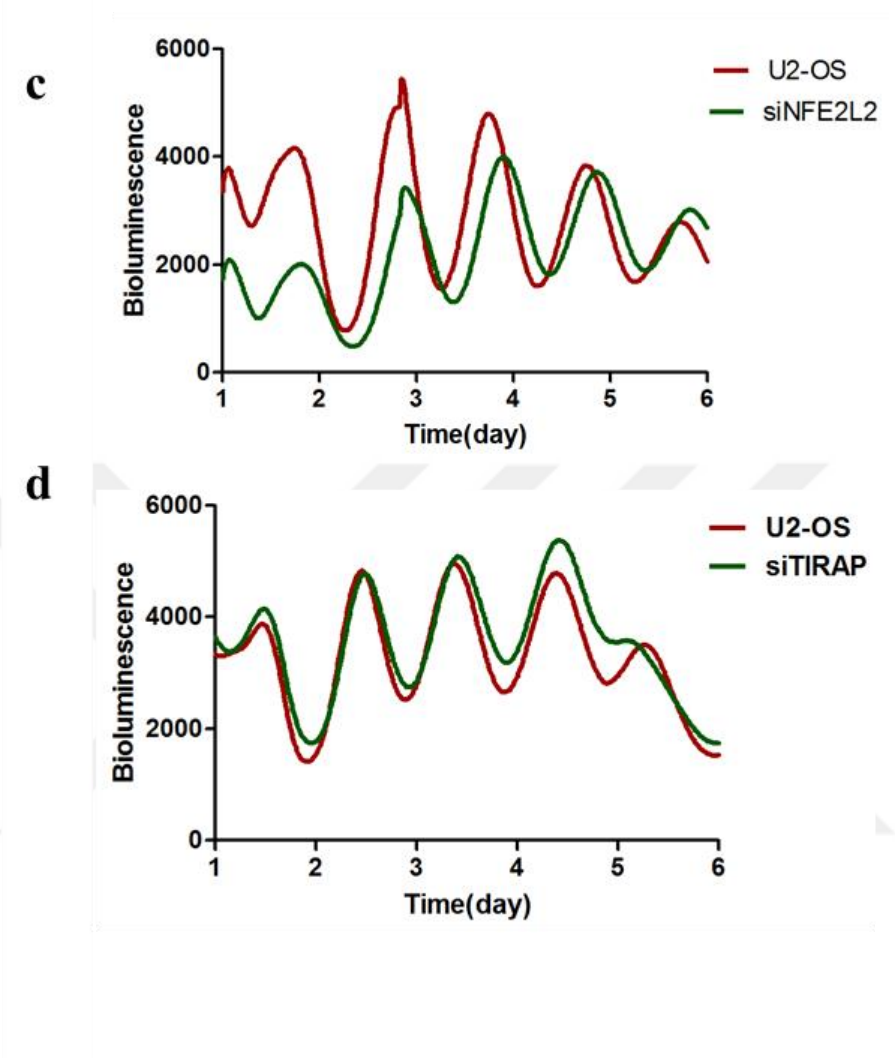


Figure S 1. Raw Data of Bioluminescence Records of U2-OS Bmal1: *dluc* cell line transfected with siRNA of (a) SSBP2, SSBP4 (b) WWTR1(c) NFE2L2 (d) TIRAP and controls

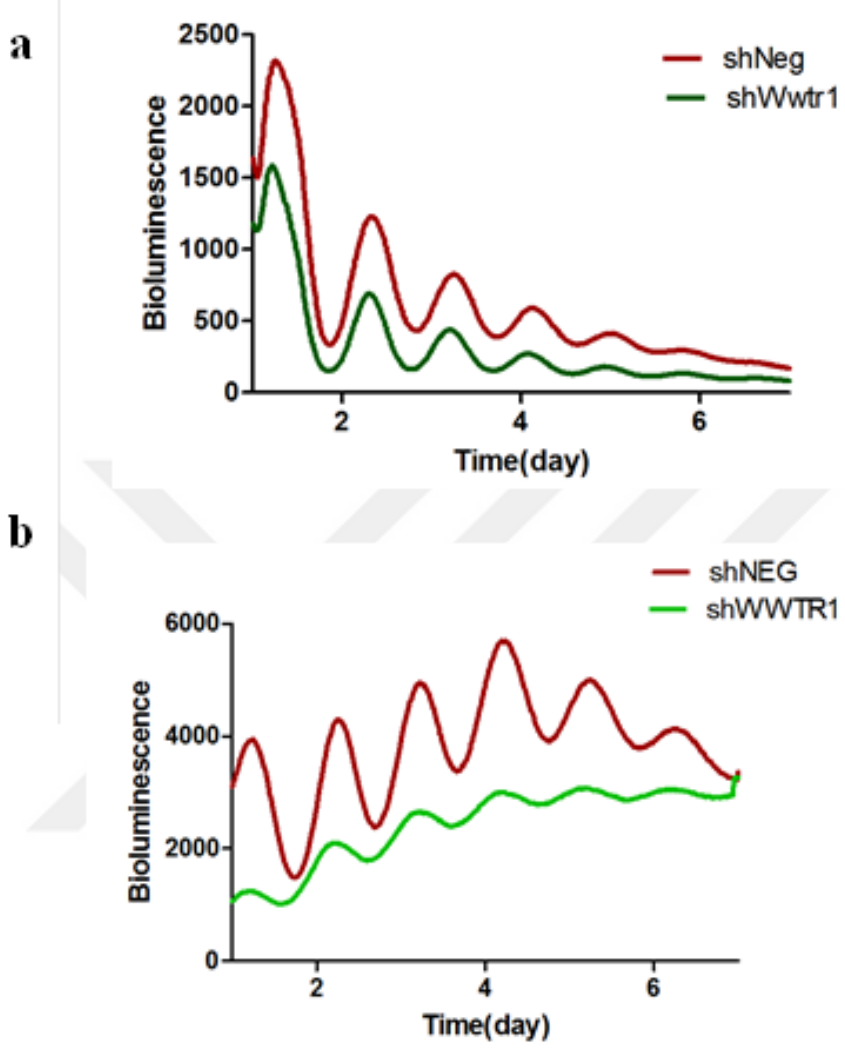
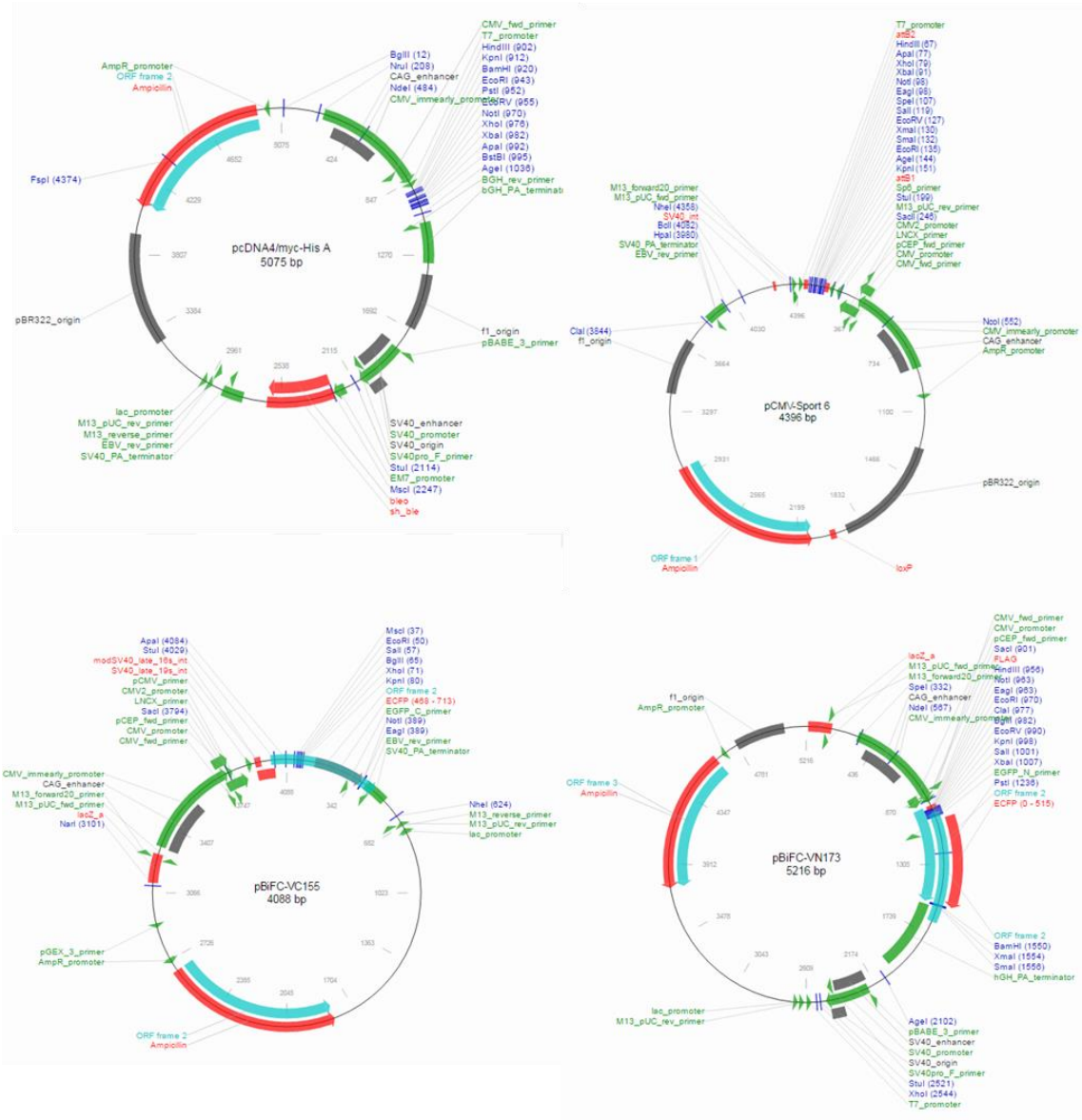
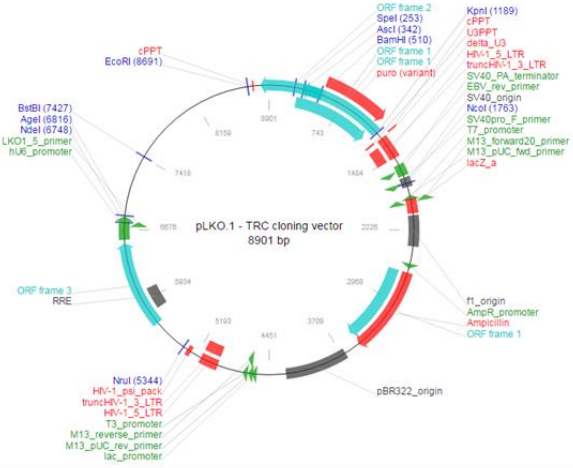


Figure S 2. Raw Data of Bioluminescence Records of NIH 3T3 Per1: *dluc* and U2-OS Bmal1: *dluc* cell lines transfected with shWwtr1 mix and shWWTR1 and shNeg control

APPENDIX C

Maps of Expression vectors

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EcoRI (8691)
BstBI (7427)
AgeI (6816)
NdeI (6748)
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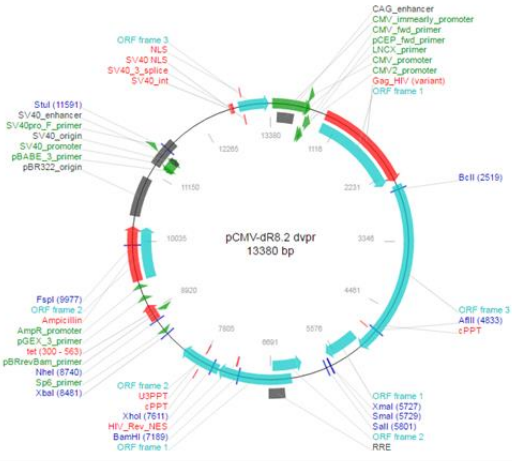
hU6_promoter
ORF frame 3
RRE
NruI (5344)
HIV-1_psi_pack
truncHIV-1_3_LTR
HIV-1_5_LTR
T3_promoter
M13_reverse_primer
M13_pUC_rev_primer
lac_promoter
pBR322_origin
f1_origin
AmpR_promoter
Ampicillin
ORF frame 1
ORF frame 2
SpeI (253)
AscI (342)
BamHI (510)
puro (variant)
KpnI (1189)
cPPT
U3PPT
delta_U3
HIV-1_5_LTR
truncHIV-1_3_LTR
SV40_PA_terminator
EBV_rev_primer
SV40_origin
NcoI (1763)
SV40pro_F_primer
T7_promoter
M13_forward20_primer
M13_pUC_fwd_primer
lacZ_a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NLS
SV40 NLS
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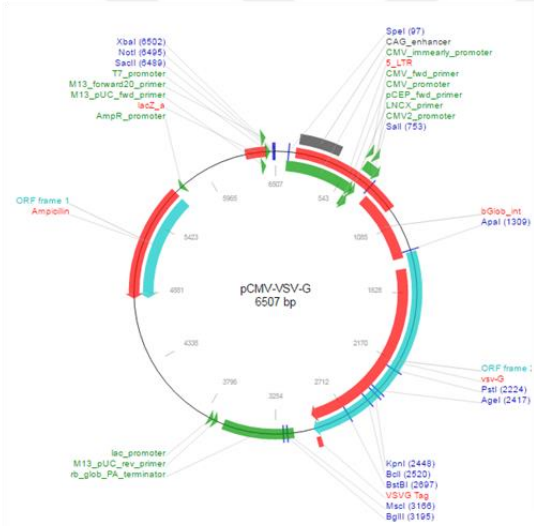
SV40_int
StuI (11591)
SV40_enhancer
SV40pro_F_primer
SV40_origin
SV40_promoter
pBABE_3_primer
pBR322_origin
FspI (9977)
ORF frame 2
Ampicillin
AmpR_promoter
pGEX_3_primer
tet (300 - 563)
pBRrevBam_primer
NheI (8740)
Sp6_primer
XbaI (8481)
ORF frame 2
U3PPT
cPPT
XhoI (7611)
HIV_Rev_NES
BamHI (7189)
ORF frame 1
CAG_enhancer
CMV_immearly_promoter
CMV_fwd_primer
pCEP_fwd_primer
LNCX_primer
CMV_promoter
CMV2_promoter
Gag_HIV (variant)
ORF frame 1
BclI (2519)
ORF frame 3
AflII (4833)
cPPT
ORF frame 1
XmaI (5727)
SmaI (5729)
SalI (5801)
ORF frame 2
RRE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XbaI (6502)
NotI (6495)
SacII (6489)
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M13_forward20_primer
M13_pUC_fwd_primer
lacZ_a
AmpR_promoter
ORF frame 1
Ampicillin
lac_promoter
M13_pUC_rev_primer
rb_glob_PA_terminator
SpeI (97)
CAG_enhancer
CMV_immearly_promoter
5_LTR
CMV_fwd_primer
CMV_promoter
pCEP_fwd_primer
LNCX_primer
CMV2_promoter
SalI (753)
bGlob_int
ApaI (1309)
ORF frame 1
vsv-G
PstI (2224)
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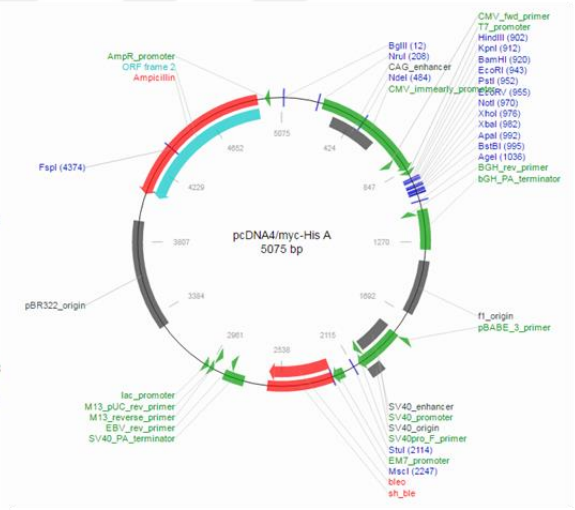
VSVG Tag
MscI (3166)
BglII (3195)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ORF frame 2
Ampicillin
FspI (4374)
pBR322_origin
lac_promoter
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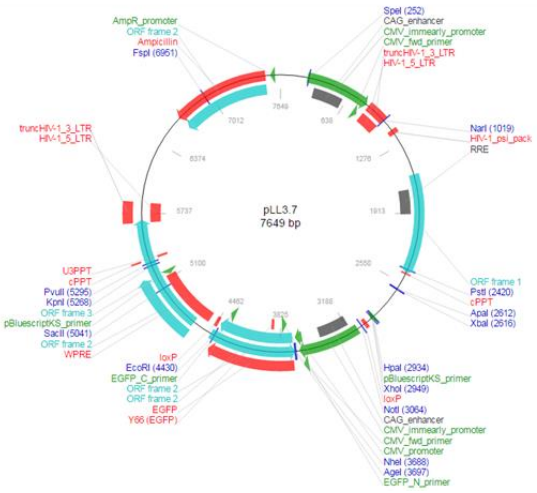
M13_reverse_primer
EBV_rev_primer
SV40_PA_terminator
f1_origin
pBABE_3_primer
SV40_enhancer
SV40_promoter
SV40_origin
SV40pro_F_primer
StuI (2114)
EM7_promoter
MscI (2247)
bleo
sh_ble
BglII (12)
NruI (208)
CAG_enhancer
NdeI (484)
CMV_immearly_promoter
CMV_fwd_primer
T7_promoter
HindIII (902)
KpnI (912)
BamHI (920)
EcoRI (943)
PstI (952)
EcoRV (955)
NotI (970)
XhoI (976)
XbaI (982)
ApaI (992)
BstBI (995)
AgeI (1036)
BGH_rev_primer
bGH_PA_terminator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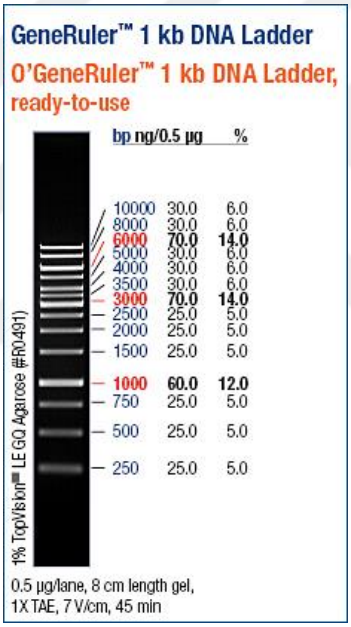
AmpR_promoter
ORF frame 2
Ampicillin
FspI (6951)
truncHIV-1_3_LTR
HIV-1_5_LTR
U3PPT
cPPT
PvuII (5295)
KpnI (5268)
ORF frame 3
pBluescriptKS_primer
SacII (5041)
ORF frame 2
WPRE
loxP
EcoRI (4430)
EGFP_C_primer
ORF frame 2
ORF frame 2
EGFP
Y66 (EGFP)
SpeI (252)
CAG_enhancer
CMV_immearly_promoter
CMV_fwd_primer
truncHIV-1_3_LTR
HIV-1_5_LTR
NarI (1019)
HIV-1_psi_pack
RRE
ORF frame 1
PstI (2420)
cPPT
ApaI (2612)
XbaI (2616)
HpaI (2934)
pBluescriptKS_primer
XhoI (2949)
loxP
NotI (3064)
CAG_enhancer
CMV_immearly_promoter
CMV_fwd_primer
CMV_promoter
NheI (3688)
AgeI (3697)
EGFP_N_primer

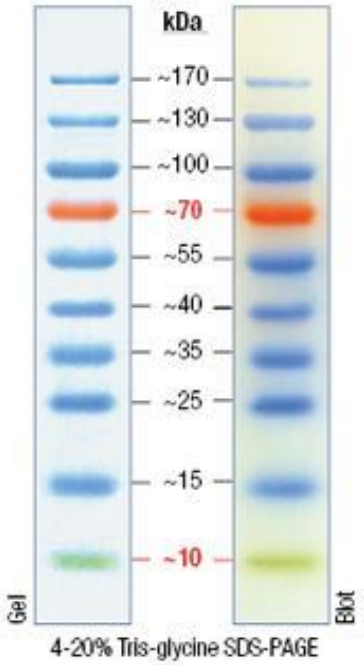
APPENDIX D

DNA and Protein Markers

DNA Molecular Weight Marker



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



PageRuler Prestained Protein Ladders

APPENDIX E

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