

Discovery and Characterization of Small Molecules Stabilizing the Cryptochrome in the Mammalian Circadian Clock

by

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To my family and friends...

ABSTRACT

Circadian clock mechanism is found in most of the living organisms and helps to maintain a daily rhythm of sleep/wake cycle, metabolism, body temperature and many other physiological processes. The mammalian circadian clock mechanism is intrinsic in organs and tissues and it is synchronized by the suprachiasmatic nuclei (SCN) located in anterior hypothalamus. Since many physiological processes are related to the circadian clock, in numerous studies it has been shown that the disruption of the clock can lead to certain diseases.

At the molecular level, circadian clock mechanism consists of a transcriptional-translational autoregulatory feedback loop. In the core loop, BMAL1 (Brain and muscle Arntl-1 like) and CLOCK (the circadian locomotor output cycles kaput), which are transcription factors, initiate the transcription of clock controlled genes including the *Cry1*, *Cry2*, *Per1*, and *Per2* genes. Afterwards, CRY (cryptochrome) and PER (period) proteins interact and then translocate back to nucleus where they repress the activity of BMAL1:CLOCK, hence repress their own expression. Ubiquitin ligases SCF-FBXL3 and SCF-FBXL21 (SKP1-CUL1-F-box protein complexes) regulate the degradation and stabilization of CRY1 and CRY2 proteins. The stability of CRY proteins is important because they can alter the period and amplitude of the circadian rhythm, in turn, affecting metabolism.

The aim of this thesis is to identify small molecules that enhance CRY stability considering they can be effective in type 2 diabetes treatment. Glucose metabolism is under the regulation of circadian clock mechanism along with other mechanisms. The crosstalk between gluconeogenesis pathway and CRY proteins can be exploited to discover small molecules for treatment of type 2 diabetes or other glucose metabolism related diseases. In previous studies, it was discovered that increase in CRY1 protein levels inhibit glucagon-induced gluconeogenesis. This inhibition is caused by CRY1 binding to the α subunit of the G protein ($G_s\alpha$) which blocks GPCR-mediated increases in cAMP.

In order to identify CRY stabilizers, around 2 million small molecules were previously screened in our laboratory for their interaction with the FBXL3/21 binding pocket *in silico*. In this thesis, 32 candidate small molecules were tested for their effects on CRY stability, circadian clock, and gluconeogenesis. Among these candidates, small molecules

TW63 and TW68 have shown promising results by increasing the stability of CRY1/2 and lengthening the period of circadian rhythm in Bmal1-dLuc U2OS cells. Moreover, TW68 caused the repression of gluconeogenic genes *Pck1* and *G6pc*, and lowered the glucose production during glucagon-induced gluconeogenesis in hepatic HepG2 cells. These results signify the therapeutic potential of TW68, a CRY stabilizer, for the treatment of type 2 *diabetes mellitus*.



ÖZET

Sirkadiyen saat mekanizması neredeyse bütün canlılarda bulunur ve uyku-uyanıklık döngüsü, metabolizma, vücut sıcaklığı gibi birçok fizyolojik sürecin günlük ritminin korunmasında görev alır. Memelilerde sirkadiyen saat mekanizması organ ve dokularda içsel olarak bulunmakla beraber hipotalamusta bulunan suprakiazmatik çekirdek (SCN) tarafından senkronize edilir. Fizyolojik süreçlerin birçoğunun sirkadiyen saat ile bağlantılı olmasından dolayı, birçok çalışmanın da gösterdiği üzere, biyolojik saatin bozulması bazı hastalıklara yol açabilmektedir.

Moleküler düzeydeki sirkadiyen saat mekanizması transkripsiyonel – translasyonel geribildirim döngülerinden oluşur. Merkezdeki döngüde BMAL1 ve CLOCK transkripsiyon faktörleri heterodimer oluşturarak *Cry1/2* (Cryptochrome) ve *Per1/2* (Period) genlerinin ekspresyonunu etkinleştirir. Daha sonra CRY ve PER proteinleri bir kompleks oluşturarak çekirdeğe geri dönerler ve burada BMAL1:CLOCK kompleksinin aktivitesini baskılayarak kendi ekspresyonlarını da baskırlar. CRY1 ve CRY2 proteinlerinin degradasyonu ve stabilitesi SCF-FBXL3 ve SCF-FBXL32 ubiquitin ligazları tarafından ayarlanır. CRY proteinlerinin stabilitesi önemlidir; çünkü sirkadiyen ritmin periyodunu ve genliğini değiştirebilir ve böylece metabolizmayı etkileyebilir.

Bu tezin amacı CRY proteinlerinin stabilitesini arttıran küçük molekül ilaçların taranarak tip 2 diyabet tedavisinde kullanılmak üzere belirlenmesidir. Glikoz metabolizması diğer birçok mekanizma gibi sirkadiyen saat mekanizmasının kontrolü altındadır. Tip 2 diyabet veya diğer glikoz mekanizması bozuklarının tedavisinde kullanılmak üzere küçük molekül ilaçların keşfi için glukoneogenez yolağına odaklanılabilir. CRY1 protein seviyesi artışının etkisiyle glukagona bağlı glukoneogenezin inhibe edildiği önceki çalışmalarda görülmüştür. Bu inhibisyonun nedeni CRY1'in G proteininin α alt ünitesine bağlanarak GPCR'a bağlı cAMP artışının engellenmesidir.

CRY proteininin stabilizörlerinin belirlenmesi için laboratuvarımızda daha önceden yaklaşık 2 milyon küçük molekül bilgisayar ortamında taranmıştır. Bu taramada moleküllerin CRY üzerindeki FBXL3/21 bağlanma bölgesi ile etkileşimine odaklanılmıştır. Bu tezin konusu olarak taramanın sonucunda elde edilen 32 tane küçük molekül ilaç adayının CRY stabilitesine,

sirkadiyen ritme ve glukoneogeneze olan etkileri test edilmiştir. Bu adaylardan TW63 ve TW68 kodlu moleküller CRY1/2 proteinlerinin stabilitesini arttırmış ve U2OS *Bmal1-dLuc* hücrelerinde sirkadiyen ritmin periyodunu uzatmıştır. Buna ek olarak, glukagona bağlı glukoneogenez sırasında TW68 uygulanan HepG2 karaciğer hücrelerinde *Pck1* ve *G6pc* glukoneogenez genleri baskılanmış ve glikoz üretiminde azalma görülmüştür. Bu sonuçlar, bir CRY stabilizörü olan TW68'in tip 2 diyabet tedavisinde terapötik potansiyelinin olduğunu göstermektedir.



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NOMENCLATURE

AC	Adenyl Cyclase
AMPK	AMP (adenosine monophosphate)-activated protein kinase
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like protein
ATM	Ataxia telangiectasia mutated serine/threonine kinase
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BMAL1	Component Brain and muscle Arntl-1 like
CaMK	Calcium/calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CCG	Clock-controlled genes
CHK2	Checkpoint kinase 2
CK1	Casein kinase 1
CLOCK	Circadian locomotor output cycles kaput
CRSD	Circadian rhythm sleep disorders
CRE	Calcium/cAMP response element
CREB	CRE-binding protein
CRY	Cryptochrome
CT	Circadian time
CUL1	Cullin-1
DBP	D-box binding protein
DMEM	Dulbecco's modified Eagle's Medium

DMSO	Dimethyl sulfoxide
DIO	Diet induced obesity
DSPD	Delayed sleep phase disorder
DYRK1A	Dual-specificity tyrosine-phosphorylated and regulated kinase 1A
EC50	Half maximal effective concentration
FAD	Flavin adenine dinucleotide
FASPS	Familial advanced sleep phase syndrome
FBS	Fetal bovine serum
FBXL	F-box/LRR-repeat protein
G6pc	Glucose-6-phosphatase
G6pt1	Glucose-6-phosphate translocase 1
Gck	Glucokinase
GPCR	G-protein-coupled receptor
GSK-3β	Glycogen synthase kinase 3 β
Glut2	Hepatic glucose transporter 2
Gys2	Glycogen synthase 2
HEK293T	Human embryonic kidney HEK293T cell line
HEPES	N-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid
HepG2	Human hepatocellular carcinoma HepG2 cell line
HFL	Hepatic leukemia factor
HRP	Horseradish peroxidase
ipRGC	Intrinsically photosensitive retinal ganglion cells

ISWR	Irregular sleep-wake rhythm
L-PK	Liver pyruvate kinase
LUC	Luciferase
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFIL3	Nuclear factor, interleukin-3
NIH3T3	Mouse embryo fibroblast NIH3T3 cell line
NPAS2	Neuronal PAS domain protein 2
PAR-bZIP	Proline and acidic amino acid-rich basic leucine zipper
PAS	Per-Arnt-Single-minded
PBS	Phosphate-buffered saline
PER	Period
PHR	Photolyase homology region
PIC	Protease inhibitor Cocktail
<i>Pck1</i>	Phosphoenolpyruvate carboxykinase
REM	Rapid eye movement
REV-ERB	Nuclear receptor subfamily 1, group D
ROR	Retinoic acid-related orphan nuclear receptors
RT-qPCR	Real time – quantitative polymerase chain reaction
SCN	Suprachiasmatic nuclei
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis

SKP1	S-Phase kinase associated protein 1
SUMO	Small ubiquitin-like modifier
TAD	Trans-activating domain
TBS	Tris-buffered saline
TDP-43	TAR (transactive response) DNA binding protein 43
TEF	Thyroid embryonic factor
TNFα	Tumor necrosis factor α
TTFL	Transcriptional-translational feedback loop
U2OS	Human osteosarcoma U2OS cell line
USP7	Ubiquitin-specific protease 7
Wee1	Wee1 G2 Checkpoint Kinase
XPA	Xeroderma pigmentosum, complementation group A
VNTR	Variable-number tandem-repeat
ZT	Zeitgeber time
β-TrCP	β -transducin repeat-containing protein

CHAPTER 1

INTRODUCTION

Most of the living organisms have a circadian rhythm oscillating with a period of 24 hours. This rhythm helps the adaptation of organisms to the environmental changes caused by the daily rotation of earth. The entrainment of the circadian clock is modulated by external cues such as light and dark cycles and food intake. In mammals, this mechanism is intrinsic in organs and tissues and its synchronization is achieved by the suprachiasmatic nuclei (SCN). Any disturbance of the circadian clock mechanism can affect various physiological processes, which can increase the risks of health issues and lead to certain diseases.

In mammals, the molecular mechanism of circadian clock consists of a positive and a negative transcriptional-translational feedback loop that are intertwined. Core clock proteins CRY1/2 and PER1/2 are repressors while BMAL1 and CLOCK are activators of this mechanism. The stability of CRY1 and CRY2 proteins is regulated by the SCF-FBXL3 and SCF-FBXL21 ubiquitin ligases which play a role in the degradation of CRY proteins. In the overall system, the stability of CRY proteins can affect the period and amplitude of the circadian rhythm.

Identifying small molecules that effect CRY stability is important because previous studies show that increasing CRY stability can be effective for type 2 diabetes treatment. Previously, in this project, certain small molecules were selected by computational docking of small molecules to FBXL3/21 and CRY1 interaction pocket. Selected candidate small molecules were screened and characterized *in vitro*. First, the suitable doses of these small molecules were determined by assessing cell viability. Afterwards, the small molecules were screened according to their effect on the half-life of CRY1 and CRY2 proteins. Two small molecule TW63 and TW68 stabilized CRY and increased the period of U2OS Bmal1-dLuc rhythm in a dose-dependent manner. They were further characterized using Western blotting and RT-qPCR to investigate the effect on levels of core clock proteins and mRNAs, respectively. The results further supported the stabilization of CRY1/2 by TW68 but not TW63.

Finally, we observed that TW68 also represses *Pck1* and *G6pc*, which are gluconeogenic genes, and lowers glucose production when gluconeogenesis was induced by glucagon. Overall, this study reveals a new CRY1/2 stabilizer, TW68, which exhibits therapeutic effects for type 2 diabetes *in vitro*.

In Chapter 2, background information on the biological rhythms which includes the molecular mechanism of the mammalian circadian clock and the diseases related to its impairment are reviewed.

In Chapter 3, the materials and methods used in this thesis is given. Statistical analysis and experimental procedures for cell culture maintenance, cell viability assessment, and biochemical methods used for characterization of small molecules are explained in detail.

The results obtained in this thesis are presented in Chapter 4 and these results are discussed in Chapter 5 which is finalized with concluding remarks.

CHAPTER 2

LITERATURE REVIEW

2.1. Overview of Biological Rhythms and Clocks

All living systems from unicellular organisms to mammals have developed an inner timing system called biological rhythm. These universal rhythms evolved in organisms to have survival advantage by helping them adapt to dynamic conditions of the earth such as light/dark cycles and temperature changes. Chronobiology is the scientific field that studies adaptations to geophysical cycles [1,2]. These geophysical cycles can be tidal (12.8 hours), circadian (daily, 24 ± 0.5 hours), lunar (29.53 days) and seasonal (365 days). The biological clocks of the many organisms are in synchrony with these cycles. These rhythms can be observed in the daily sleep-wake cycles, feeding patterns, lunar ovarian cycles, seasonal hibernation and migration of organisms [3].

Biological rhythms have been classified into three categories according to their periodicity: circadian rhythms, infradian rhythms and ultradian rhythms [1]. Circadian rhythms are approximately 24-hour cycles that are synchronized by the earth's daily rotation. Several examples of circadian rhythms sleep-wake cycle, body temperature, feeding pattern, metabolism, hormonal regulation and locomotor activity [4,5]. Ultradian rhythms cycle with a period less than 24-hour. Breathing, blood circulation, REM (rapid eye movement) and hormone secretion cycles are all ultradian rhythms that occur in the range of seconds, minutes or hours. The swimming activity of the beach hoppers is a circatidal rhythm which is a subtype of ultradian rhythms. Moreover, in some psychological disorders such as bipolar disorder patients have mood oscillations that are ultradian [6,7,8]. Infradian rhythms oscillate with a period more than 24-hour, ranging from days to years. Ovarian cycles show circalunar rhythm, a subtype of infradian rhythms. Other examples include seasonal migration, hibernation and mating patterns of many species [6,9,10].

2.2. Circadian Rhythms and Clocks

Circadian rhythms are approximately 24-hour cell-autonomous oscillations that are believed to have evolved as a result of earth's daily rotation. These rhythms help organisms to adapt to light/dark cycle influenced events that recur daily such as food availability [11]. Vitaterna, Takahashi, and Turek describe the circadian rhythms as follows, "One of the most dramatic features of the world in which we live is the cycle of day and night. Correspondingly, almost all species exhibit daily changes in their behavior and/or physiology. These daily rhythms are not simply a response to the 24-hour changes in the physical environment imposed by the earth turning on its axis but, instead, arise from a timekeeping system within the organism." [12].

In Latin, *circa* means "approximately" and *dies* means "a day". An important feature of a circadian rhythm is that its period is slightly more than 24-hour. The circadian rhythm is endogenous since it persists even when there are no external cues, i.e. under constant light or darkness. However, this endogenous mechanism is able to synchronize itself to 24 hours. The earth's 24-hour rotation results in periodic changes in environmental cues, called "zeitgebers", such as light and temperature which entrains the circadian clock mechanism [6,13,14].

From unicellular organisms to mammals, most organisms harbor a circadian clock mechanism. Nitrogen-fixing cyanobacteria have a circadian oscillation of nitrogenase enzyme, allowing it to perform nitrogen fixation during the nighttime and photosynthesis during the day [15]. In *Arabidopsis*, chloroplast movement, stromal opening and leaf movement show a circadian rhythm allowing the plant to utilize photosynthesis better during the day [16]. Mating activities, feeding patterns, and sleep cycles show a circadian rhythm both in *Drosophila* and in mammals [13,17].

2.2.1. History

The internal biological timekeeping mechanism of the organisms is believed to be evolved 2.5 billion years ago during the Great Oxidation Event which may have led to the circadian oscillations in reactive oxygen species and detoxification mechanisms [18]. The earliest written information about the biological clock was the observation of leaf movements and birdsong rhythms by Greek natural historians, however, they believed these events were merely a direct response to stimuli rather than an internal timekeeping mechanism [1].

In 1729, for the first time, biological rhythm was noted as an innate property by Jean Jacques d'Ortois de Marian, a French astronomer. He conducted experiments to study leaf movements of *Mimosa* by placing the plant in total darkness for several days and observed that the leaves still opened at daytime and closed at nighttime without external stimulus. He concluded that the leaf movement should be the plant's innate property and not a response to environmental stimuli [1,19]. Two centuries later, in 1962, French scientist and cave explorer Michel Siffre lived in a cave for two months without environmental cues and discovered that his sleep-wake cycle had a period of 24 hours 30 minutes which is slightly longer than the normal period of 24-hour [20]. In 1965, Jürgen Aschoff, one of the founders of chronobiology, published the findings of his experiment on more than 150 human subjects that stayed in isolation inside an underground bunker. He monitored the body temperature, sleep-wake cycle and urinary excretion of these subjects, finally concluded that these measurements all had an average period of 25 hour [4].

The discovery of molecular components of the circadian clock started with the identification of period (*per*) gene in *Drosophila melanogaster*. In 1971, Ronald J. Konopka and Seymour Benzer discovered *per* gene by screening mutant flies for clock genes. Three mutants showed abnormal circadian rhythm, one with a shorter period of 19 hour, one with a longer period of 28 hour and one arrhythmic phenotype with no circadian rhythm. These three mutations all mapped to the same gene on X chromosome, which was named period [21]. Following this discovery, another important core clock component named “the circadian locomotor output cycles kaput” (*Clock*) was identified in 1994 by Joseph S. Takahashi and his group. The group screened mutant mice and *clock* gene was characterized from a period-lengthening mutation [22,23]. In 1997, Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL) which is commonly known as component Brain and muscle Arntl-1 like (BMAL1) protein was discovered by John B. Hogenesch and his group [24,25].

One of the core clock components, cryptochromes have been long known as a blue light photoreceptor in plants while it was identified in humans in 1996 by Aziz Sancar and his group [26,27]. In 1999, the importance of *Cry1* and *Cry2* genes in mammals was shown by Gijsbertus van der Horst and his colleagues with the observation that *cry1* null-mutant mice have a rhythm with a decreased period (22.51 hour) and *cry2* null-mutant mice have an increased period (24.63 hour) compared to wild type mice (23.77 hour). Following this work,

another study revealed the role of mCRY1 and mCRY2 in the negative arm of the circadian clock feedback loop [28,29].

2.2.2. Characteristics and Basic Concepts

Rosbash and Hall describe the circadian rhythms: “Circadian rhythms have, by definition, periods of about 24 hr. They persist (“free-run”) under constant conditions with respect to principal environmental time cues (zeitgebers) and are thus endogenous rhythms rather than driven ones (e.g., those that are repeatedly stimulated by a cyclically changing environment). They are entrained by light and temperature cycles, and in some cases by other less prominent time cues. Entrainment normally serves to synchronize these not quite 24 hr biological rhythms to the precise daily rhythm of the environment.” [14].

One characteristic of circadian rhythm is its innate nature. In all organisms studied until now, there is an intrinsic rhythm that persists in the absence of any input from the environment. This persistence comes from the constant period sustained which is different than the expected periods of about 24 hours. This free-running and self-sustained rhythms have been shown to persist under constant darkness and temperature conditions. This persistent rhythm is even present in the isolated cells from the brain [30]. Although there is an intrinsic rhythm, this rhythm is not synchronized, i.e. it is in a different phase in individual cells in the body [31,32].

Moreover, circadian rhythms can be synchronized meaning that the whole organism's circadian clock is in the same phase. This synchronization process is called “entrainment” of the clock. Environmental cues are external inputs (zeitgebers) of the circadian clock that align the phase and period of the intrinsic rhythm with the external rhythms such as light-dark cycles [31,32]. The main zeitgeber with the most effective synchronizing ability is light and others are temperature changes [33], feeding time [34], physical activity [35], social interactions [36] and sleep deprivation [37,38].

Robustness is an essential characteristic of the circadian clock which protects the mechanism from both intrinsic and external perturbations. The definition of robustness in this concept is the ability to maintain constant period, phase and amplitude during daily oscillations. The intrinsic perturbations such as molecular noise and external perturbations are overcome by the entrainment of the clock. Jetlag, for example, occurs as a result of our

robust timekeeping mechanism, where adjusting to the new condition takes some time [39,40].

Circadian time (CT) is a special timing system commonly used in chronobiology. It is used to describe the free running intrinsic timing of the organism irrespective of the environmental cues (zeitgebers). In humans, for example, the active phase starts at CT 0 which is the light phase while for nocturnal animals like rodents, the active phase starts at CT 12 which is the dark phase. Zeitgeber time (ZT) is another system which is defined relative to the environmental cues. This system starts with the sunrise (ZT 0) which is the light phase and continues with sunset (ZT 12) which is the dark phase [1].

2.2.3. Features of Circadian Rhythm: Period, Amplitude and Phase

Biological rhythm can be demonstrated by plotting a particular measure (e.g. body temperature) with changing time. These wave-shaped plots reveal three key parameters of a biological rhythm: period, amplitude and phase (Figure 2.1). Amplitude is defined as the difference between the highest level (peak) and the lowest level (trough) of the measure. The phase of the rhythm is the relationship between a reference time in the cycle with another cycle or a fixed event. For example, in Figure 2.1 the phase is the time of the peak relative to the beginning of the light condition. The time it takes to complete one cycle is defined as the period which can be calculated from the time difference between two consecutive peaks [1,12].

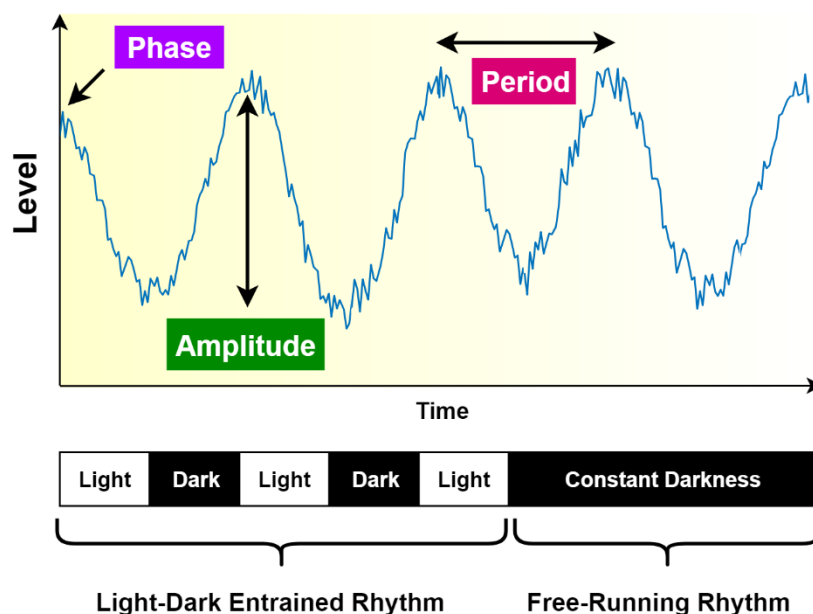


Figure 2.1. Rhythm parameters shown on a schematic representation of circadian oscillations for the entrained system followed by the free-running system. Adapted from [12] and [41].

For decades, these parameters (especially period) have been used to identify new clock genes. When a circadian clock gene is mutated, the phenotype is usually observed as a change in one of the features of the circadian rhythm [42,12]. Several examples of the phenotypic changes in mutant clock genes are given in Table 2.1. The molecular mechanism of the circadian clock is given in Section 2.4.1.

Table 2.1. Effect of mutations in mammalian clock genes on circadian phenotype

Mutated Gene	Change in Circadian Phenotype
<i>mPer1</i>	Shorter period, dampened amplitude, or arrhythmic [43]
<i>mPer2</i>	Shorter period, arrhythmic [43]
<i>mPer3</i>	Marginally shorter period [44]
<i>mCry1</i>	Shorter period [28]
<i>mCry2</i>	Longer period [28]
Both <i>mCry1</i> and <i>mCry2</i>	Arrhythmic [28]
<i>Clock</i>	Longer period, arrhythmic in constant conditions [22]
<i>Bmal1</i>	Arrhythmic [45]
<i>Dbp</i>	Marginally shorter period [46]
<i>Rev-erba/β</i>	Arrhythmic [47]
<i>CK1ε</i>	Longer period [48]

2.2.4. Biological Importance

Circadian rhythms are evolutionarily conserved in almost all living organisms; therefore, these rhythms are thought to have biological importance that increases the fitness of the organism. The dynamic environment of the earth is most distinctly characterized by day and night cycle which causes a sharp change in both the amount of light and temperature. The circadian clock is thought to evolve as a response mechanism in order to prepare for these environmental changes. When there is a disturbance in the environmental conditions, such as constant darkness, it is an advantage that the internalized circadian rhythms are not disturbed substantially. In cyanobacteria, it was shown that mutants with impaired circadian clock have significantly lower fitness under competition with wild type strains [11,49].

In mammals, the impairment of circadian rhythm also increases fitness. For example, a study in chipmunks demonstrated that chipmunks lacking circadian rhythm were more susceptible to be the prey of predators [50]. Many behavioral and physiological processes such as locomotor activity, energy metabolism, sleep-wake cycles, endocrine secretion, cardiovascular activity [17,51,52,42], blood pressure, physical strength, and mental alertness [32,53] are controlled by the circadian clock.

2.3. The Central and Peripheral Circadian Clock in Mammals

The mammalian circadian clock mechanism is made up of three components: inputs, outputs and a pacemaker (Figure 2.2). As previously mentioned, inputs are environmental cues (zeitgebers) such as temperature and light; whereas outputs are physiological and behavioral responses. The circadian pacemaker that connects the input to output is the suprachiasmatic nucleus (SCN) in the hypothalamus. SCN contains ~20,000 neurons that are individual cell-autonomous circadian oscillators. Their intercellular coupling result is a synchronized and robust oscillation. Circadian clocks of the peripheral tissues are synchronized by the neural and humoral signals from SCN in accordance with the environmental cues. [5,54,55].

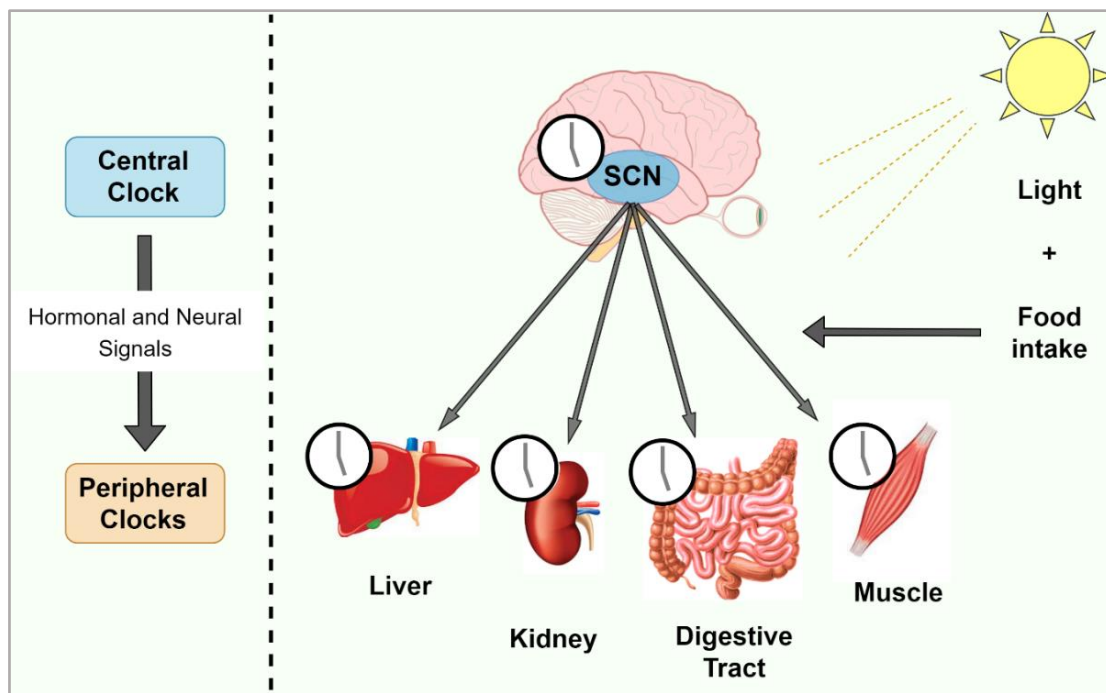


Figure 2.2. Schematic representation of the synchronization of peripheral clocks by the central clock. Adapted from [56] and [57].

Light is the main zeitgeber providing the strongest synchronization. SCN receives light signals coming from the eye through the retinohypothalamic tract. A photoactive protein, melanopsin located in intrinsically photosensitive retinal ganglion cells (ipRGCs) of the eye plays an important role in circadian photoreception [32,58]. When the signal is received via Ca^{2+} influx to the SCN, mitogen-activated protein kinase (MAPK) and calcium/calmodulin-dependent kinase (CaMK) phosphorylates and activates calcium/cAMP response element-binding protein (CREB) [59,60]. In the morning when the sun rises, the phosphorylated CREB binds to calcium/cAMP (cyclic adenosine monophosphate) response element (CRE) sequences that induce the expression of *Per* genes. Starting with the sunset at night, expression levels of *Per* decrease. Hence, the synchronized circadian oscillation is established in the SCN [59,61].

The peripheral clocks are synchronized by SCN via control of neural and hormonal processes such as melatonin, prolactin, cortisol and growth hormone secretion, bowel movement, core body temperature, and immune system [54,62]. Besides the SCN, other synchronization mechanisms include food intake and temperature fluctuations. For example, the liver regulates circadian clock via glucocorticoid hormone secretion depending on food intake. It has been reported that decreased availability of food can reset peripheral circadian clocks and this resetting is not dependent on SCN [63,64].

2.4. Molecular Components of the Mammalian Circadian Rhythm

2.4.1. Transcriptional and Translational Feedback Loops

In mammals, the circadian clock is a cell-autonomous mechanism generated by a positive and a negative transcriptional-translational feedback loop (TTFL) that are connected to each other (Figure 2.3) at the molecular level [13,65]. The positive feedback loop contains two transcription factors BMAL1 and CLOCK, belonging to basic helix-loop-helix; Per-Arnt-Single-minded (bHLH-PAS) family. BMAL1 and CLOCK heterodimer binds to E-box or E'-box elements (5'-CACGTG-3' or 5'-CACGTT-3', respectively) in the promoter region of the target genes [66,67]. This complex initiates clock-controlled genes (CCG) including the *Per* and *Cry* genes, which form negative feedback loop of the circadian clock mechanism. In the cytoplasm, PER and CRY proteins accumulate to form a heterodimer and translocate back to the nucleus where they interact with BMAL1:CLOCK and repress the transcription of the CCG genes [29,68,69]. Microarray and RNA-seq studies revealed that more than 20% of mammalian genes are under the control of circadian clock [70,71,72] including *Dbp* (D-box binding protein) and *Rev-erba/β* (Nuclear receptor subfamily 1 group D) [73,74]. This elegant mechanism of negative feedback loop causes genes and proteins to oscillate every ~24 hours [75]. However, it is also subject to fine-tuning by a positive feedback loop and many post-translational modifications [76].

The positive TTFL is essential for the regulation of BMAL1 expression and other clock-controlled genes. BMAL1:CLOCK complex drives the transcription of the retinoic acid-related orphan nuclear receptors (RORs - α and β homologs) and REV-ERBs (α and β homologs) by binding to E-box elements in their promoters. RORs and REV-ERBs are transcription factors competing to bind to ROR response elements (RORE) in the promoter region of *Bmal1* [77,78,79]. This leads to the repression of *Bmal1* by REV-ERBs and the activation of *Bmal1* transcription by RORs [80]. A study suggested that the promoter region of the *Clock* gene also contains a REV-ERB binding site, indicating CLOCK expression may be regulated by REV-ERBs [81].

The positive and negative loops are also connected through D-box elements [82]. Transcription factors from PAR-bZIP family such as DBP, HFL (hepatic leukemia factor), TEF (thyroid embryonic factor) bind to D-box elements to transactivate the clock genes [83]. On the other hand, NFIL3 (nuclear factor, interleukin-3) occupies the D-box elements and

represses clock genes [84,85]. These transcription factors are also indirectly affected from the circadian expression of clock-controlled genes since they either have RORE or E-box element in their promoter region (refer to Figure 2.3) [86].

Another layer of regulation of the circadian clock mechanism is facilitated by post-translational modifications. Phosphorylation, acetylation, SUMOylation, and ubiquitination provide additional precision to the system [87,88]. The cellular localization, stability, and activity of the circadian clock proteins are regulated by phosphorylation. In order to maintain the oscillation, the effect of CRY:PER repression should decrease via degradation mechanisms. PER proteins are phosphorylated by casein kinase 1 epsilon (CK1 ϵ) and casein kinase 1 gamma (CK1 γ), allowing PER proteins to be ubiquitinated by β -transducin repeat-containing protein (β -TrCP) for 26S proteasomal degradation [89,90]. BMAL1 is phosphorylated by casein kinase 2 alpha (CK2 α) leading to the accumulation of BMAL1 in the nucleus [91]. Additionally, BMAL1 is subject to SUMOylation which is necessary for its interaction with CLOCK which effects its turnover rate directly [92,93]. CLOCK-mediated BMAL1 acetylation is also important for the repressive activity of CRY:PER on E-box bound BMAL1:CLOCK because acetylated BMAL1 recruits CRY1 to the complex [94]. CLOCK protein is also a histone acetyltransferase that acetylates the H3 and H4 in the promoter regions of circadian genes regulated by BMAL1:CLOCK [95]. This process is vital for circadian oscillations since it enables the transactivation of these genes [96]. The phosphorylation and degradation of CRY proteins will be discussed thoroughly in the following section (Section 2.4.2).

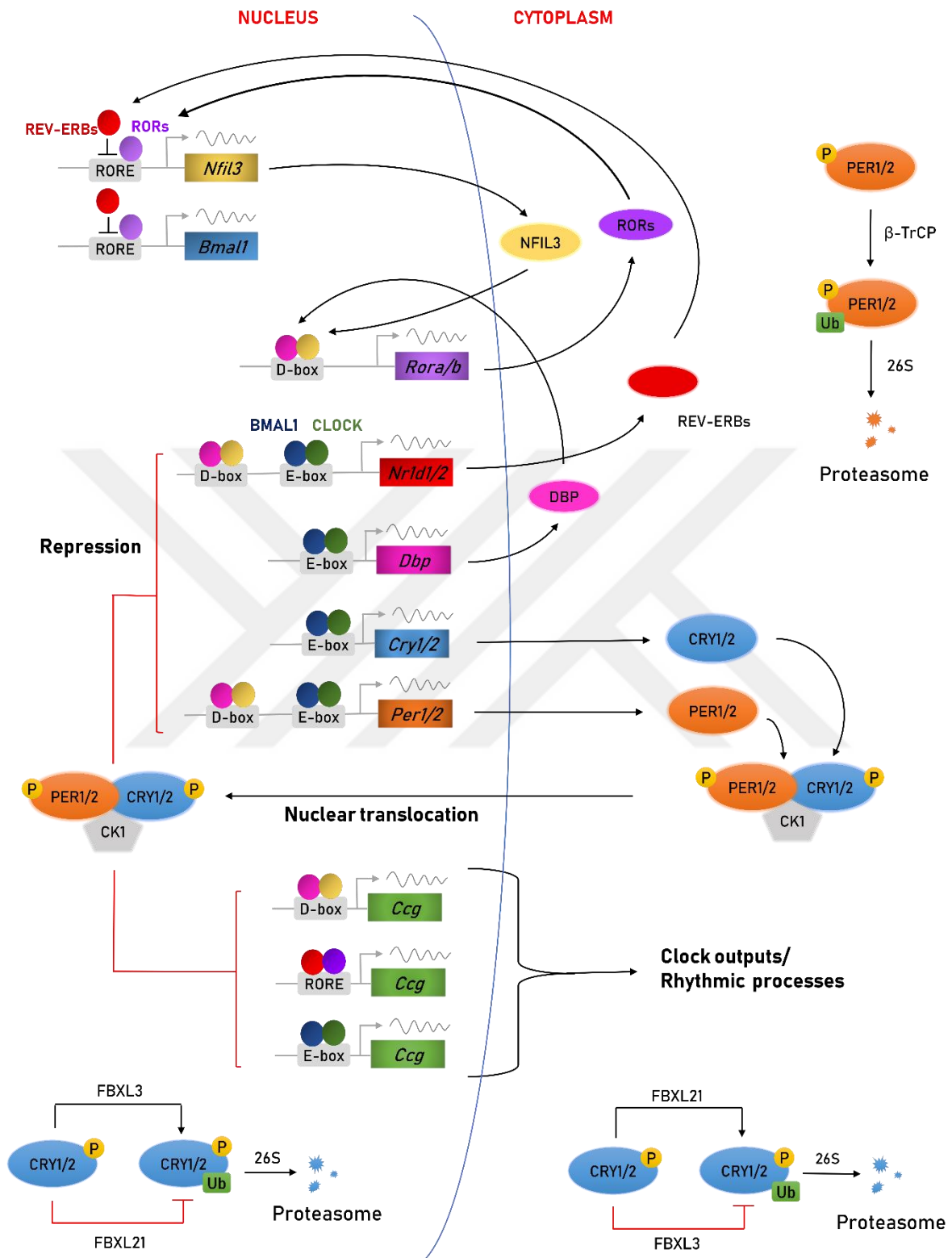


Figure 2.3. Transcriptional and translational feedback loops constituting the molecular mechanism of mammalian circadian clock. Adapted from [71] and [97].

2.4.2. Stability and Degradation of Cryptochrome 1 & 2

The phosphorylation and ubiquitination of CRY proteins are very important to sustain the oscillation of the circadian clock. Following the repression by CRY:PER complex, these proteins should be degraded in order to start the new cycle. Degradation mechanism of PER proteins was explained in the previous section (Section 2.4.1); however, degradation mechanism of CRY proteins should be elaborated in more detail since repression by CRY proteins is significantly stronger than PER proteins [98,99].

CRY1 and CRY2 proteins are both phosphorylated by AMP-activated protein kinase (AMPK) at the residues S71 and S280 for CRY1 [100]. Another phosphorylation mechanism specific to CRY2 is glycogen synthase kinase 3 β (GSK-3 β) mediated phosphorylation following the phosphorylation by dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) [99]. The degradation and stability of CRY proteins are mainly controlled by ubiquitination catalyzed by SKP1–CUL1–F-box protein complexes that function as E3 ubiquitin ligases: SCF^{FBXL3} and SCF^{FBXL21}. The phosphorylation by AMPK enhances the interaction of CRY proteins with FBXL3, leading to the polyubiquitination and 26S proteasomal degradation [101,87,102].

Another F-box protein, FBXL21, increases the stability of CRY proteins by functioning antagonistically with FBXL3. The paralogue genes *Fbxl3* and *Fbxl21* both degrade CRY, however FBXL21-mediated degradation is much slower than FBXL3. Therefore, FBXL21 protects CRY proteins from faster degradation leading to their stabilization. It is also suggested that while FBXL3 ubiquitinates CRY on multiple lysine residues, FBXL21 specifically ubiquitinates K11 and K107 residues [97,103]. In 2013, the groups of Fukada and Takahashi independently discovered the stabilizing effect of FBXL21 on CRY. They investigated the function of FBXL3 and FBXL21 at different subcellular compartments: nucleus and cytoplasm (Figure 2.4). Fukada group concluded that FBXL21 stabilized CRY both in the nucleus and cytoplasm [97]. Takahashi group observed the stabilizing effect of FBXL21 in the nucleus, but not in the cytoplasm [103]. More recently, using proteomics analysis of CRY interactome, ubiquitin-specific protease 7 (USP7) and TAR DNA binding protein 43 (TDP-43) were discovered as CRY stabilizers [104].

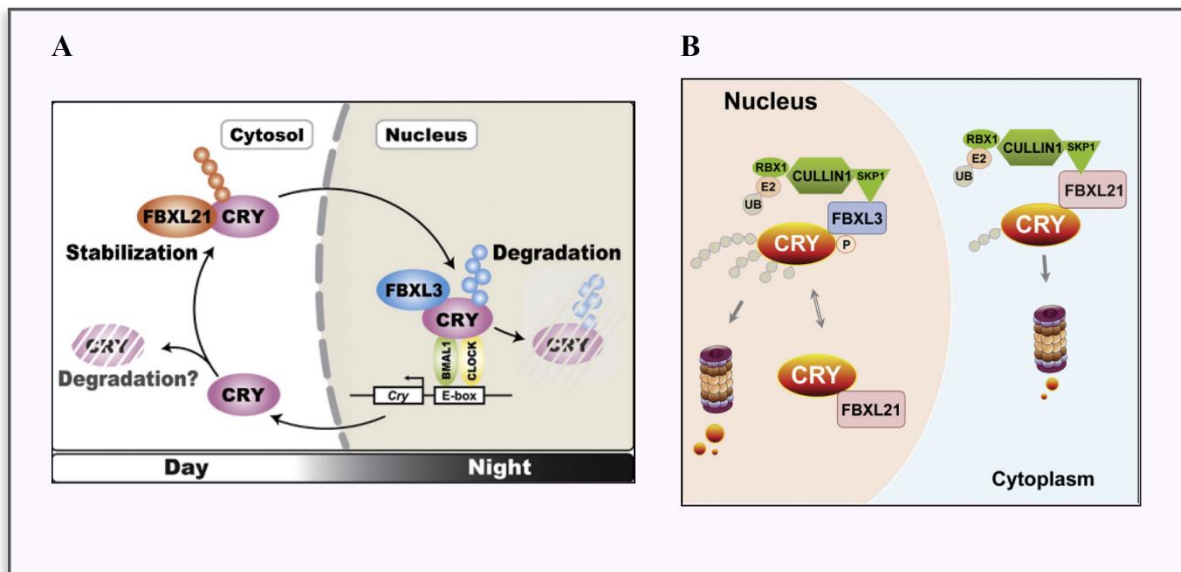


Figure 2.4. Suggested mechanisms of CRY ubiquitination and stabilization by FBXL3 and FBXL21. **A)** Retrieved from [97]. **B)** Retrieved from [103].

*Permission to reuse these figures are given in Appendix E.

2.4.3. Structural Implications of Core Clock Components

Core clock proteins contain highly similar domains that provide interaction of these proteins with DNA and each other. BMAL1, CLOCK and PER proteins contain PAS domains, while ROR α and REV-ERB α have the same common nuclear receptor structure. However, CRY proteins have a distinct structure consisting of a photolyase homology region (PHR) and CC helix which includes highly conserved α -helical coiled coil structure [105,106].

BMAL1 and CLOCK proteins both contain four similar domains: basic helix loop helix (bHLH) domain followed by tandem PAS domains (PAS-A and PAS-B) and finally a trans-activating domain (TAD). The BMAL1:CLOCK heterodimer is shown in Figure 2.5. bHLH domain is an E-box binding domain providing the DNA binding of BMAL1:CLOCK heterodimer. PAS domains of these proteins interact with each other which enables BMAL1:CLOCK heterodimer formation. Interaction of CRY:PER complex with BMAL1:CLOCK is required for repression and is achieved through the binding of CRY proteins to the TAD domain and C-terminal region of BMAL1 (Figure 2.5B) [105,107,108].

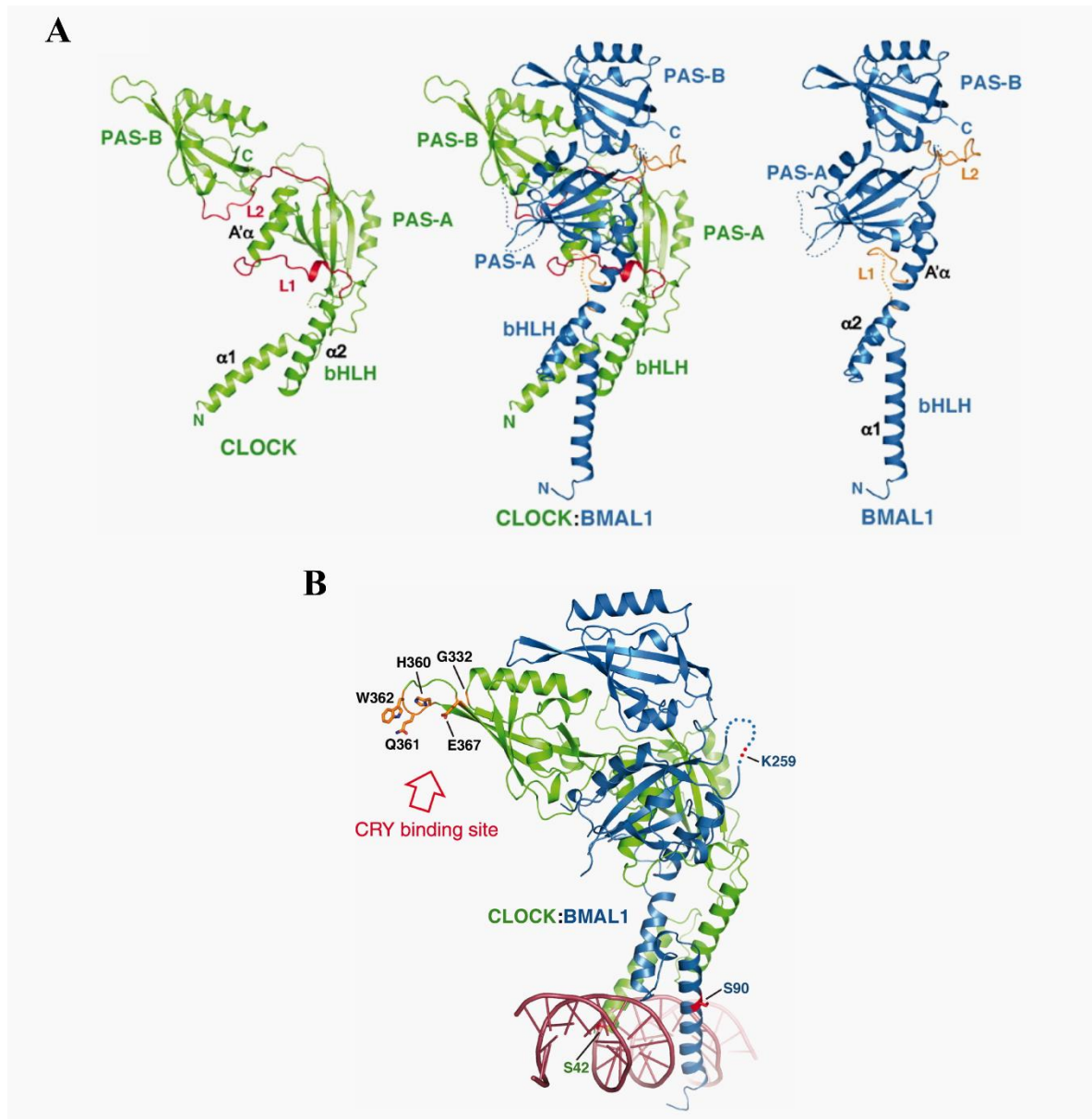


Figure 2.5. Crystal structure of BMAL1:CLOCK heterodimer. Retrieved from [108].

*Permission to reuse these figures are given in Appendix E.

In mammals, PHR domain together with the CC helix of CRY protein interacts with the C-terminal of PER proteins enabling CRY:PER heterodimer formation (Figure 2.6) [109,110,111,112]. CRY:PER binding is stabilized by a zinc ion (Zn^{2+}) that interacts with C432 and H491 residues of CRY2 together with C1210 and C1213 residues of PER2 [110]. Another important function of CRY:PER complex is the repressor activity. C-terminal tail and CC helix of CRY provides a site for interaction with the C-terminal of BMAL1. Also, the secondary pocket of CRY (shown in Figure 2.6A) interacts with PAS domains of CLOCK. Both of these interactions constitute the interaction of CRY:PER complex with BMAL1:CLOCK to repress E-box mediated transcription [110,113].

Moreover, FBXL3 and FBXL21 interact with CRY at the FAD-binding pocket in the PHR domain (Figure 2.6) [101,114]. The interaction sites of PERs and FBXLs coincide in some regions as shown in Figure 2.6B [110]. As a result of these shared sites, PER binding reduces CRY degradation. When AMPK phosphorylates S71 of CRY1, interaction with PER2 is weakened and FBXL3 binding is enhanced, which in turn, destabilizes CRY1 [100].

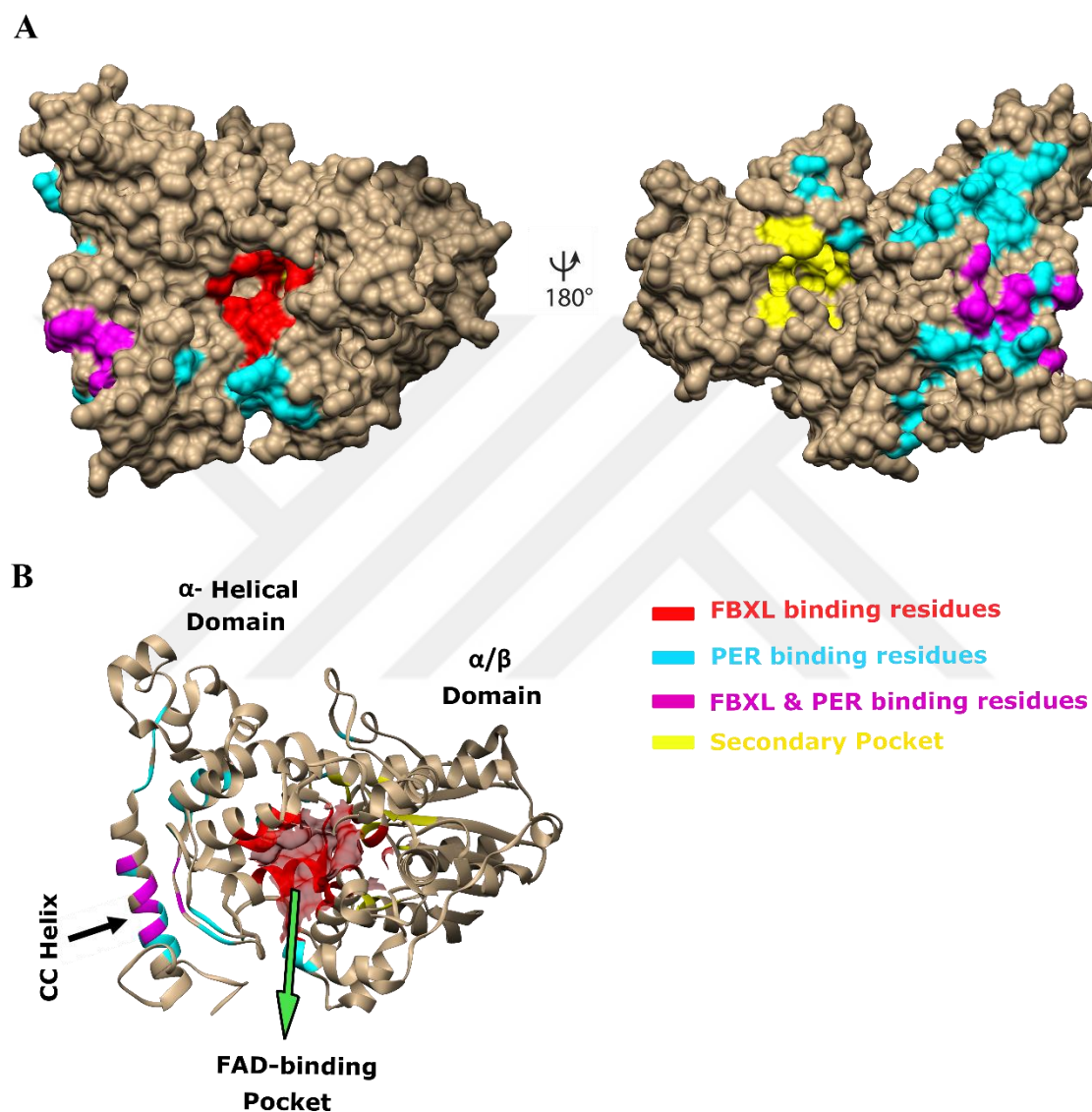


Figure 2.6. Crystal structure of mCRY2 (PDB ID: 4i6j). **A)** Surface representation with FBXL binding residues (red) [101], PER binding residues (cyan), both FBXL & PER binding residues (magenta) [110], secondary pocket (yellow) [115]. **B)** Ribbon representation with main domains and FAD-binding pocket.

*Molecular graphics prepared with UCSF Chimera [116].

2.5. Circadian Regulation of Glucose Metabolism

The circadian clock regulates glucose homeostasis in several ways. Basal glucose levels are directly regulated by the SCN independent of feeding pattern, which was demonstrated in a study where SCN-lesioned rats lost circadian rhythm of basal glucose and insulin levels [117]. Glucose homeostasis is also affected from pineal melatonin hormone. Melatonin has a circadian secretion rhythm which is under the control of SCN [118]. Analysis of mice with melatonin receptor knockout revealed enhancement of PER1 protein expression and an increase in insulin secretion from pancreatic islets [119].

Core clock components have also been shown to affect several proteins and pathways of glucose metabolism. Glycogen synthase 2 (*Gys2*), found mostly in liver, is the key enzyme for glycogen biosynthesis. Clock mutant mice have attenuated circadian oscillation of *Gys2* mRNA levels, protein levels and glycogen content in liver [120]. Several hepatic glucose regulatory genes including glucose-6-phosphate translocase 1 (*G6pt1*), glucokinase (*Gck*), liver pyruvate kinase (*L-PK*), and hepatic glucose transporter 2 (*Glut2*), which have circadian expression patterns in WT mice, lost their rhythm in *Bmal1*^{-/-} knockout mice [121]. Phosphoenolpyruvate carboxykinase 1 (*Pck1*) is a rate limiting hepatic enzyme in the gluconeogenesis pathway. When *Pck1* expression was induced with a glucocorticoid receptor agonist, dexamethasone, *Cry1*^{-/-} *Cry2*^{-/-} double knockout mice have increased expression of *Pck1* gene [122].

2.5.1. Interaction of Cryptochrome with Gluconeogenesis Pathway

Gluconeogenesis pathway is required to maintain blood glucose level by synthesizing glucose from non-carbohydrate sources during fasting [123]. The mechanism by which circadian clock regulates gluconeogenesis has been elucidated in a joint study of Montminy's group and Kay's group. In this study, overexpressed hepatic *Cry1* and *Cry2* caused a significant decrease in blood glucose levels, reduction of *Pck1* and glucose-6-phosphatase (*G6pc*) gene levels, and reduced insulin resistance in diabetic (db/db) mice. They revealed that overexpression of CRY proteins decreases gluconeogenesis by blocking the GPCR-initiated (G-protein-coupled receptor) cAMP increase. Specifically, when induced with glucagon, CRY1 inhibits the G_sα subunit of the G-protein. Gluconeogenesis regulation mechanism of cryptochrome is illustrated in Figure 2.7 [124].

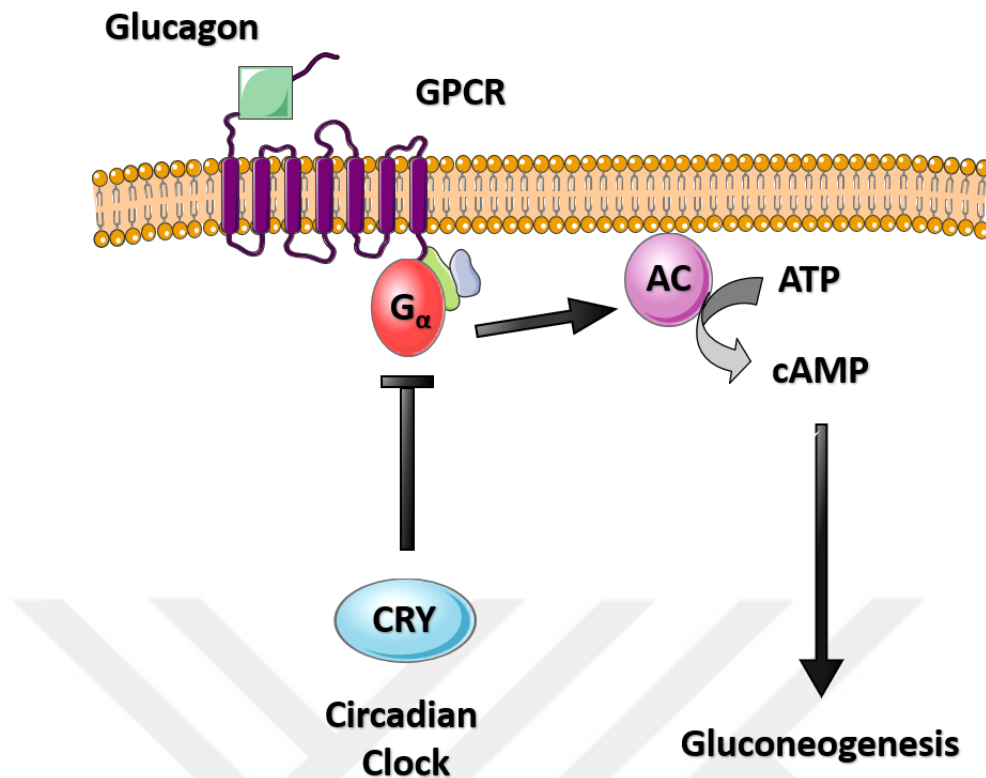


Figure 2.7. Crosstalk between the circadian clock and gluconeogenesis pathway modulated by CRY. Adapted from [124].

*This figure is created using <http://smart.servier.com/> (Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License)

2.6. Circadian Rhythm and Diseases

Many physiological processes such as metabolism, feeding behavior, sleep and wake cycles, locomotor activity and hormonal activity are linked to the circadian rhythm. Therefore, when the circadian rhythm of an organism is disrupted, there is a high probability that disease-related pathways are also affected [11,125]. The other way around is also valid. The diseases themselves can also affect the circadian rhythm [126]. As an example, several studies on obese Zucker rats reveal that circadian rhythm of activity and body temperature is dampened [127,128]. A list of changes in metabolic symptoms is given in Table 2.2 for mice with altered circadian clock genes. In the next subsections, several diseases linked to circadian clock such as sleep disorders, metabolic disorders, and cancer will be explained in detail.

Table 2.2. Changes in metabolic symptoms in mice with altered circadian clock genes

Gene	Organ / Cell Type	Change in Metabolic Phenotype
<i>Cry1</i> overexpression	Liver	Reduced gluconeogenesis, lowered blood glucose level, protected from insulin resistance [124]
<i>Cry1</i>^{-/-} <i>Cry2</i>^{-/-}	Global	Decreased body weight [129], increased cortisone levels leading to glucose intolerance [122]
<i>Per1</i>^{-/-} <i>Per2</i>^{-/-}	Global	Increased bile acid levels in serum causing hepatic cholestasis [130]
<i>Clock</i> mutant	Global	Developed metabolic syndrome with obesity, insulin resistance and higher blood glucose levels [131]
	Pancreas / β -cells	Decreased insulin secretion and weakened glucose tolerance which can lead to <i>diabetes mellitus</i> [132]
<i>Bmal1</i>^{-/-}	Global	Decreased gluconeogenesis, lower blood glucose level, attenuated blood triglyceride, and glucose rhythm [133]
	Liver	Lower fasting blood glucose level, faster glucose clearance [121]
	Pancreas / β -cells	Decreased insulin secretion and weakened glucose tolerance which can lead to <i>diabetes mellitus</i> [132]
<i>Rev-erba</i>^{-/-}	Global	Elevated serum triglycerides [134]
<i>Rev-erba</i>^{-/-} <i>Rev-erbβ</i>^{-/-}	Liver	Increased blood triglyceride and glucose levels, disturbed lipid metabolism [47]

2.6.1. Sleep Disorders

Circadian clock dysregulations cause chronic sleep-wake cycle disturbances leading to circadian rhythm sleep disorders (CRSDs). CRSDs caused by an alteration of the endogenous circadian clock are delayed sleep phase disorder (DSPD), advanced sleep phase disorder (ASPD) irregular sleep-wake rhythm (ISWR), and non-24-h sleep-wake disorder [125,135]. Delayed sleep phase disorder patients suffer from insomnia and their sleep-wake timings are delayed more than 2 hours recurrently every day [136]. Rare familial cases of DSPD have been identified and associated with variable-number tandem-repeat polymorphism (VNTR) in the *PER3* gene [137]. In advanced sleep phase disorder, the sleep-wake timings are shifted 3-4 hours earlier. Familial advanced sleep phase syndrome (FASPS)

results from S662G mutation in *hPER2* found in the CK1 ϵ binding region [138] or from T44A point mutation in CK1 δ causing loss of function [139].

Another cause for circadian rhythm sleep disorders is the change in environmental and social factors while the endogenous clock is unaffected. The main factors are shift-work and jetlag [136]. For frequent travelers, jetlag can cause and chronic sleep, mood and appetite disturbances [140]. Shift-workers are subject to sleep disturbances that may impair the circadian clock [141]. Studies indicate that night shift-workers have an increased risk of developing cardiovascular diseases [142,143], metabolic diseases including metabolic syndrome [144,145], obesity [146], diabetes [147] and some types of cancer such as breast cancer [148,149] and endometrial cancer [150,151].

2.6.2. Metabolic Syndrome, Obesity and Type 2 *Diabetes Mellitus*

There is a strong relationship between circadian clock and metabolism which helps balance the tissue homeostasis and physiological processes [106,152,153]. In liver, around 50% of metabolites mostly consisting of nucleotide and amino acid metabolism display circadian patterns [154]. A study revealed that the circadian regulation of glucose and insulin levels were independent of the feeding patterns, which indicates that SCN plays an important role in the control of glucose metabolism [155].

Metabolic syndrome is characterized by several characteristics such as obesity, high circulation glucose, and high cholesterol levels. Clock mutant mice with loss of function mutation are obese with dampening in diurnal feeding rhythm and eventually develop metabolic syndrome [131]. Another study on pancreatic β -cells concluded that both Clock mutant and Bmal1 knockout mice have decreased insulin secretion and weakened glucose tolerance which can lead to *diabetes mellitus* [132].

Type 2 *diabetes mellitus* is the most common type of diabetes. Patients have insulin deficiency, and insulin resistance, but the insulin secretion is not totally impaired [156]. Treatment strategies for this disease include lowering blood glucose level with medication and low glucose diet [157]. In humans, a genetic association study found a link between BMAL1 variants and susceptibility to type 2 diabetes [158]. In 2010, a study revealed that hepatic *Cry1* overexpression caused a significant decrease in blood glucose level and reduced insulin resistance in mice [124]. Another study concluded that *Cry1*^{-/-} *Cry2*^{-/-} double knockout mice develop glucose intolerance and have increased fasting glucose levels in blood [122].

2.6.3. Cancer

In studies of night shift workers, it has been shown that circadian rhythm disruption can make humans more susceptible to some types of cancer: breast cancer [148,149] and endometrial cancer [150,151]. Epidemiological studies have shown that breast cancer risk is increased for women with *Per3* [159] and *Npas2* [160] gene variants. Another study performed on Chinese population found that some *CRY2* SNPs are associated with prostate cancer and insulin resistance [161]. Interactions between the circadian clock and DNA damage response mechanisms such as DNA repair and DNA damage checkpoints can be one of the underlying reason for susceptibility to cancer [162,163]. Interestingly, nucleotide excision repair is under the control of circadian clock in mammals through DNA damage recognition and repair factor XPA protein [164,165].

Many core clock proteins have been related to cancer [166,167]. A study revealed that PER1 interacts with ATM and Chk2 which are DNA damage checkpoint proteins. Moreover, human cancer cells were sensitized to DNA damage-induced apoptosis when Per1 was overexpressed [168]. Another study showing that *Per2* mutant mice have enhanced tumor growth suggests that *Per2* gene also has a role in tumor suppression through DNA damage response mechanisms [169]. In mice, *Bmal1* knockdown increased the tumor growth and cell proliferation while decreasing the expression of *Wee1* and *p53* genes which play a role in apoptosis [170]. It has been shown that BMAL1 deregulates C-myc proto-oncogene [169] and both ROR α and REV-ERB β control N-myc proto-oncogene [171].

Interestingly, *Cry* mutations do not predispose mice to cancer [163]. Contrary to expectations, *p53*-null mutant mice that are prone to cancer are protected from the early onset of cancer by *Cry* mutations. These *p53*^{-/-} *Cry1*^{-/-} *Cry2*^{-/-} triple knockout mice have significantly increased lifespan of 28.1 weeks compared to only *p53*^{-/-} knockout mice (18.6 weeks) [172]. A study following these findings revealed that (TNF α)-initiated apoptosis is favored in *Cry* double knockout and *p53* mutant cells. GSK3 β , a kinase that functions in phosphorylation of core clock proteins, interferes with NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway which leads to apoptosis [173].

2.7. Small Molecule Modifiers of Mammalian Circadian Clock

Circadian clock mechanism interacts with important physiological and behavioral processes such as metabolism, hormonal regulation, locomotor activity, and sleep-wake cycle. It has been shown that impaired circadian rhythm can lead to various diseases including sleep disorders, glucose metabolism disorders, cardiovascular diseases, and cancer. Correcting the impairments of the circadian rhythm can be a strategy for the treatment and prevention of these diseases. In recent years, many studies focused on the discovery of small molecules interacting with circadian clock proteins for the treatment of these diseases [174,175].

2.7.1. Screening Methodologies

Small molecules are chemical compounds that have low molecular weight; hence, they can easily diffuse across the cell membrane and target proteins inside the cell. Various methods are used to identify small molecules interacting with the target protein. Mainly, These methods are mainly high-throughput chemical library screens grouped as unbiased screens and target-based screens [174].

Unbiased screens were performed based on circadian phenotyping and/or mechanisms such as transactivation activity of BMAL1:CLOCK. Circadian phenotyping method consists of monitoring circadian genes with reporters [174]. Most commonly used cell-based assays utilize U2OS cells and NIH3T3 cell lines stably expressing luciferase fused with *Bmal1* [176,177] and *Per2* promoter genes, respectively [178,179,180]. This method begins with synchronization of cells with dexamethasone or forskolin and the rhythmic expression of the bioluminescent genes are recorded for several days. Then, cells are treated with molecules and then molecules that effect the period, amplitude and/or phase are selected for further characterization. Another method is the mechanism-based screen which is again a reporter-based assay. Small molecules have been screened for their effect on BMAL1:CLOCK transactivation on *Per1-Luc* construct [181,182].

Target-based screens can focus on protein modifications, protein-protein interactions and ligand binding of a specific protein. For circadian clock proteins, phosphorylation is vital for protein stability and the robustness of the rhythm. Small molecules that are already known kinase inhibitors have been screened for their effect on CK1 and GSK-3 β [183]. The studies also focused on targeting the nuclear receptors RORs and REV-ERBs since they regulate transcriptional activation of *Bmal1* and various genes functioning in cell metabolism,

proliferation, and inflammation. Small molecules that are already known nuclear receptor ligands have been screened for their effect on ROR α/γ [184] and REV-ERB α [185,186].

Structure-based virtual screening is an unexploited area for the identification of small molecules modifying circadian clock. Virtual screening is performed using molecular docking software programs in order to calculate the binding affinity of many different small molecules to a druggable binding sites of a protein target. 3D structure of many core clock proteins including CRYs, PERs, BMAL1, and CLOCK are already available on Protein Data Bank [187]. Binding interfaces of these structures can be used as a target binding pocket. A specific example would be targeting FBXL3 and FBXL21 binding pocket on CRYs for the discovery of small molecules stabilizing or destabilizing CRYs. The candidate molecules with the highest binding affinity from the *in silico* screening can be evaluated further using *in vitro* and *in vivo* assays [188].

2.7.2. Modifiers of CRYs

Cryptochrome proteins found in the negative arm of the circadian clock mechanism have been associated with cancer [161] and glucose metabolism disorders [122]. Moreover, CRYs interact with (TNF α)-initiated apoptosis [173] and gluconeogenesis pathway [124] (refer to Section 2.6). In light of these findings, several studies focused on discovering small molecule modifiers of CRYs.

Cryptochrome stabilizers KL001 and its derivatives have been discovered by Steve Kay and his group [180,189]. KL001 is a carbazole derivative that interacts with CRY by binding to its FBXL3 interaction site at the FAD-binding pocket [190]. This interaction reduces the ubiquitination of CRY by FBXL3 and its proteasomal degradation, hence KL001 stabilizes the CRY proteins. It was observed that these CRY stabilizers increase the period of the circadian rhythm. Moreover, the treatment of primary hepatocytes with KL001 leads to the inhibition of glucagon-mediated gluconeogenesis. Therefore, KL001 can be utilized in the treatment of type 2 diabetes [180].

Small molecule KS15 is a derivative of 2-ethoxypropanoic acid and it has been identified as an inhibitor of repressor activity of CRY proteins. KS15 reduces the binding of CRYs on BMAL1:CLOCK transcriptional activator complex, therefore repression of E-box mediated transcription is impaired. Treatment of cells with KS15 decreased the amplitude of circadian rhythm significantly while having no effect on the period [182,191]. Further studies

revealed that KS15 has anti-proliferative effects and anti-tumor activity on human breast cancer MCF7 cell line [192].

2.7.3. Other Modifiers

In many of the high-throughput chemical screening studies of circadian clock, CK1 inhibitors have been observed to alter the period of the circadian clock most drastically. When CK1 is inhibited, the PER protein turnover slows down and translocation of CRY:PER complex is impaired which leads to the lengthening of the period [89,90,175]. Some of these CK1 ϵ/γ inhibitors that lengthen the period are IC261 [89], CKI-7 [193], CK01 [194], TG003, PF-5006739, and Cmpd-1,2,3 [179]. TG003 has the most drastic effect on the period since it increases the period of U2OS *hPer2-Luc* cells to 48 hours at 25 μ M [195]. A study focusing on therapeutic effects of PF-5006739 revealed that this small molecule improves glucose tolerance in two diabetic mouse models of obesity which are diet induced obesity (DIO) and leptin-deficient genetic obesity (ob/ob) models. Hence, these CK1 inhibitors can be therapeutics of glucose metabolism diseases type 2 diabetes and obesity [196].

Several selective GSK-3 β inhibitors have been discovered and these inhibitors shorten the period of the circadian clock. GSK-3 β phosphorylates core clock proteins CRY2 and PER2 to modulate their proteasomal degradation and localization. GSK-3 β inhibitors with period shortening effects are SB216763 [197], Indirubin-3'-oxime, and Kenpaullone [176]. These small molecules have effects on cancer pathways since GSK-3 β has a role in cancer progression; however, their therapeutic potential through circadian clock has not been explored yet [198].

Moreover, there are several amplitude altering small molecules that enhance or dampen the amplitude of the circadian rhythm. Four compounds identified Cmpd-8, 9, 10 and 11 enhance the amplitude and decrease the period, but their mechanism of action is not known yet [179]. Another small molecule Nobiletin, a polymethoxylated flavonoid, which is a natural compound also has amplitude enhancement and period shortening effects. Nobiletin directly targets RORs and has shown therapeutic effects for the treatment of metabolic syndrome in diabetic mice models [199]. Inversely, small molecules targeting REV-ERBs have been observed to dampen the amplitude. SR9011 and GSK4112 are REV-ERB agonists that decrease the amplitude of the circadian rhythm. In liver cells, GSK4112 reduced glucose levels and the expression of gluconeogenic genes *Pck1* and *G6pc* [200]. Treatment of obese (DIO) mice with SR9001 led to decreased fat mass and improved hyperglycemia [201].

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Culture

HEK293T, U2OS, NIH3T3 and HepG2 cell lines were used for different experiments. The cells were maintained in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 100 µg/ml streptomycin and 100 µg/ml penicillin (Gibco). The cell cultures were grown in an 85% humidified incubator at 37°C with 5% CO₂. To subculture the cells when they reach confluency, first the medium was removed, and the cells were washed with 5mL PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). Then, cells were detached by adding 1mL Trypsin/EDTA (0.25%:0.02%, PAN Biotech) and resuspended with 4 mL fresh medium. Finally, in a new 10cm plate (NEST Biotechnology), 1 mL of the resuspended cells were mixed with 9 mL fresh medium.

3.2. Cell Viability Assessment (MTT Assay)

For determining the non-toxic doses of small molecules, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, AppliChem) cell viability assay was employed. This assessment was performed for both U2OS and HEK293T cells. The cells were seeded in a 96-well plate at a density of 4×10^3 cells per well. After 48 hours of incubation, small molecules dissolved in dimethyl sulfoxide (DMSO) were added in 3 different doses: 2.5 µM, 10 µM, 20 µM. Another 48 hours later, the medium was removed and 80 µL DMEM mixed with 20 µL MTT solution (final concentration: 1 mg/mL) was added to each well. The cells were incubated for 4 h in a humidified incubator at 37°C with 5% CO₂. Then, the solution was vacuumed and the formazan crystals that formed on the bottom of the plate were dissolved in 1:1 mixture of DMSO and ethanol. Using Synergy H1 Microplate Reader (Bio-Tek Instruments), the absorbance values (A) were measured at 600nm and 690nm which is the reference wavelength. The cell viability was calculated using Equation 3.1.

$$\% \text{ Cell Viability} = \left[\frac{(A_{600nm} - A_{690nm})_{\text{sample}}}{(A_{600nm} - A_{690nm})_{\text{DMSO}}} \right] \times 100 \quad (3.1)$$

3.3. Protein Degradation Assay

Protein degradation assay was performed with transiently transfected HEK293T cells. Reverse transfection was performed with different plasmids depending on the experiment: 40 ng of C-terminally luciferase-fused CRY1 (CRY1-LUC in pcDNA), 10ng of CRY2-LUC or 4ng luciferase (LUC) plasmid. For site directed mutagenesis experiments, we transfected 40 ng CRY1-LUC and 40 ng CRY1-LUC with W399L mutation (CRY1-W399L-LUC in pcDNA). Using 6 μ L polyethyleneimine (PEI), HEK293T cells were transfected at a density of 4 $\times$ 10⁴ cells per well on a 96 well white flat bottom plates (Corning Costar). After 24 hours, small molecules (final 0.8% DMSO) were added at doses indicated in Figure 4.4, 4.5, 4.6, 4.13, and A.1. After another 24 hours, HEPES-NaOH (final concentration: 10 mM, pH 7.2) and luciferin (final concentration: 1 mM) was supplemented to the medium. In order to stop protein synthesis, cycloheximide was added with a final concentration of 30 μ g/ml. Immediately, the plate was sealed and placed in Synergy H1 Microplate Reader (Bio-Tek Instruments) and every 20 min, the luminescence was measured for 24 hours. Using GraphPad Prism 5 software, the half-life was calculated from fitting the data to one phase exponential decay curve. In dose-response curves, using GraphPad Prism 5 sigmoidal curve fitting was applied to calculate EC50 (half maximal effective concentration) and R² (coefficient of determination).

3.4. Cell Based Circadian Assay

Oscillations in circadian genes can be observed using stable cell lines harboring luciferase reporter. U2OS *Bmal1-dLuc* cells were seeded on 35-mm dishes at a density of 3.5 $\times$ 10⁵ cells/plate to monitor circadian rhythm of *Bmal1*. NIH3T3 *mPer1-dLuc* cells were seeded at a density of 4 $\times$ 10⁵ cells/plate to monitor circadian rhythm of *mPer1*. When cells reached 80-90% confluency (after ~24 hours), the complete medium was replaced with growth medium (DMEM, 10% FBS, 100 μ g/ml penicillin-streptomycin) containing 100 nM dexamethasone. For synchronization, the cells were incubated with dexamethasone for 2 hours. Then, the medium was replaced with Lumicycle medium supplemented with freshly

added 0.1mM luciferin. Lumicycle medium without phenol red was prepared from DMEM powder (Sigma D-2902), 3.5 gr D(+) glucose powder (cell culture grade, Sigma), 0.35 gr sodium bicarbonate (cell culture grade, Sigma), 50 mL FBS (final 5%), 10 mL HEPES buffer (final 10 mM), 10 mL penicillin-streptomycin (final 100 µg/ml) completed up to 1 L with sterile milliQ water. The small molecules (final 0.4% DMSO) were added at doses indicated in Figure 4.7, 4.8, 4.9, and A.2. Plates were placed in a luminometer at 37°C (LumiCycle 32, Actimetrics) where the bioluminescence was measured every 10 minutes for 7 days. Data analysis was performed with LumiCycle Analysis software (Actimetrics).

3.5. SDS-PAGE and Western Blotting

U2OS cells were seeded on 35mm plates with a density of 3.5×10^5 cells/plate and incubated at 37°C with 5% CO₂. After 24 hours, small molecules (final 0.4% DMSO) were added at various doses. The cells were incubated for another 24 hours, and then washed and harvested with ice-cold PBS using a cell scraper (Nest Scientific). Harvested cells were centrifuged at 3000 x g, 4°C for 7 minutes and then supernatant was removed. For cell lysis, RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.5% SDS) is prepared with freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1X Protease Inhibitor Cocktail (PIC, Thermo Scientific). While keeping the cells on ice, 60 µL RIPA buffer was added to cell pellets and samples with buffer were pipetted up and down every 5 minutes for 20 minutes. Then, the lysed cells were centrifuged at 13,000 x g, 4°C for 15 minutes to eliminate cell debris. The supernatants were collected, and total protein concentration of the samples were measured using Pierce Protein Assay (Thermo Scientific). The protein samples were mixed with 16.7µL 4X Laemmli Buffer (0.1% bromophenol blue, 8% SDS, 400 mM β-mercaptoethanol, 40% glycerol). Then, the samples were incubated in 95°C for 10 minutes and finally put on ice.

For quantification of proteins, the proteins were separated according to their molecular weight using SDS-PAGE gel electrophoresis and then detected by Western blotting with corresponding antibodies. The samples were loaded to 10% SDS-PAGE gel. After the gel was run at 100 mV for 90 minutes, the proteins in the gel were transferred to polyvinylidene difluoride membrane (0.45 µm pore size Hydrophobic PVDF Transfer Membrane, Merck-Millipore) by electrophoresis. The membranes were blocked by incubation with 5% fat free milk powder in TBS-0.015% Tween 20 solution for 1 hour with gentle shaking at room temperature. Following this step, the membranes were incubated with primary antibody for

18 hours with gentle shaking at 4°C and consecutively incubated with secondary antibody for 1 hour with gentle shaking at room temperature. The primary antibodies used for detection of proteins are: Anti-CRY1 (Bethyl), Anti-CRY2 (Bethyl), Anti-PER2 (Bethyl), and Anti- β -ACTIN (Cell Signaling). Anti-rabbit-HRP antibody (Cell Signaling) was used for CRY1, CRY2, and PER2 as the secondary antibody. Anti-mouse-HRP antibody (SantaCruz) was used for β -ACTIN as the secondary antibody. Finally, the proteins were visualized with ECL (Enhanced Chemiluminescence) HRP substrate (Advansta) using ChemiDoc Imaging System (Bio-Rad).

3.6. RNA Isolation and cDNA Preparation

To investigate the effect of small molecules on expression levels of circadian genes, we utilized U2OS cell line whereas, for genes involved in gluconeogenesis we used HepG2 cell line. U2OS cells were harvested using the methodology described in Section 3.5. HepG2 cells were treated and harvested as described in Section 3.8. Total RNA isolation from the samples was performed with the NucleoSpin RNA kit (Macherey-Nagel) including DNase treatment. RNA is eluted with 50 μ L DNase RNase free water. Using NanoDrop 2000 (Thermo Scientific) the approximate concentration and purity of RNA was measured. The integrity of the eluted RNA was confirmed by agarose gel electrophoresis where 1% agarose gel in prepared using DEPC (diethylpyrocarbonate) treated water.

First step of cDNA synthesis is denaturation and annealing of template RNA and oligo(dT)₂₃VN (New England Biolabs). For this purpose, a mixture of 500 ng of RNA, 2 μ L of oligo(dT)₂₃, 1 μ L of 10 mM dNTP mix was completed to 10 μ L with DNase RNase free water. The mixture was incubated at 65°C for 5 minutes and consecutively placed on ice (+4°C). The second step is the extension of cDNA where 2 μ L from 10X M-MuLV buffer (New England Biolabs), 1 μ L from M-MuLV Reverse Transcriptase (200 U/ μ L), 0.2 μ L from RNase Inhibitor (40 U/ μ L) and 6.8 μ L DNase RNase free water were added into the RNA-oligo(dT) mixture in the tube. The samples were kept at 42°C for 1 hour for extension reaction and then at 65°C for 20 minutes for inactivation of the enzyme. Produced cDNA samples were diluted 1:5 with DNase and RNase free water for storage at -20°C and serially diluted 1:25 to be used in quantitative PCR reactions.

3.7. Real Time – Polymerase Chain Reaction (RT-PCR)

Real time – quantitative PCR method was implemented for detecting the effect of small molecules on expression levels of core clock genes and gluconeogenic genes. Primer sequences are listed in Appendix B. The reference gene used in this project was ribosomal protein, large, P0 (*Rplp0*) [202]. The reaction mixture consisted of 8 μ L SYBR Green (Bioline), 8 μ L DNase free water, 1 μ L primer mixture (forward and reverse primers: 10pmol each), and 3 μ L cDNA obtained as described in Section 3.6. After 4 minutes of polymerase activation at 95°C, the cycling protocol is 8 seconds at 95°C (denaturation), 10 seconds at 60°C (annealing), 20 seconds at 72°C (elongation) using CFX Connect Real-Time PCR detection system (Bio-Rad). The product of qPCR was also analyzed by melt curve (T_m) and agarose gel imaging for checking the quality.

3.8. Gluconeogenesis Experiments

The effect of small molecules on glucagon-induced gluconeogenesis was determined by comparing the expression of gluconeogenic genes using RT-qPCR and the glucose levels performing glucose production assay. HepG2 cells were seeded on 12-well plates with a density of 2×10^5 cells/well in low glucose (1000 mg/L) DMEM with 10% FBS, 100 μ g/ml streptomycin and 100 μ g/ml penicillin. The medium was supplemented with 2 μ g/ml insulin and 100nM dexamethasone. Cells were incubated for 3 hours, then the medium was exchanged with low glucose DMEM without FBS. Subsequently, the small molecules (final 0.4% DMSO) were added at desired concentrations. After 18 hours, the cells were treated with 10nM glucagon. Cells used in RT-qPCR analysis were harvested after 2 hours with ice-cold PBS using a cell scraper. After 3 hours, the cells used in glucose production assay were washed with warm glucose-free Krebs-Ringer bicarbonate buffer (118.5 mM NaCl, 4.74 mM KCl, 23.4 mM NaHCO_3 , 1.18 mM KH_2PO_4 , 1.18 mM MgSO_2 , 2.5 mM CaCl_2 , and 25 mM HEPES – pH 7.6) containing 1% BSA, 100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 0.29 mg/ml L-glutamine. Then, the cells were incubated in this buffer additionally containing 2 mM sodium pyruvate 20 mM sodium lactate for 4 hours. After the collection of the buffer, the glucose levels were measured using Glucose and Sucrose Assay Kit (Sigma, MAK013).

3.9. Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5 software. In the comparison of a single dose of the small molecule with control (DMSO), Student's t-test was

employed to calculate significance (p-value). For dose-dependent experiments, as a group all doses were compared, and p-value was calculated by employing one-way ANOVA (analysis of variance) followed by Dunnett's multiple comparisons test. Dunnett's multiple comparisons test compares each dose with the control (DMSO) separately in a pair-wise manner.



CHAPTER 4

RESULTS

4.1. Cell Viability Assessment

The stabilization and degradation mechanisms of CRY by ubiquitination is an important factor affecting the pace of the mammalian circadian clock. Therefore, we focused on CRY and FBXL interaction sites for the discovery of small molecules stabilizing CRYs. Small molecules with highest binding energies were identified by molecular docking studies on the CRY1/2 and FBXL3/21 interaction sites. This part of the study was performed by Şeref Gül in Kavaklı Laboratory. From the *in silico* screening, he selected top 32 small molecules to be tested. The toxicity of these top 32 molecules were evaluated on cells prior to *in vitro* screening. These small molecules were coded as TW38-70. We performed the cell viability assessment on U2OS cells using MTT assay for three different doses of small molecules: 2.5 μ M, 5 μ M, 20 μ M. Small molecules were dissolved in dimethyl sulfoxide (DMSO) which was used as a control in the experiment. The results of the cell viability assessment for 32 molecules is given in Figure 4.1.

For further testing, the small molecule doses yielding more than 90% cell viability and with the highest possible dose were selected. Small molecules yielding less than 90% cell viability were eliminated because they would be toxic to the cells and reduce the reliability of the following *in vitro* experiments. The highest possible dose is selected in order to prevent elimination of a small molecules having the desired effect at higher doses.

Table 4.1 shows the non-toxic small molecules with their selected doses. These selected doses were used in the subsequent *in vitro* experiments.

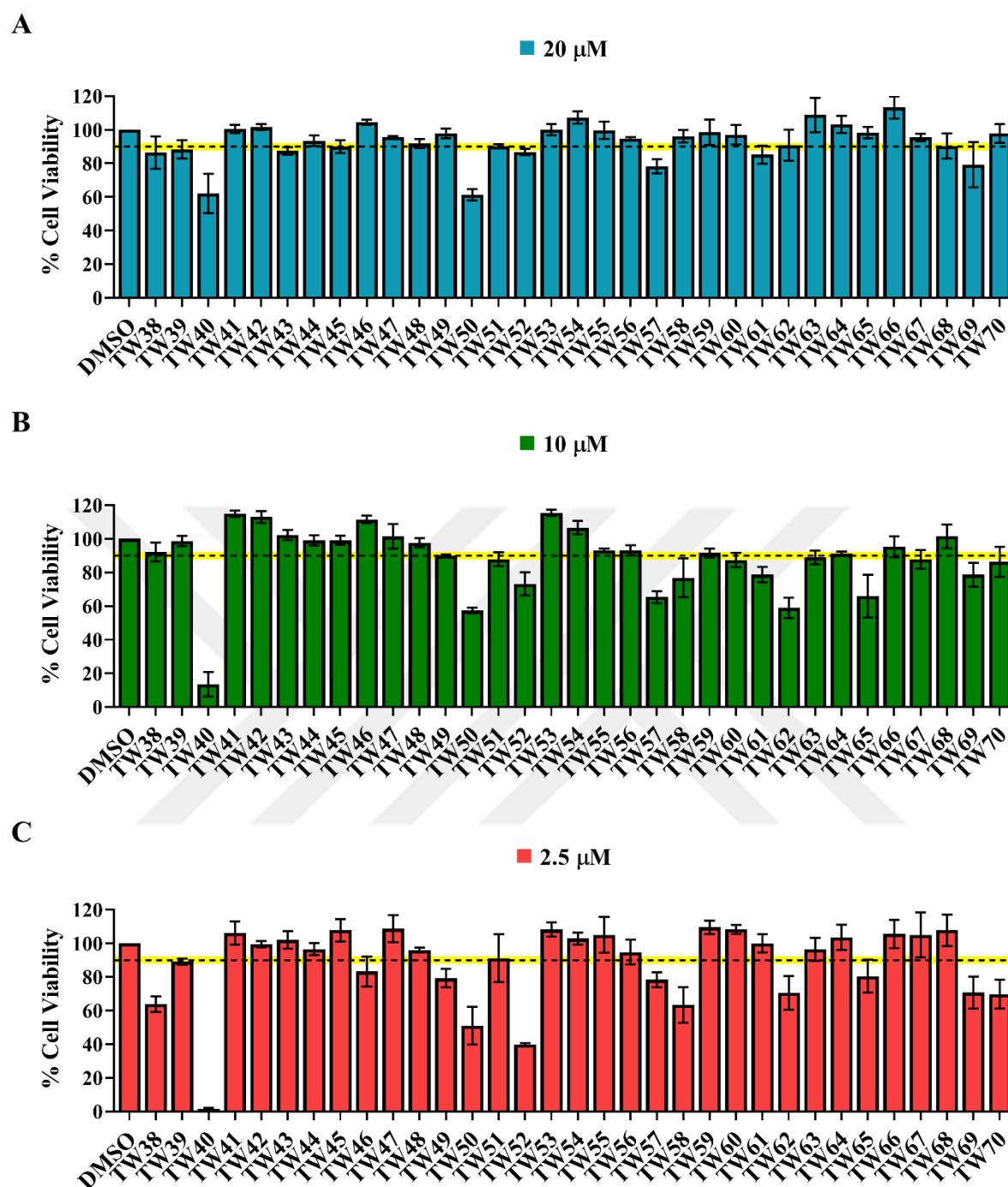


Figure 4.1. Cell viability assessment of HEK293T cells treated with small molecules TW38-70 at doses of **A)** 20 μ M **B)** 10 μ M, and **C)** 2.5 μ M. Dashed line indicates 90% cell viability. Data are given as mean $\pm$ SEM from at least 3 individual experiments.

Table 4.1. Non-toxic doses of small molecules TW38-70.

Small Molecule	Dose (μ M)	Small Molecule	Dose (μ M)
TW38	10	TW55	20
TW39	10	TW56	20
TW40	Toxic	TW57	Toxic
TW41	20	TW58	2.5
TW42	20	TW59	20
TW43	20	TW60	20
TW44	20	TW61	20
TW45	20	TW62	Toxic
TW46	10	TW63	20
TW47	20	TW64	20
TW48	20	TW65	20
TW49	10	TW66	20
TW50	Toxic	TW67	20
TW51	20	TW68	20
TW52	Toxic	TW69	2.5
TW53	20	TW70	2.5
TW54	20		

4.2. Screening of Small Molecules using CRY Half-Life

Stability of a protein can be quantified by measuring its half-life using protein degradation assay. In line with our aim of identifying small molecules stabilizing CRY1/2, we performed this assay to screen for molecules increasing the half-life of CRY1/2. For this purpose, HEK293T cells were transfected with either CRY1-LUC, CRY2-LUC or only LUC plasmid. Then, cells were treated with cycloheximide which inhibits protein synthesis [203] and luciferase readings were recorded for 24 hours. Some small molecules showed a

significant effect on LUC half-life; hence, they were not investigated further (Figure 4.2). Among 20 small molecules screened which do not effect LUC half-life, TW60 and TW68 stabilized both CRY1 and CRY2 significantly. TW63 specifically increased the half-life of CRY1 while showing no effect on CRY2 and LUC half-life (Figure 4.3).

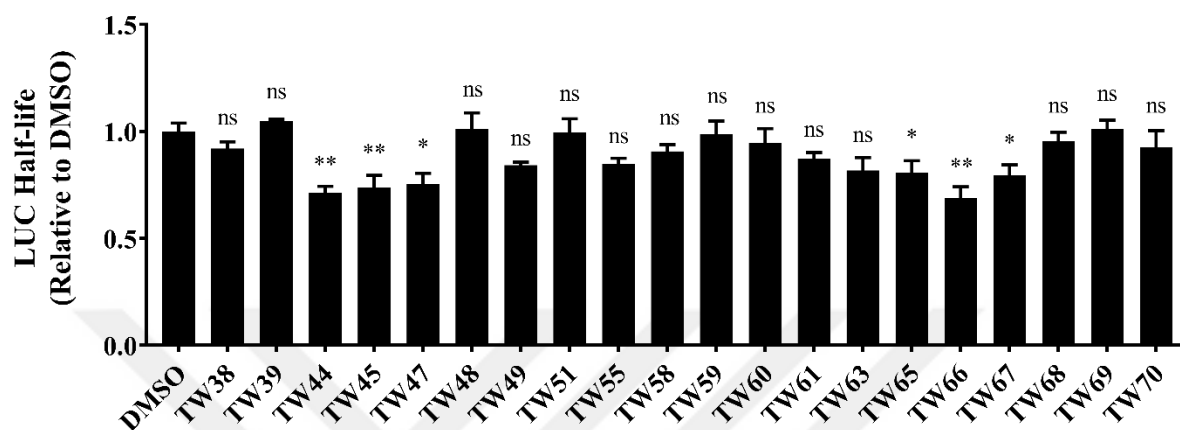


Figure 4.2. Effect of small molecules on LUC half-life relative to DMSO. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant).

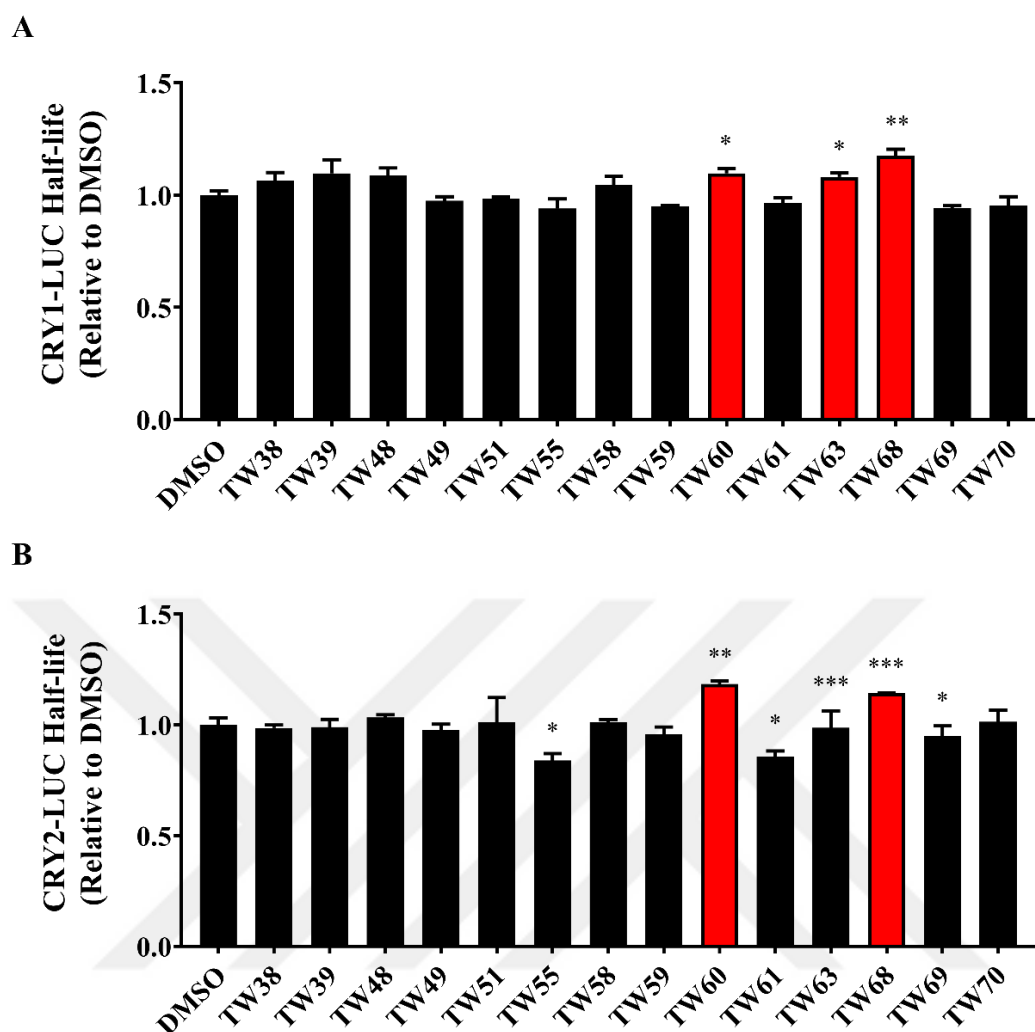


Figure 4.3. Effect of small molecules on **A)** CRY1-LUC and **B)** CRY2-LUC half-life relative to DMSO. Small molecule stabilizer candidates are shown in red. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, all others are non-significant).

4.3. Effect of TW63 and TW68 on CRY Half-Life

Small molecules enhancing CRY half-life were further investigated for their dose-dependent effects. When a small molecule binds to the protein target, it should show an effect that increases consistently with increasing dose and finally reach a saturation point where all the binding sites of the proteins are occupied with the molecule [204]. We performed CRY1-LUC degradation assay for TW60, TW63, and TW68 in a dose dependent manner. TW60 lacked dose-dependent activity (Figure A.1 in Appendix A). Both TW63 and TW68 significantly increased the CRY1-LUC half-life dose-dependently (Figure 4.4).

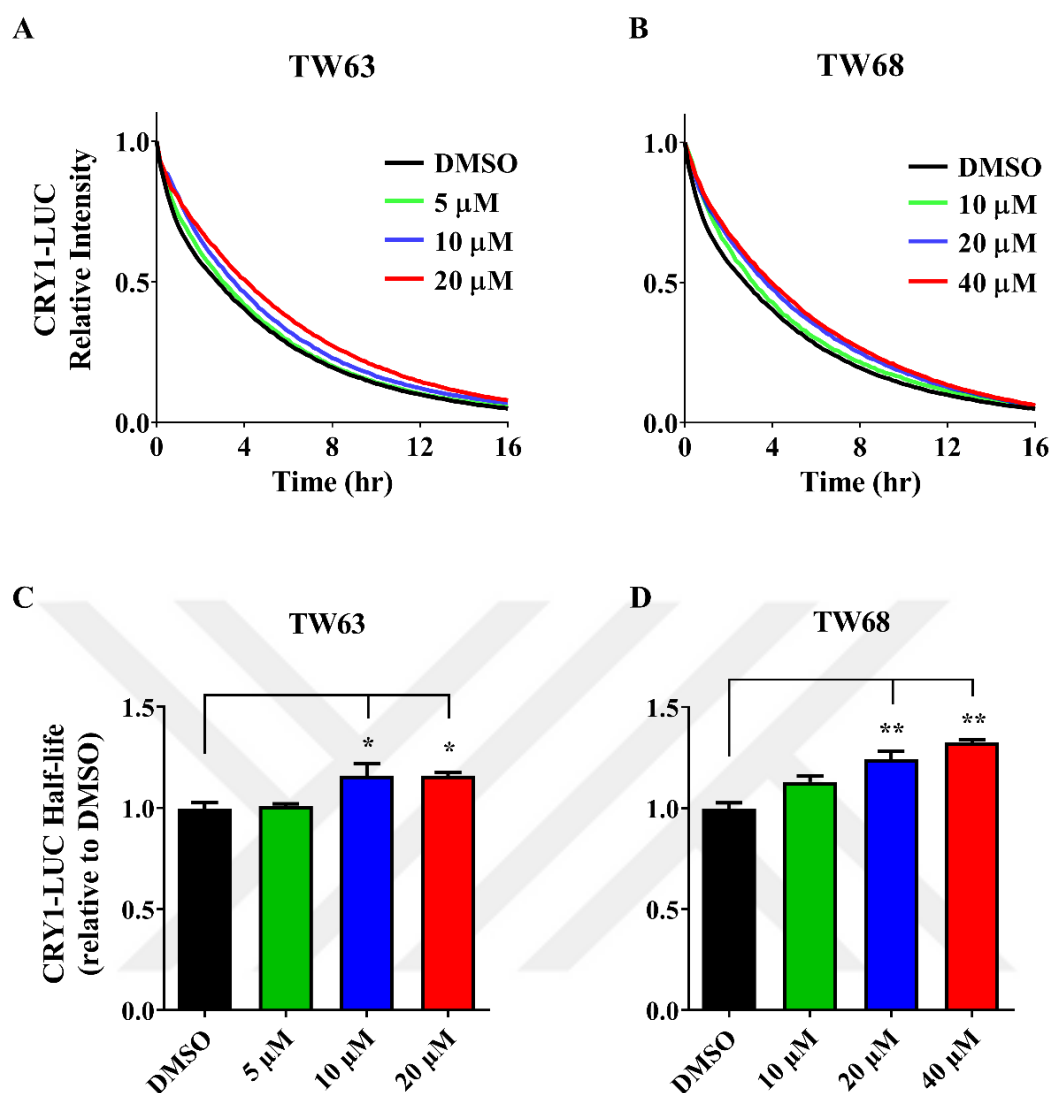


Figure 4.4. Dose-dependent effects of TW63 and TW68 on CRY1-LUC stability (A and B) and half-life relative to DMSO (C and D). Data are given as mean $\pm$ SEM from at least 4 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test (* p <0.05, ** p <0.01, all others are non-significant).

Next, CRY2-LUC degradation assay was performed for TW60, TW63, and TW68. TW60 lacked dose-dependent activity (Figure A.1 in Appendix A). In agreement with the data in Figure 4.3B, TW63 did have dose-dependent effect on CRY2-LUC half-life. TW68 increased CRY2-LUC half-life significantly in a dose-dependent manner as shown in Figure 4.5. It can be concluded from Figure 4.4 and 4.5 that TW60 does not show any dose-dependent effect on CRYs half-lives, TW63 has a specific and dose-dependent effect on CRY1-LUC half-life by enhancing it, and TW68 increases both CRY1-LUC and CRY2-LUC half-life dose-dependently.

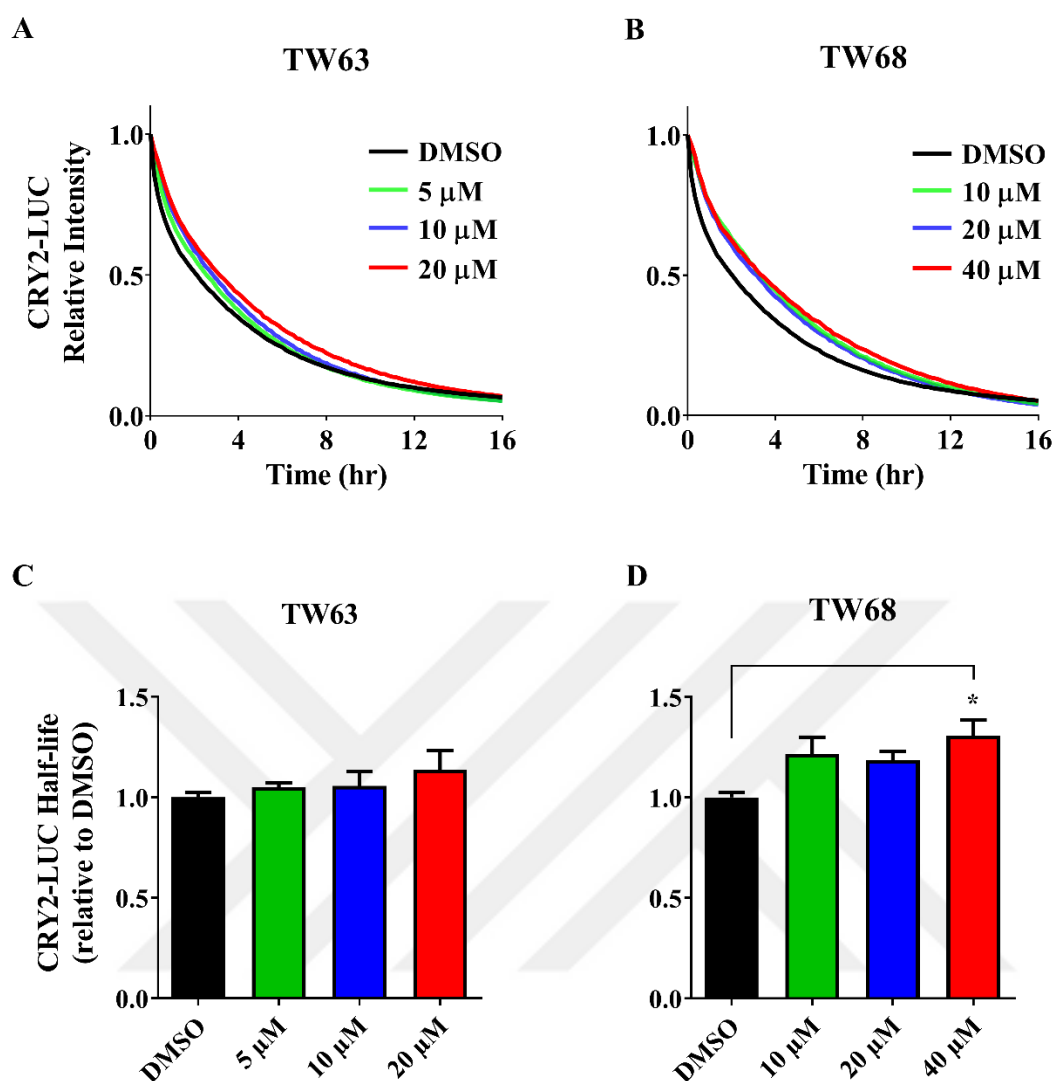


Figure 4.5. Dose-dependent effects of TW63 and TW68 on CRY1-LUC stability (A and B) and half-life relative to DMSO (C and D). Data are given as mean $\pm$ SEM from at least 4 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test (* $p < 0.05$, all others are non-significant).

In drug discovery studies, the dose of the small molecule that shows the half of the maximum effect is named EC_{50} (half maximal effective concentration). This dose can be calculated by plotting dose-response curves [205,206]. Small molecules were tested at 0, 2.5, 5, 10, 20, 40 and 80 μ M for their effect on CRY1-LUC half-life. Dose-response curves plotted for TW63 and TW68 are given in Figure 4.6. EC_{50} value for TW63 was determined as ~ 15 μ M, so in the following experiments we used 7.5 μ M, 15 μ M, 30 μ M doses for TW63.

EC₅₀ value for TW68 was determined as ~10 μ M, hence in the following experiments we used 10 μ M, 20 μ M, 40 μ M doses for TW63.

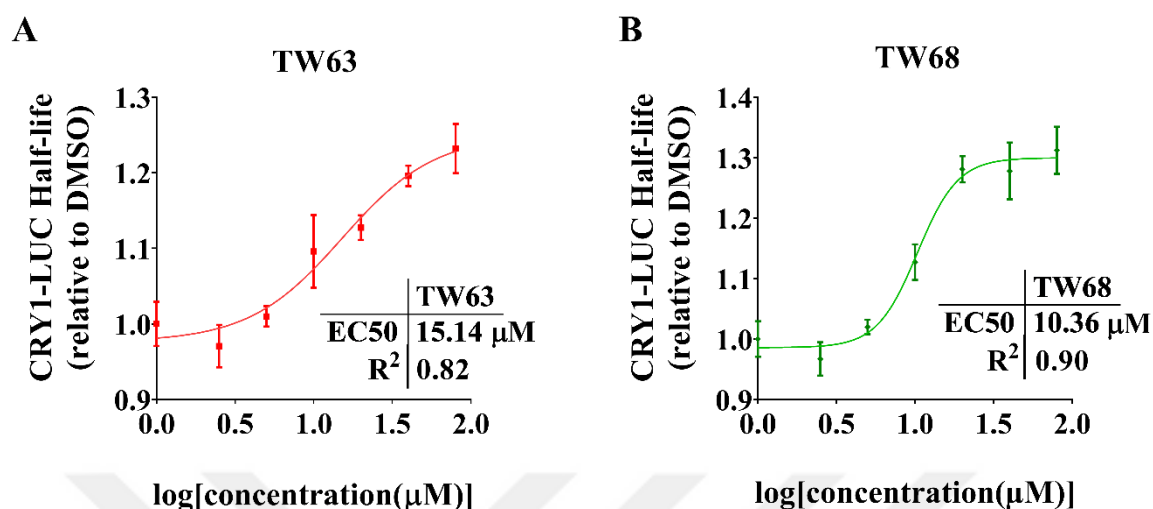


Figure 4.6. Dose-response curves of **A)** TW63 and **B)** TW68 on HEK293T cells. Half maximal effective concentration dose (EC₅₀) and the coefficient of determination (R²) values are given on the corresponding plot. Data are given as mean $\pm$ SEM from at least 4 individual experiments.

4.4. Effect of TW63 and TW68 on Circadian Rhythm

We performed cell based circadian assay in order to determine the effects of small molecules TW63 and TW68 on circadian rhythm. Bioluminescence rhythms of U2OS *Bmal1-dLuc* cells were recorded and analyzed. The period and the amplitude of the circadian rhythm was calculated from data fitted to 2nd order polynomial. Small molecule TW60 did not affect the period and amplitude of the rhythm in U2OS *Bmal1-dLuc* cells (Figure A.2 in Appendix A). Due to its inability to stabilize CRY dose-dependently and to effect the circadian rhythm, TW60 was not investigated further. On the other hand, TW63 treatment of U2OS *Bmal1-dLuc* cells significantly lengthened the period and reduced the amplitude dose-dependently (Figure 4.7).

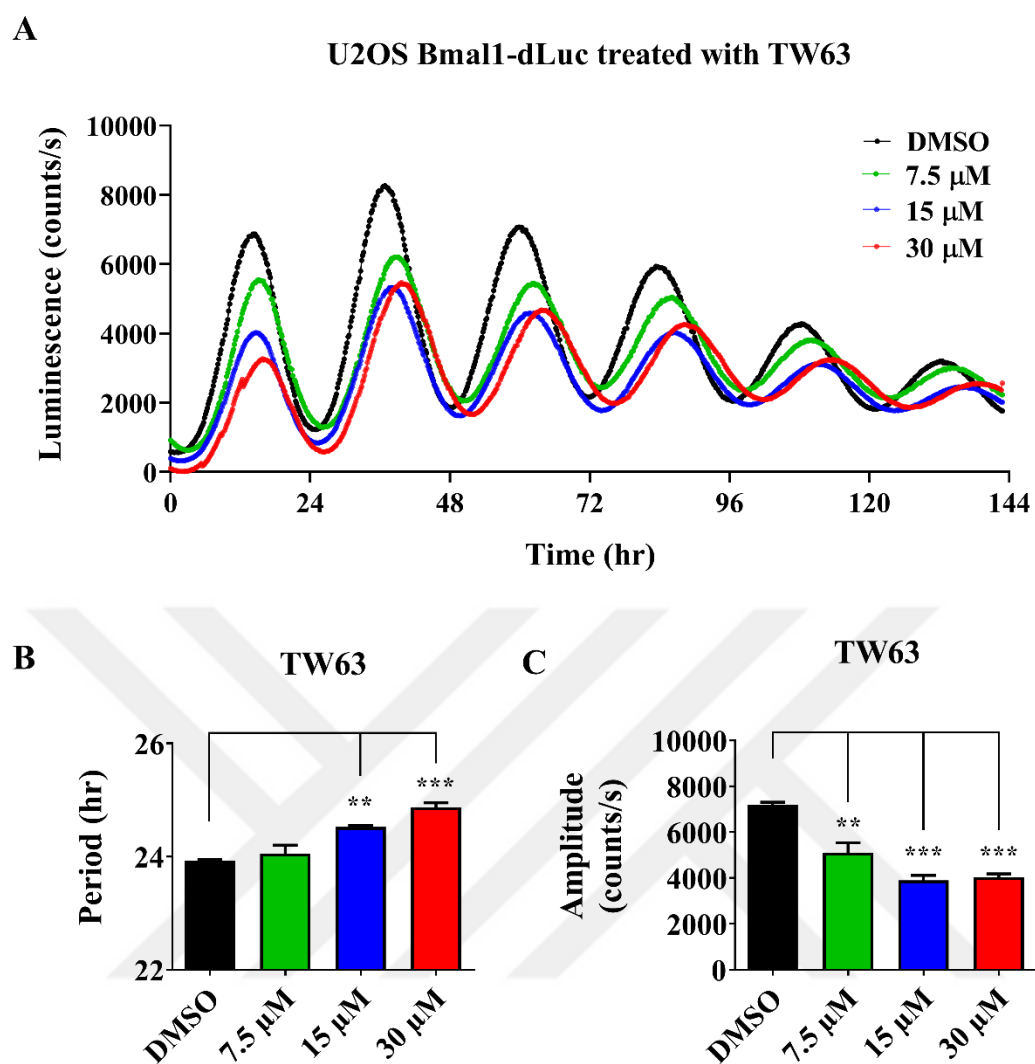


Figure 4.7. Bioluminescence rhythms of U2OS *Bmal1-dLuc* cells treated with various doses of TW63. **A)** Raw data recorded for 7 days. **B)** Dose-dependent effect of TW63 on period (hr) and amplitude (counts/s) of the oscillation. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test (** $p < 0.01$, *** $p < 0.001$, all others are non-significant).

Moreover, when cells were treated with the small molecule TW68, the period increased and the amplitude reduced significantly in a dose-dependent manner (Figure 4.8). TW68 treatment shows stronger effect of period lengthening (~ 3 hours) compared to TW63 (~ 1 hour). Hence, to test the effect of TW68 on different genetic background to confirm it has effect on circadian rhythm NIH3T3 *mPer1-dLuc* cells was subjected to different concentration of TW68. Results showed that it greatly affected period lengthening and amplitude reduction was observed on this system. Especially at 40 μ M, TW68 caused

arrhythmicity on NIH3T3 *mPer1-dLuc* cells as shown in Figure 4.9. These results suggested that TW68 affect circadian possibly modulating the activity or level of CRYs.

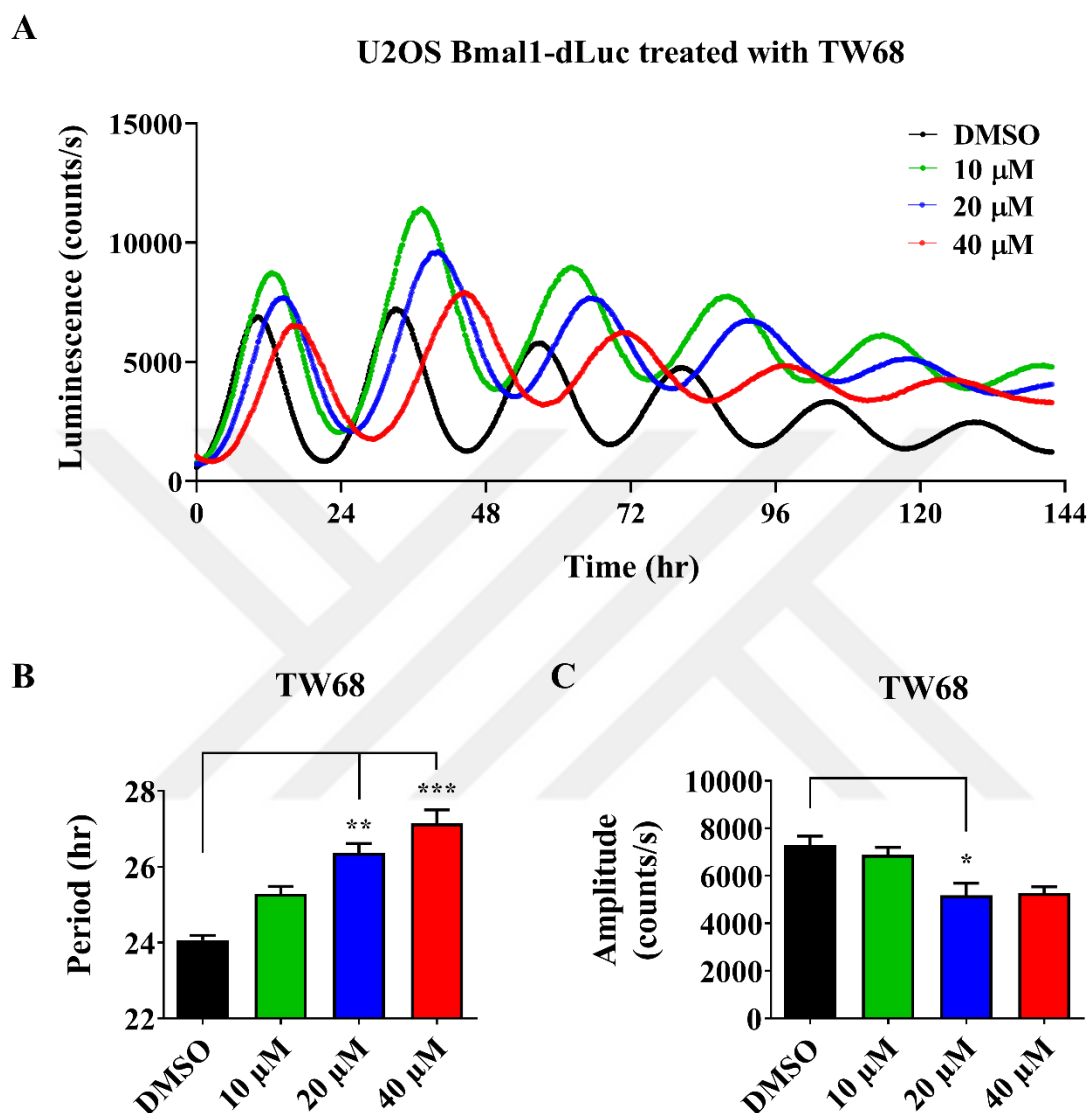


Figure 4.8. Bioluminescence rhythms of U2OS *Bmal-dLuc* cells treated with various doses of TW68. **A)** Raw data recorded for 7 days. **B)** Dose-dependent effect of TW68 on period (hr) and amplitude (counts/s) of the oscillation. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test ($p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$, all others are non-significant).

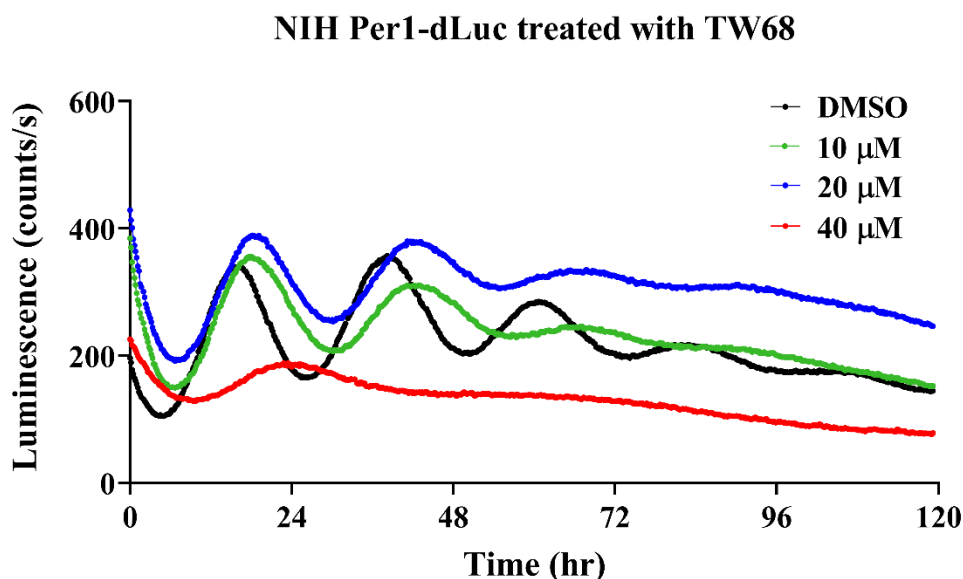


Figure 4.9. Bioluminescence rhythms of NIH3T3 *mPer1-dLuc* cells treated with various doses of TW68. Data are given for one experiment.

4.5. Analysis of CRYs and Other Clock Components

Biochemical and molecular methods were performed in order to characterize the effect of TW63 on protein and mRNA levels of circadian clock components using Western blot and RT-qPCR. I hypothesized that small molecules that stabilize CRY1/2 are expected to cause an increase in the amount of CRY1/2 at the protein level. When CRY is stabilized, CRY:PER complex will repress BMAL1:CLOCK transactivation more strongly causing a decrease in the expression of *Cry1/2*, *Per1/2*, *Dbp*, and *Rev-erba* genes since they have E-box elements in their promoter region.

4.5.1. Effect of TW68 on Protein Levels

After preparation of cell lysate from U2OS cells treated with molecule and DMSO, samples were subjected to the SDS-PAGE followed by Western blot analysis using anti-CRY1 and anti-CRY2. TW63 was eliminated in this study because it lacked dose-dependent activity (Figure A.3 in Appendix A). Cells treated with TW68 had significantly increased amount of both CRY1 and CRY2 in a dose-dependent manner. This suggested that TW68 stabilizes CRY1/CRY2 but being more effective on CRY1 stabilization compared to CRY2. At the highest dose (40 μ M), the amount CRY1 doubled, whereas CRY2 amount increased 1.5-fold (Figure 4.10A-B).

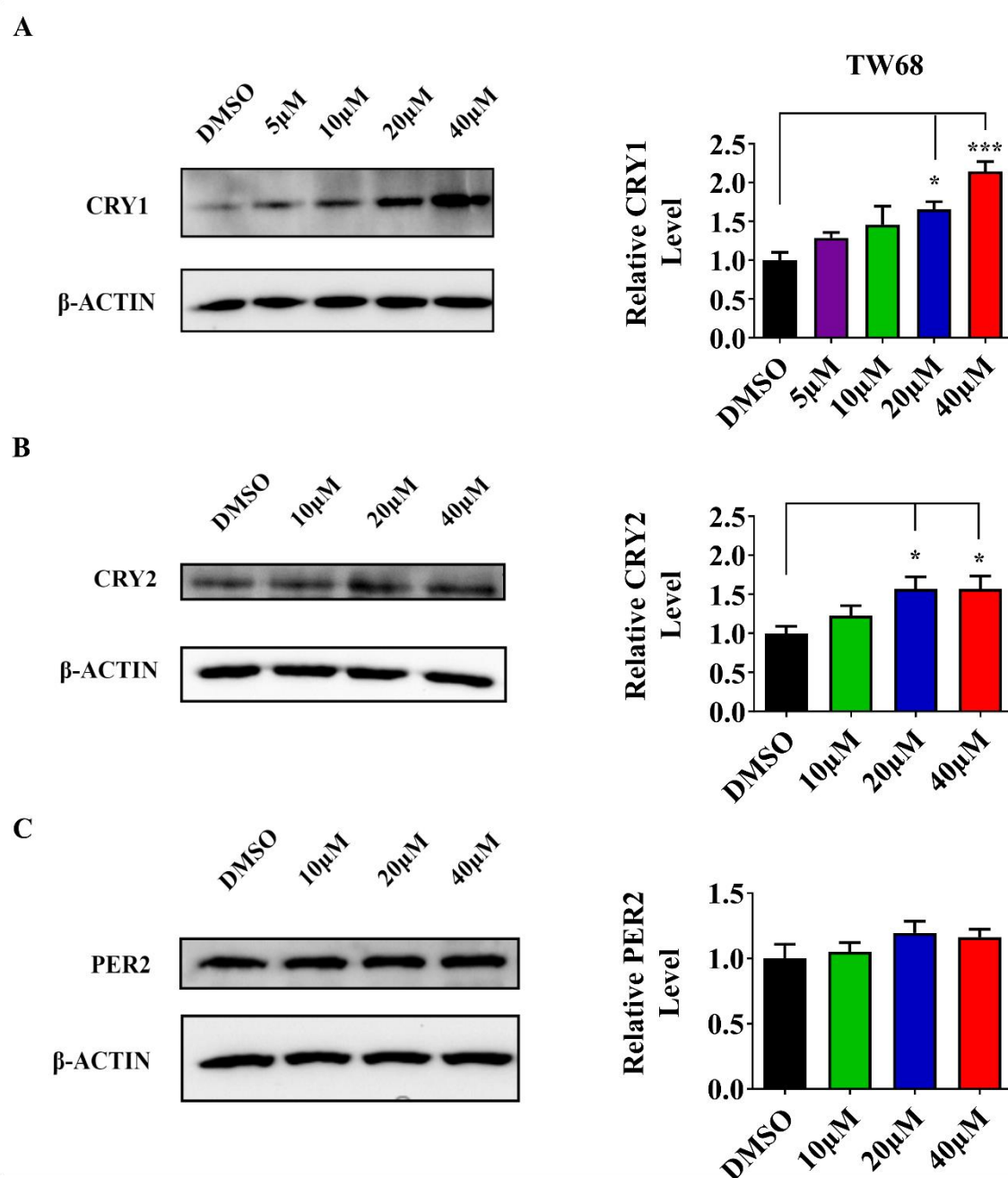


Figure 4.10. Western blot analysis of unsynchronized U2OS cells treated with TW68. **A)** CRY1, **B)** CRY2, and **C)** PER2 protein level normalized with β-ACTIN. Data are given as mean ± SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test (* $p < 0.05$, *** $p < 0.001$, all others are non-significant).

In the meanwhile, PER2 protein levels were not altered by TW68 treatment (Figure 4.10). Western blot analysis of unsynchronized U2OS cells treated with TW68. A) CRY1, B) CRY2, and C) PER2 protein level normalized with β -ACTIN. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test (* $p < 0.05$, *** $p < 0.001$, all others are non-significant). PER2 levels were not affected in TW68 cells probably due to the opposite effects of CRY stabilization on PER2. CRY stabilization leads to more CRY:PER complex which stabilizes PER because there is less degradation of PER when it interacts with CRY. On the other hand, CRY stabilization represses *Per* which means less PER protein is produced [207].

4.5.2. Effect of TW68 on mRNA Levels of Output Genes

In order to assess the effect of the TW68 on output genes we treated U2OS cells with TW68/DMSO control and determined the mRNA levels of clock genes using RT-qPCR method. mRNA levels were normalized with respect to *Rplp0* gene expression because its expression level is independent from circadian genes [202]. In line with stabilization of CRY proteins, TW68 treatment resulted in significant decrease of *Cry1*, *Cry2*, *Per2*, *Dbp* and *Rev-erba* expression in a dose-dependent manner. The reduction in the expression of these genes was expected when CRY is stabilized, because they are under the control of CRY:PER repression of BMAL1:CLOCK transactivation. On the other hand, *Bmal1* and *Clock* expression was not affected from TW68 treatment. These results show that TW68 is a CRY1 and CRY2 stabilizer and this stabilization strengthens the repression in the negative arm of the circadian clock feedback loop (Figure 4.11).

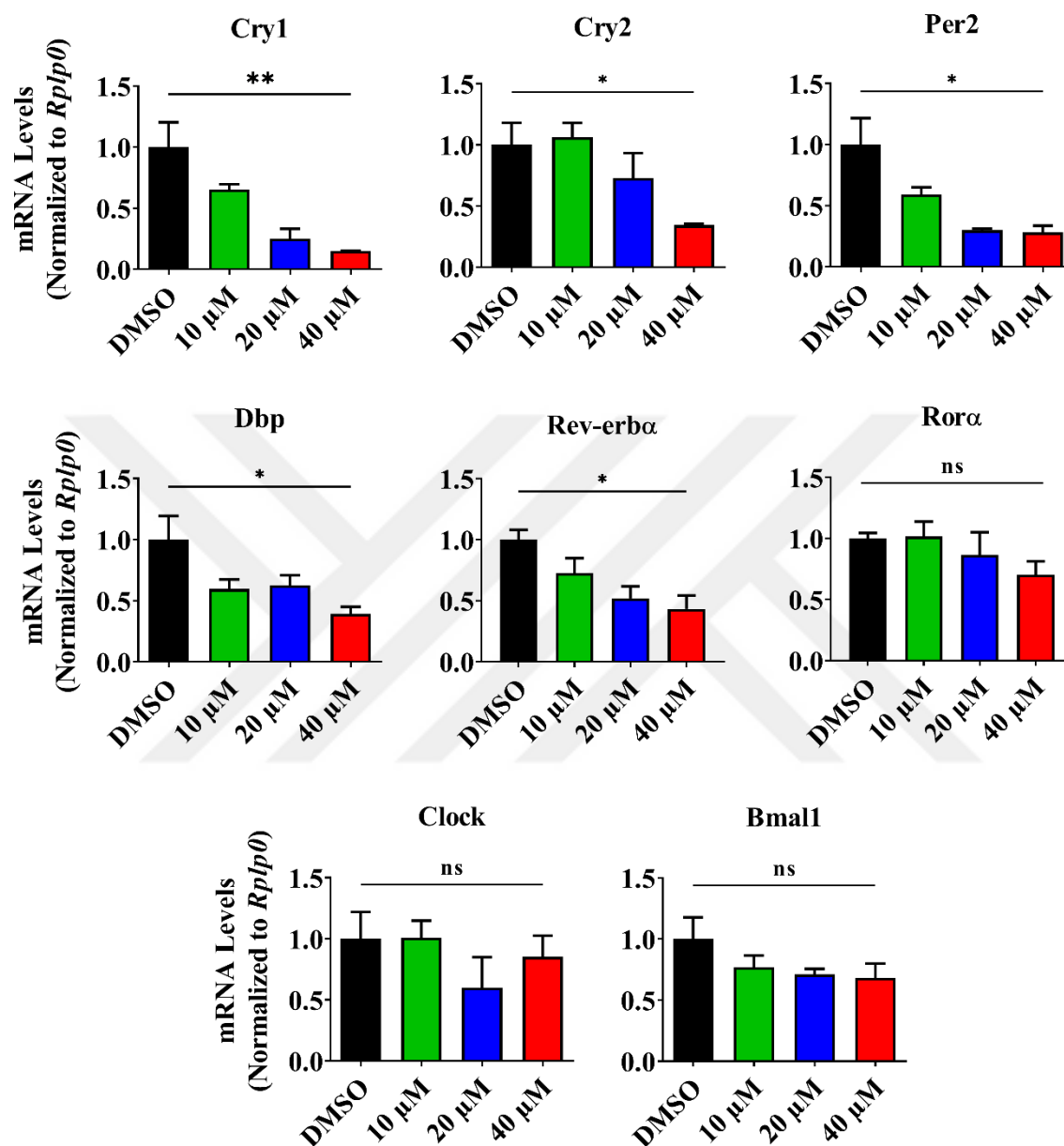


Figure 4.11. RT-qPCR analysis of unsynchronized U2OS cells treated with TW68. mRNA levels of *Cry1*, *Cry2*, *Per2*, *Dbp*, *Rev-erba*, *Rora*, *Clock*, and *Bmal1* relative to *Rplp0*. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA (*p<0.05, **p<0.01, ns: non-significant).

4.6. Detailed Computational Analysis Between TW68 and CRY1

Molecular docking results of TW68 to CRY1 revealed several residues on FBXL3/21 binding site that possibly interact with TW68. TW68 forms π - π interactions with two residues W399 (W417 in CRY2) and W292 (W310 in CRY2). These residues are found in the FAD binding pocket where FBXLs bind for the ubiquitination of CRY [101,110]. According to the molecular docking result, possible binding position of TW68 on CRY1 is shown in Figure 4.12.

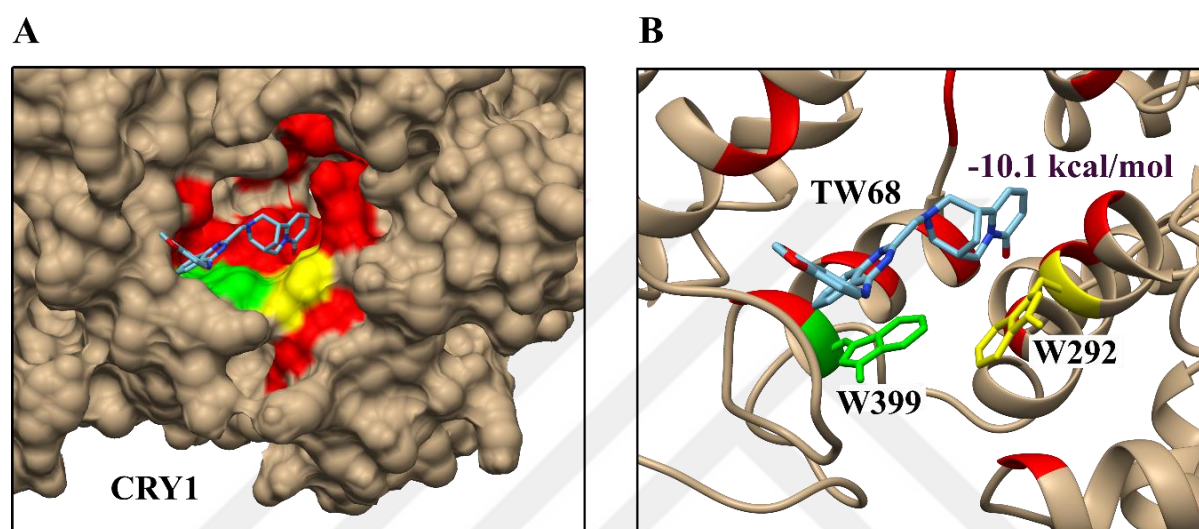


Figure 4.12. Predicted TW68 binding site in CRY1. **A)** Surface representation of FAD and FBXL binding pocket (red) shown together with TW68 (blue) and interacting residues W399 (green) and W292 (yellow). **B)** Ribbon representation of (A).

*Molecular graphics prepared with UCSF Chimera [116].

Docking results suggest that W399 amino acid residues in the CRY1 contributes to stable binding with energy of -10.1 kcal/mol. If this prediction is true, replacing the amino acid residue to another amino acid should eliminate binding of the CRYs to TW68. To test this prediction, Tryptophan (W) residue in this site was replaced with Leucine (L) in wild type *Cry1-Luc* plasmid using site-directed mutagenesis (provided by Şeref Gül in Kavaklı laboratory). The repression activity of the W399L-CRY1 mutant protein was retained compared to wild type CRY1 which shows that the mutant is functional (Figure A.4 in Appendix A). This mutant was subjected to the luciferase study to see the effect of TW68. Protein degradation assay was performed by transfecting HEK293T cells with CRY1-LUC and W399L-CRY1-LUC followed by TW68 treatment. The half-life of CRY1-LUC increased dose-dependently; whereas, W399L mutant CRY1-LUC half-life was not affected

in the presence of the TW68 (Figure 4.13). In other words, stabilization effect of TW68 on CRY was diminished when W399 residue was mutated, suggesting that W399 may be critical for the binding of TW68. Hence, TW68 possibly stabilizes CRY by interacting with its FBXL binding residues.

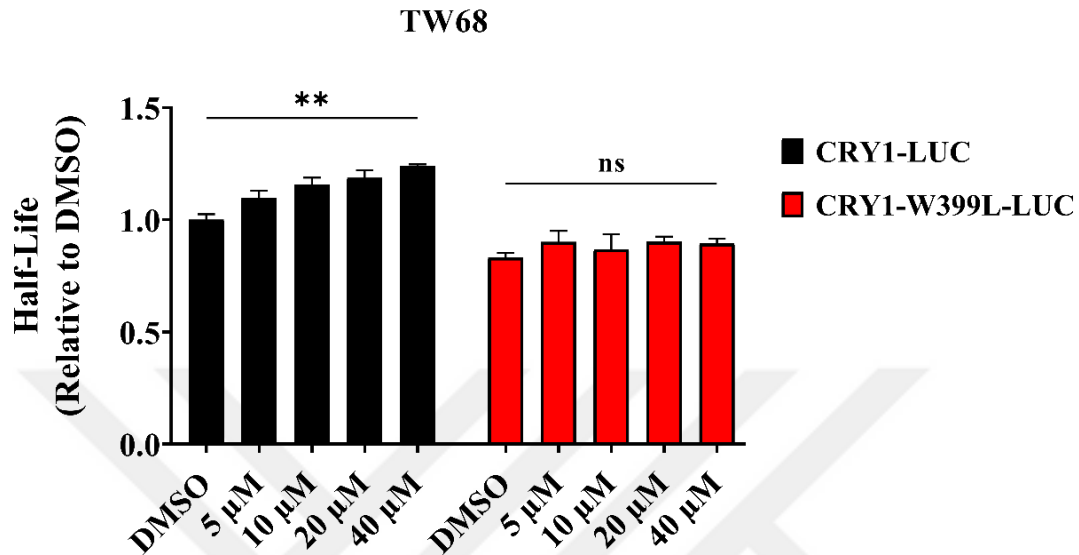


Figure 4.13. Dose-dependent effects of TW68 on CRY1-LUC and CRY1-W399L-LUC mutant half-life relative to DMSO. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA (** $p < 0.01$, ns: non-significant).

4.7. Effects of TW68 on Gluconeogenesis

In Section 2.5.1, the interaction between the circadian clock and gluconeogenesis through the CRY proteins is explained in detail. Previous studies have revealed that glucagon-mediated expression of gluconeogenic genes *Pck1* and *G6pc* reduce when CRY1/2 is either adenovirally overexpressed in mice [124] or stabilized by a small molecule in mouse hepatic cells [180]. Moreover, glucose levels were also reduced in these studies. We performed qPCR and glucose production assay to test whether the stabilization of CRY1/2 by TW68 also represses the glucagon-mediated gluconeogenesis. HepG2 human hepatocellular carcinoma cells were used in gluconeogenesis experiments.

The gluconeogenic genes *Pck1* and *G6pc* were repressed significantly in a dose-dependent manner when TW68 treated cells were induced with glucagon (Figure 4.14A). The repression of these gluconeogenic genes were specific to glucagon-induction since there was no significant difference in the expression of both genes for samples without glucagon-

induction. In line with this observation, treatment of cells with TW68 resulted in significantly decreased glucose production in a dose-dependent manner (Figure 4.14B). Again, there was no significant change in glucose level genes for samples without glucagon-induction. Overall, TW68 represses glucagon-induced gluconeogenesis in HepG2 cells possibly by stabilizing CRY and hence reducing GPCR-mediated cAMP levels.

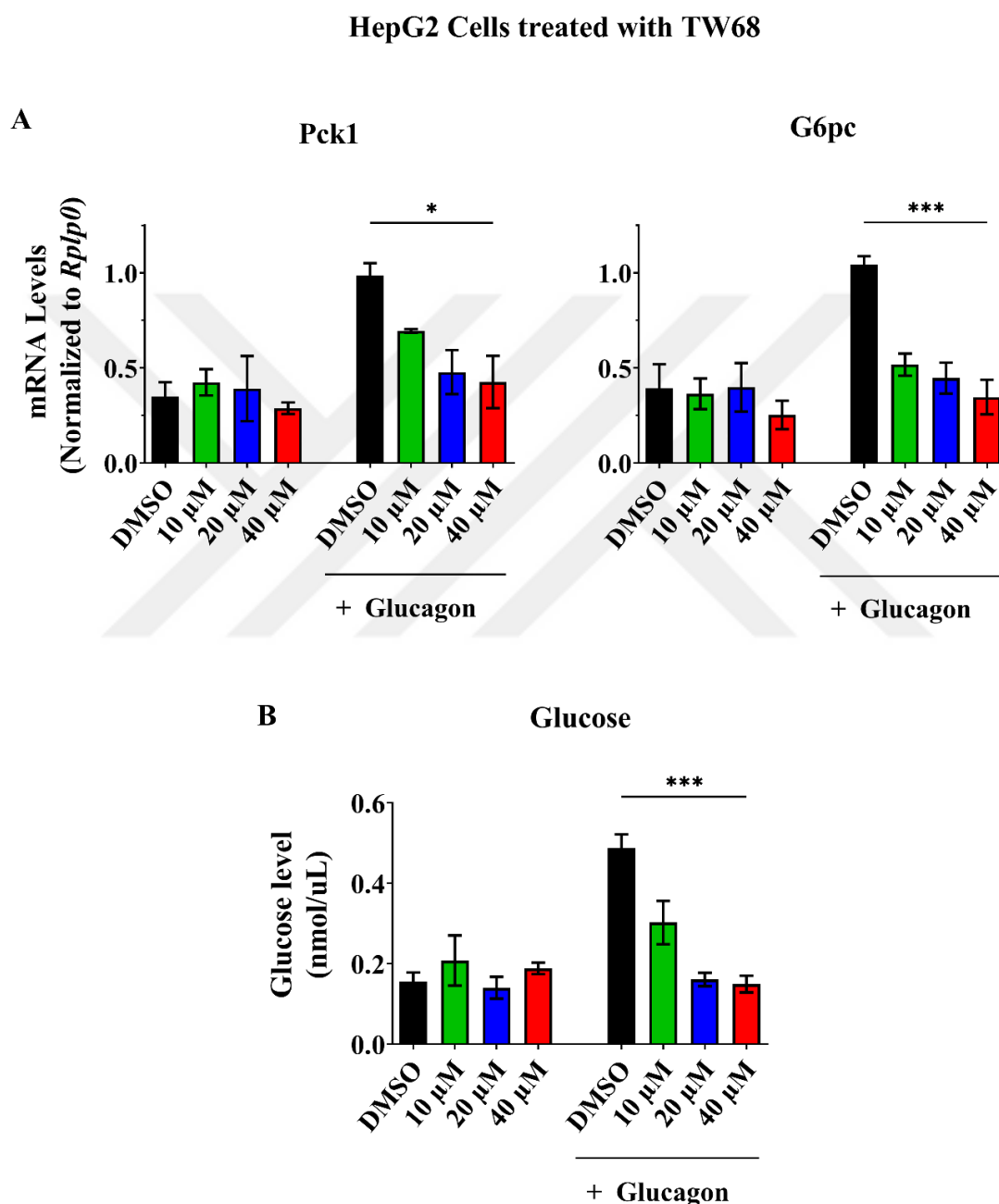


Figure 4.14. Effect of TW68 on gluconeogenesis in HepG2 cells. **A)** RT-qPCR analysis of gluconeogenic *Pck1* and *G6pc* genes when cells were treated with TW68 and induced with glucagon. **B)** Glucose production amount of cells treated with TW68 and induced with glucagon. Statistical analysis performed using one-way ANOVA (* $p < 0.05$, *** $p < 0.001$).

CHAPTER 5

DISCUSSION

The connection between circadian clock and disease mechanisms have been extensively studied in the last two decades. Many different studies on circadian clock focused on these disease mechanisms including sleep disorders, glucose metabolism disorders, cardiovascular diseases, and cancer [62,126]. Gluconeogenesis is one of the mechanisms that is related to glucose metabolism disorders such as type 2 diabetes. The relationship between gluconeogenesis and circadian clock has been revealed in a study which concluded that core clock protein CRY inhibits glucagon-mediated gluconeogenesis and also lowers blood glucose levels [124]. Therefore, CRY can be used as a therapeutic target for the treatment of type 2 diabetes.

Another hot topic in the circadian clock field is the discovery of small molecules interacting with circadian clock proteins which can be utilized the treatment of these diseases [174,175]. Drug discovery studies have recently gained speed using time-saving computational methods such as structure-based virtual screening. After *in silico* screening, the small molecules can be further characterized using *in vitro* and *in vivo* for their effect on the circadian clock and the related disease [188]. However, this method has not been applied for circadian clock protein targets for the identification of small molecules modifying circadian clock.

Our aim was to identify new small molecules that stabilize CRY using *in silico* screening followed by characterization in human cell lines. As far as we know, this is the first study that uses *in silico* screening for the identification of small molecules affecting the circadian clock. Steve Kay and his group previously identified a CRY stabilizer (KL001) using high-throughput circadian assay. We identified another CRY stabilizer, TW68, that shows similar effects with KL001 on circadian clock and gluconeogenesis. Our findings indicate that *in silico* screening can be utilized in circadian clock field as a time-saving and reliable method.

In this thesis, we focused on the *in vitro* screening and characterization of the small molecules stabilizing CRY1/2 for therapeutics of type 2 diabetes. These small molecules were previously identified by Şeref Gül in Kavaklı laboratory based on a molecular docking study focusing on CRY1/2 and FBXL3/21 interaction site which is important for the stability of CRY. The purpose of this thesis was to further screen and characterize the top 32 small molecules selected from the *in silico* screening. First, the non-toxic doses of these molecules were determined using MTT cell viability assay. As a result, we eliminated 12 small molecules due to their toxicity on cells or solubility issues. Then, the effect of these small molecules on CRY1/2 stability was evaluated by determining their effect on the half-life of CRY1/2. Among 20 small molecules screened, TW63 stabilized only CRY1 while TW60 and TW68 stabilized both CRY1 and CRY2 significantly. These candidate CRY stabilizer molecules had led to around 10-20% increase in CRY1 or CRY2 half-life. Therefore, we wanted to further investigate the dose dependent effect of candidate molecules on CRY stability and circadian rhythm.

Observing a dose-dependent effect is important for identifying small molecules that actually bind to the target protein [206]. In our case, we expect the stabilization of CRY to be enhanced when the dose of the small molecules increases and saturate at a certain dose. The dose-response curves revealed that TW63 and TW68 stabilize CRY1 significantly in a dose dependent manner with EC₅₀ values of 15 μ M and 10 μ M, respectively (Figure 4.6). On the other hand, TW60 did not show a dose dependent effect. Moreover, we wanted to detect the changes in the circadian rhythm when CRY is stabilized by these small molecules. TW63 and TW68 treated U2OS *Bmal-dLuc* cells displayed dose-dependent and significant lengthening of the period (for TW63: ~1 hour, for TW68: ~3 hours) and reduction of the amplitude of the circadian rhythm (Figure 4.7 and 4.8). Since TW68 displayed more drastic altering of the period, we tested this small molecule on NIH3T3 *mPer1-dLuc* cells and found an even more drastic amplitude reduction. For example, TW68 caused arrhythmicity on NIH3T3 *mPer1-dLuc* cells at 40 μ M (Figure 4.9). These results are in parallel with KL001's period lengthening and amplitude dampening effect on circadian rhythm.

TW68 has stronger effect than TW63 on the stabilization of CRY and the circadian rhythm. Therefore, we decided to continue characterizing TW68 via biochemical methods that measure protein and gene expression levels. In line with its stabilization effect on CRY, TW68 treated cells increased CRY1/2 protein levels significantly in a dose dependent manner, causing 1.5-fold increase in both CRY1 and CRY2 protein levels at 20 μ M (Figure

4.10). The increase in CRY levels led to the reduction in the expression of *Cry1/2*, *Per1/2*, *Dbp*, and *Rev-erba* genes (Figure 4.11). This result was expected since the stabilization of CRY would strengthen the repression of BMAL1:CLOCK transactivation by CRY:PER complex which results in the decrease in the expression of genes having E-box elements in their promoter region. Also, when we checked PER2 levels, there was no significant effect of TW68 on PER2 protein levels (Figure 4.10). These results clarify that TW68 does not act by stabilizing PER2 stabilizing effects but rather acts by stabilizing CRY1/2. Also, the stabilization of CRY1/2 by TW68 results in stronger repression of *Cry1/2*, *Per1/2*, *Dbp*, and *Rev-erba* genes.

In order to investigate the molecular interaction of TW68 with CRY1, first we analyzed the molecular docking results. These results revealed that residues W399 (W417 in CRY2) and W292 (W310 in CRY2) may form strong bonds with TW68 through π - π interactions. W399 and W292 residues are found in the FAD binding pocket where FBXLs bind and ubiquitinate CRY [101,110]. Protein degradation assay using mutated CRY1-W399L-LUC and control CRY1-LUC results have shown that half-life of CRY1 did not change significantly when we used increasing doses of TW68 (Figure 4.13). Hence, W399 residue could be important for TW68 binding because the effect of TW68 on CRY stabilization disappeared for W399L mutant. It is also known from a structural (crystal structure) that KL001 binds to CRY2 at the FBXL3/21 binding pocket [190]. These results also indicate that TW68 possibly stabilizes CRY via interacting with the binding site of FBXL3/21.

We screened small molecules that stabilize CRY in order to discover molecules that would be candidate for drug development in the treatment of type 2 diabetes [124,180]. Therefore, the final step of this thesis study was to test the effects of the candidate small molecule, TW68, on glucagon-induced gluconeogenesis. When gluconeogenesis was induced with glucagon in HepG2 cells, TW68 caused the significant repression of gluconeogenic genes *Pck1* and *G6pc*, and also significant reduction in the glucose production levels dose-dependently (Figure 4.14). KL001 also exhibits these effects on gluconeogenesis. These results show that TW68 can reduce glucagon-induced gluconeogenesis *in vitro*.

In summary, in this thesis study we have identified a small molecule, TW68, that can stabilize CRY1/2 and also reduce glucagon-mediated gluconeogenesis *in vitro*. There is strong evidence from mutant CRY half-life data that TW68 interacts with CRY1/2 in the FBXL3/21 interaction site. Therefore, this study suggests that the stabilization effect of

TW68 on CRY might be causing the reduction in glucagon-induced gluconeogenesis. The possible mechanism of action of TW68 on circadian clock and gluconeogenesis is demonstrated in Figure 5.1.

Further characterization of TW68 is necessary including *in vitro* and *in vivo* studies. The interaction of TW68 and CRY should be verified using affinity based methods such as pull down assay using biotin-conjugated TW68. Also, exact mechanism by which TW68 stabilizes CRY proteins should be investigated. Our results indicate that the stabilization might be through ubiquitination mechanism of CRY by FBXL3/21; hence, *in vitro* or *in vivo* ubiquitination assay can be performed. Finally, after the *in vitro* characterization of TW68 is completed, the future perspective of this study is the investigation of the effect of TW68 on circadian clock and gluconeogenesis *in vivo* by treating C57BL/6J wild-type (WT) and diabetic (db/db) mice with TW68.

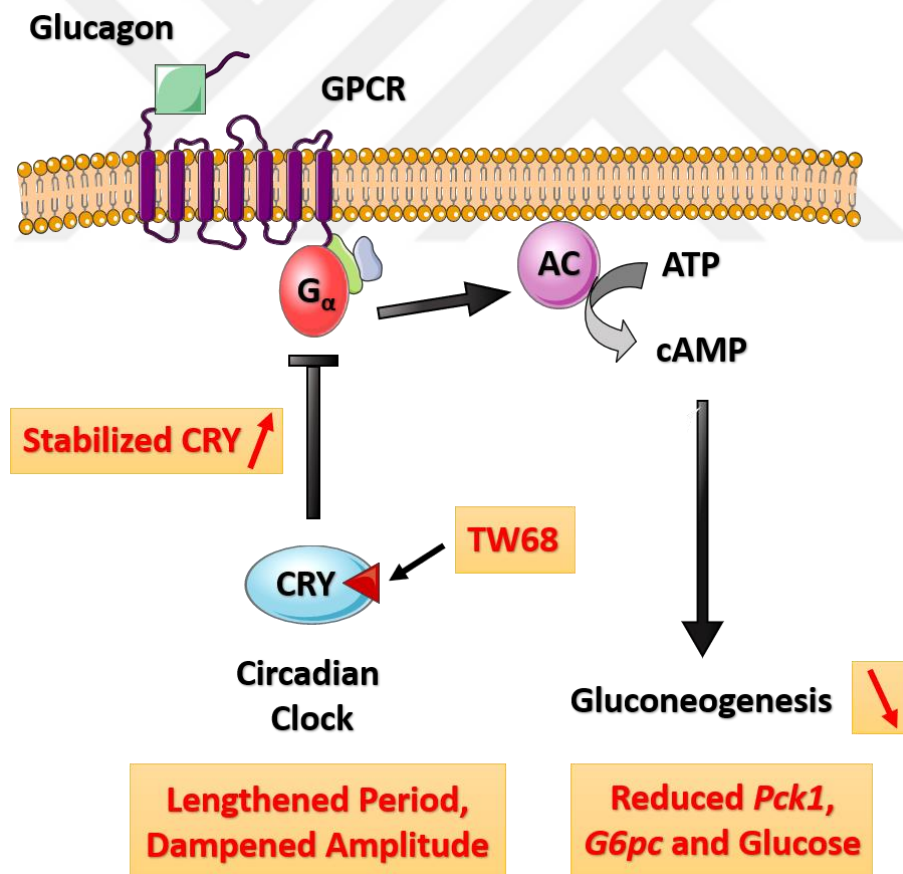


Figure 5.1. Action of TW68 on the circadian clock and gluconeogenesis pathway.

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VITA

Çağla Ergün was born in Istanbul, Turkey on May 21, 1993. She received her B.Sc. Degree in Chemical Engineering from Boğaziçi University in 2017. She continued her education at M.Sc. Program in Chemical & Biological Engineering at Koc University where she worked as a research and teaching assistant at Prof. İ. Halil Kavaklı's Biotechnology and Circadian Clock Laboratory from February 2017 to August 2019. During her M.Sc. studies, she has worked on "Discovery and Characterization of Small Molecules Stabilizing the Cryptochrome in the Mammalian Circadian Clock".

APPENDIX A: SUPPLEMENTARY DATA

Effect of TW60 on CRY Half-Life

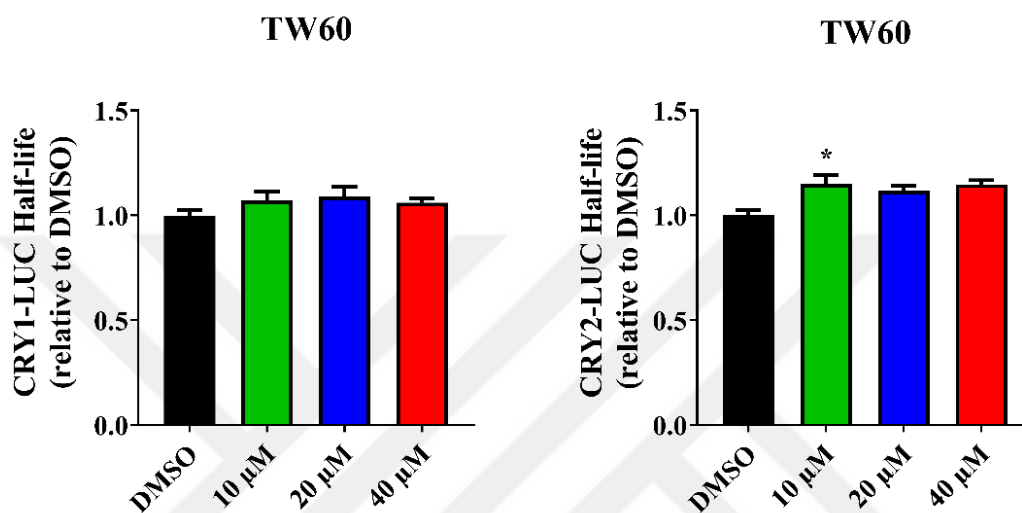


Figure A.1. Dose-dependent effects of TW60 on CRY1-LUC and CRY2-LUC stability half-life relative to DMSO. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test (* $p < 0.05$, all others are non-significant).

Effect of TW60 on Circadian Rhythm

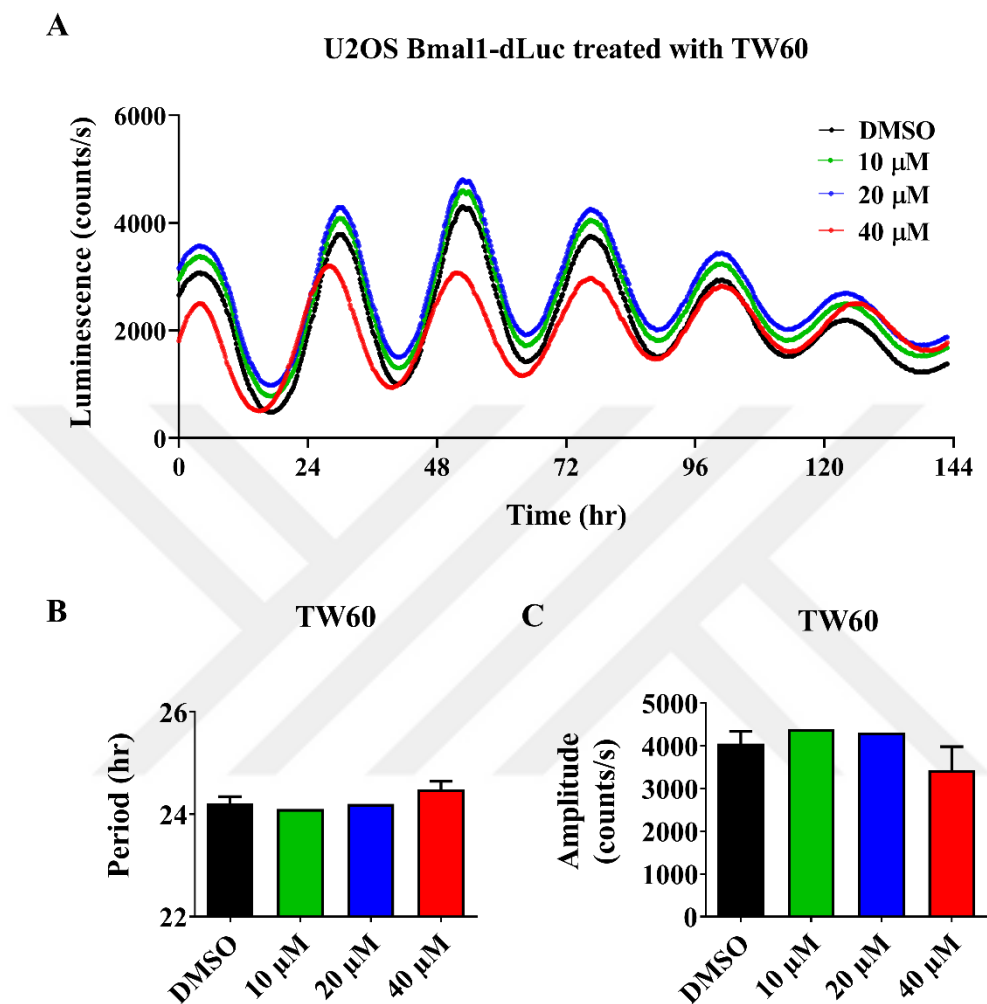


Figure A.2. Bioluminescence rhythms of U2OS Bmal-dLuc cells treated with various doses of TW60. **A)** Raw data recorded for 7 days. Dose-dependent effect of TW60 on **B)** period (hr) and **C)** amplitude (counts/s) of the oscillation.

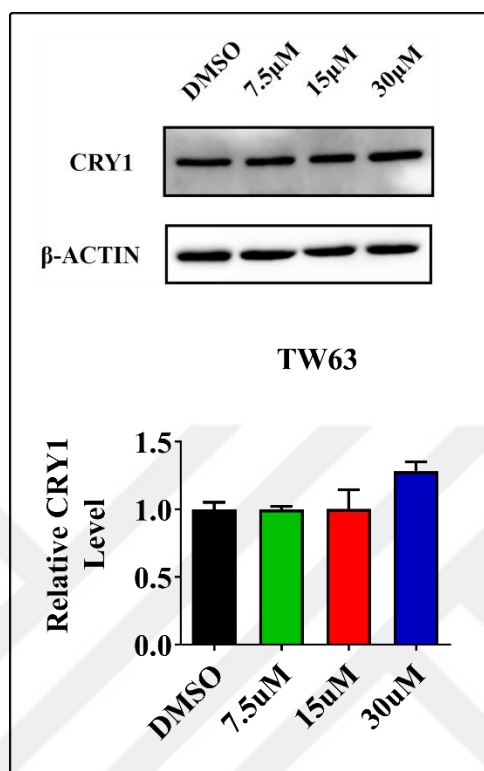
Effect of TW63 on CRY1 Protein Level

Figure A.3. Western blot analysis of unsynchronized U2OS cells treated with TW63. CRY1 level is normalized with β -ACTIN. Data are given as mean $\pm$ SEM from at least 3 individual experiments. Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test (all are non-significant).

Repression Activity of W399L-CRY1 Mutant

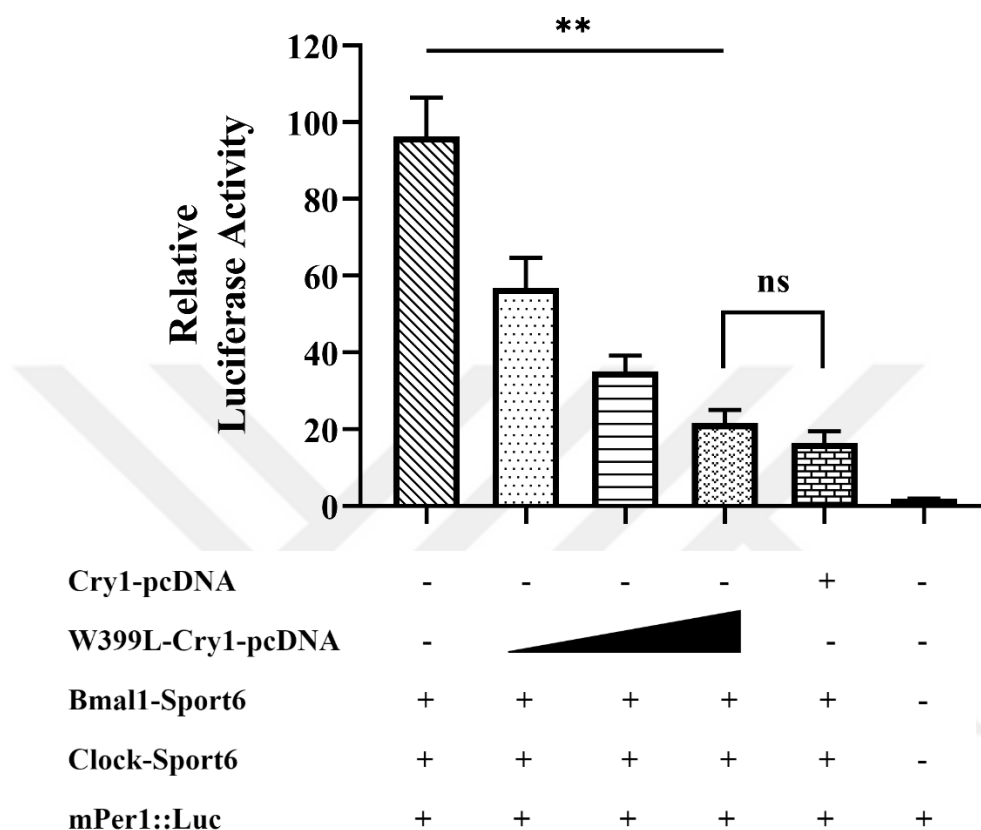


Figure A.4. Repression activity of W399L-CRY1 mutant at increasing dose (0.625 ng, 1.25 ng, 2.5 ng) compared to wild type CRY1 (2.5 ng). Data are given as mean $\pm$ SEM from 2 individual experiments. Statistical analysis performed using one-way ANOVA with Tukey's multiple comparisons test (**p<0.01, ns: non-significant).

APPENDIX B: PRIMER SEQUENCES

RT-qPCR Primers

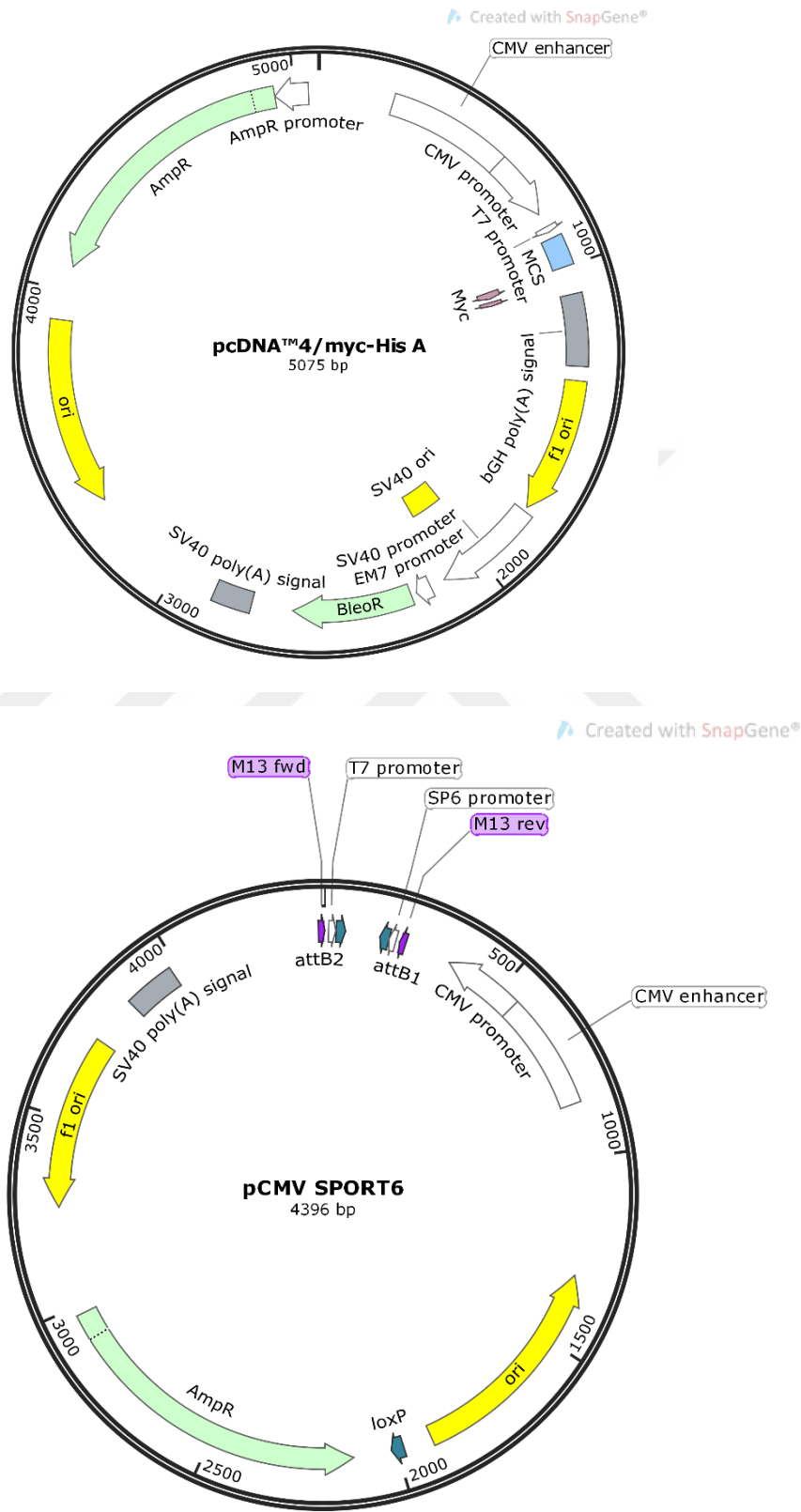
Primer Name*	Sequence
Hs-Cry1-RT-fwd	ACAGGTGGCGATTTTGTCTTC
Hs-Cry1-RT-rev	TCCAAAGGGCTCAGAATCATACT
Hs-Cry2-RT-fwd	CGTGTTCCCAAGGCTGTTCA
Hs-Cry2-RT-rev	CTCCGTCACTACTTCCACACC
Hs-Per1-RT-fwd	CCTCTTCCAGGATGTGGATGAA
Hs-Per1-RT-rev	CAACTGCAGAATCTTCTTGTGGA
Hs-Dbp-RT-fwd	GAGGAACTTAAGCCCCAGCC
Hs-Dbp-RT-rev	CTCGTTGTTCTTGTACCGCC
Hs-Bmal1-RT-fwd	GCCCATTGAACATCACGAGTAC
Hs-Bmal1-RT-rev	CCTGAGCCTGGCCTGATAGTAG
Hs-Clock-RT-fwd	GGCACCACCCATAATAGGGTA
Hs-Clock-RT-rev	TGTTGCCCCTTAGTCAGGAAC
Hs-Rora-RT-fwd	ACTACACACCAGCATCAGGC
Hs-Rora-RT-rev	GCAGGTTTCCAGATGCGATT
Hs-Nr1d1-RT-fwd	AGACATGACGACCCTGGACT
Hs-Nr1d1-RT-rev	ATGGTGGGAAGTAGGTGGGA
Hs-RPLP0-RT-fwd	TGTGGGAGCAGACAATGTGG
Hs-RPLP0-RT-rev	CATTCCCCCGGATATGAGGC
Hs-Pck1-RT-fwd	AGGTGTCACCATCACGTCCT
Hs-Pck1-RT-rev	AGGTCTACGGCCTCCAAAGA

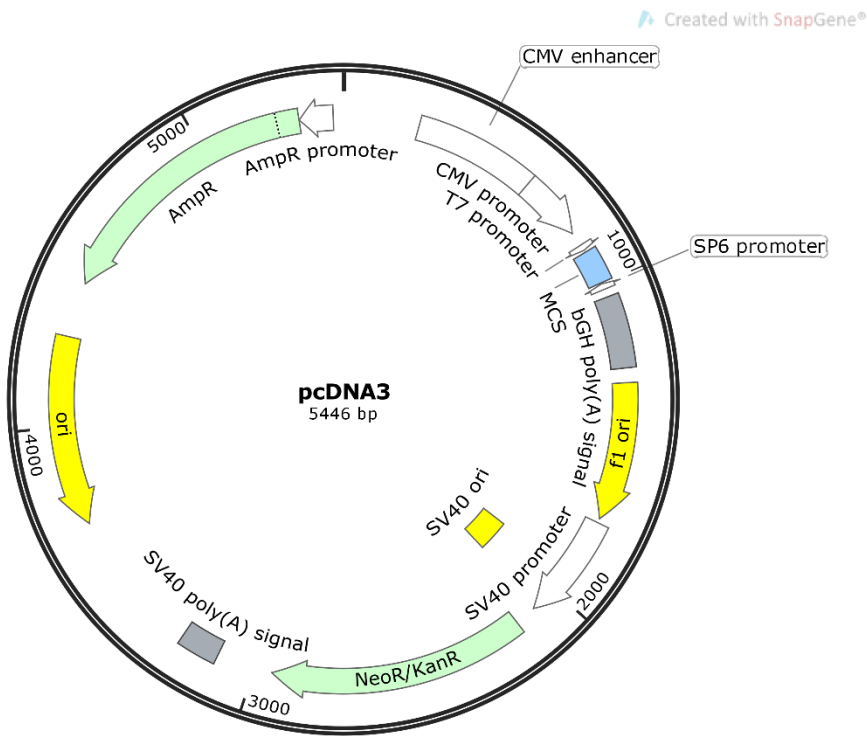
Hs-G6pc-RT-fwd	TTCAGGAAGCTGTGGGCATT
Hs-G6pc-RT-rev	CTTTCCTGGTCCAGTCTCAC

* Hs: homo sapiens, RT: real time, fwd: forward, rev: reverse



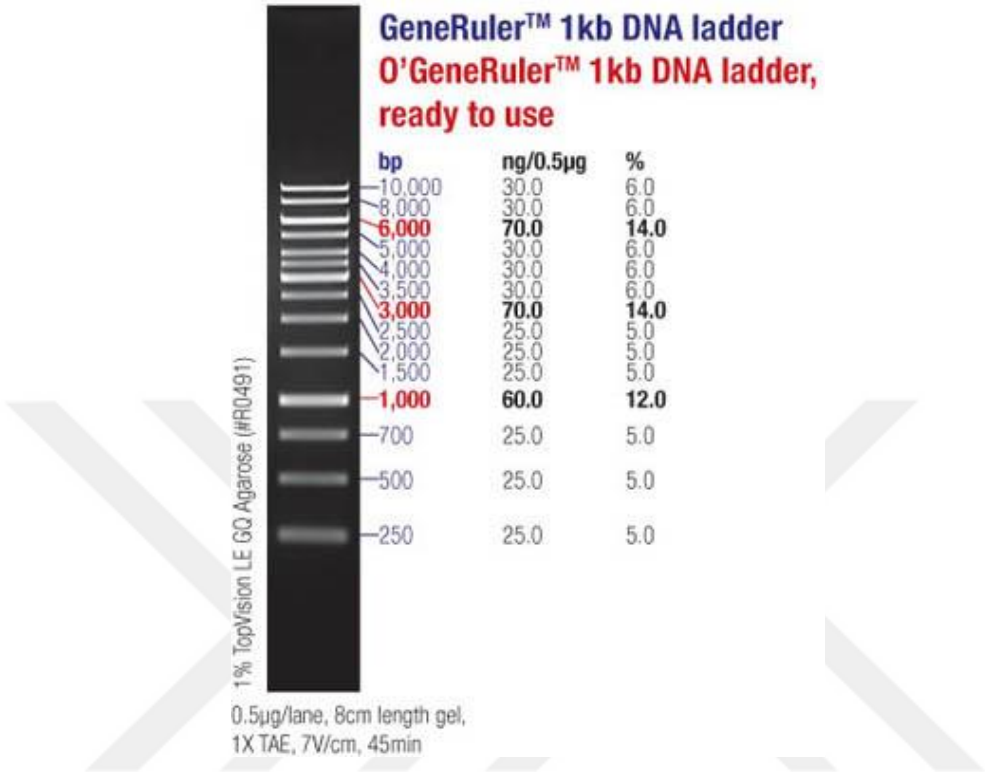
APPENDIX C: PLASMID MAPS





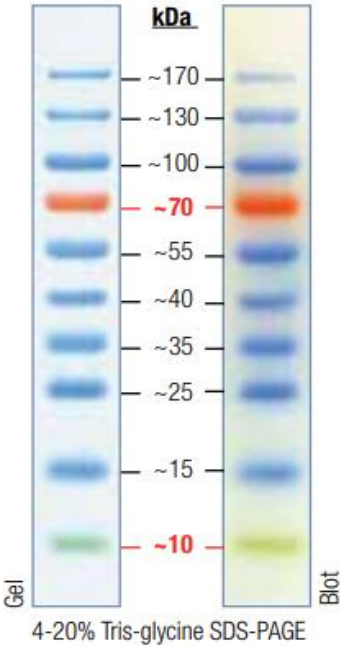
APPENDIX D: PROTEIN AND DNA MARKERS

DNA Size Marker:



Protein Molecular Weight Marker:

PageRuler™ Prestained
Protein Ladder, #SM0671



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Editor of portion(s)	N/A
Author of portion(s)	Seung-Hee Yoo Jennifer A. Mohawk 7 Sandra M. Siepka 7 Yongli Shan Seong Kwon Huh Hee-Kyung Hong Izabela Kornblum Vivek Kumar Nobuya Koike Ming Xu Justin Nussbaum Xinran Liu 8 Zheng Chen Zhijian J. Chen Carla B. Green Joseph S. Takahashi
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APPENDIX F: LABORATORY EQUIPMENT

Autoclaves:	CL-40S/SDP (60L) ALP autoclave
Centrifuges:	Microfuge 1-14K, Sigma Laboratory Microfuge 1-16K (refrigerated), Sigma Laboratory Allegra X-15R, Beckman Coulter
Deep freezes and refrigerators:	Premium U570 Ultra-Low Temperature Freezer, New Brunswick Scientific 1061 M refrigerator, Arcelik
Electrophoresis equipment:	Mini-Sub Cell GT, Bio-Rad. Mini-PROTEAN 3 Cell and Single-Row AnyGel Stand, Catalog # 165-3321, Bio-Rad
Gel documentation system:	ChemiDoc Touch Imaging System, Bio-Rad BlueLight Table (LED transilluminator), SERVA
Ice Machine:	AF 10, Scotsman.
Shaker:	Innova 4300 incubator shaker
Magnetic stirrer:	Heidolph MR 3001
Pipettes:	Finnpipette F1 1-10 μ l, F1 10-100 μ l, F1 100- 1000 μ l, ThermoScientific
pH meter:	SevenCompact pH/Ion meter S220, Mettler Toledo
Power supply:	PowerPac Basic (300V, 400mA, 75W), Bio-Rad
Pure water systems:	DV25 Pure Lab Option, ELGA GenPure ultrapure water system, ThermoScientific
Microplate Reader:	Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek
Vortex machine:	Reax Top, Heidolph
CO2 Incubator:	Memmert INC108med
Incubator:	Incucell 111
Lumicycle:	LumiCycle 32 luminometer (Actimetrics)

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