Identification of ABE2A as a Circadian Amplitude Enhancer with Drug-like Potency

by

Başak Velioğlu Ulubaş

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Koç University

Graduate School of Sciences and Engineering

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examining committee have been made.

Committee Members:

_____

Prof. İbrahim Halil Kavaklı

_____

Assist. Prof. Onur Öztaş

_____

Prof. Mahmut Çalışkan

Date: 20.06.2023

To my parents İdil and Mehmet,
to my husband, best friend, and companion
Sergen Ulubaş
and Mia, my child and love
for their endless support

ABSTRACT

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Many species have an adaptation system to the period of the solar day. This endogenous mechanism is called the circadian clock, which can be defined as an autonomous 24h cycle controlling daily rhythms in physiology and behaviour. Thus, a robust circadian clock is required for many processes in our body to work smoothly. Our body's control center of circadian clock operation is SCN (suprachiasmatic nucleus), which uses the phoetic input sensed by intrinsically photosensitive retinal ganglion cells (ipRGCs). SCN regulates cellular clocks and peripheral tissues and coordinates them with the help of neural or hormonal signals. This oscillatory system depends upon transcriptional translational regulatory feedback loops (TTFLs). The main loop consists of CLOCK, BMAL1 (positive arm), and CRYs, PERs (negative arm). Mechanistically, CLOCK and BMAL1 interact with each other in the cytosol. Then this heterodimer translocates into the nucleus and binds to E-Box elements within the promoter of *CRYPTOCHROME* (*CRY*) and *PERIOD* (*PER*). Cryptochrome (CRY), PER, and the epsilon isoform of casein kinase I (CK1ε) form a complex in the cytosol that enters the nucleus and inhibits their own transcription regulated by CLOCK and BMAL1 heterodimer. Any disruption in this mechanism may result in diseases or disorders. Small molecule modifiers are efficient tools to repair this perturbed mechanism and have many advantages over other therapeutic approaches.

In my study, I characterized a novel small molecule called ABE2A, 100 times more effective derivative of the CLK8 molecule, which increases the amplitude of circadian rhythm by specifically interacting with CLOCK and decreasing the interaction between CLOCK and BMAL1. Further experiments revealed that ABE2A decreases the nuclear abundance of CLOCK, BMAL1 and increases the cytosolic abundance of BMAL1. Additionally, it reduces the total cell protein level of CLOCK and increases PER2. ABE2A also affects the transcriptional levels of E-box and D-box-regulated genes. For instance, it drops the expression of core clock genes such as *DBP, BMAL1,* and *PER2*.

Overall, similar to CLK8, ABE2A reduces the positive arm components in the nucleus, limiting the transcription of the negative arm. Thus, the repression activity in the TTFL is stabilized and the amplitude of the circadian rhythm enhances. For these reasons, and also by being effective at nanomolar doses, ABE2A has a drug-like potency against various health problems related to decreased amplitude, such as some metabolic diseases, mood disorders, and accelerated aging.

ÖZETÇE

Bir Sirkadiyen Saat Genliği Arttırıcısı Olarak
Potansiyel bir İlaç Adayı Olan ABE2A'nın Tanımlanması
Başak Velioğlu Ulubaş
Moleküler Biyoloji ve Genetik, Yüksek Lisans
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Birçok canlı türünün evrimsel olarak dünyanın kendi çevresindeki dönüş süresine uyum sağlama sistemi bulunmaktadır. Bu endojen mekanizmaya sirkadiyen ritim denir. Bu ritim fizyolojiyi ve davranışı kontrol eden otonom 24 saatlik bir döngü olarak tanımlanabilir. Dolayısıyla, vücudumuzdaki birçok sürecin düzgün çalışması için sağlam bir sirkadiyen saat gereklidir. Vücudumuzdaki sirkadiyen saat işleyişinin kontrol merkezi, ipRGC'ler tarafından algılanan ışığı baz alan SCN (suprakiazmatik çekirdek)'dir. SCN, hücresel saatleri ve periferik dokuları düzenler ve nöral veya hormonal sinyallerin yardımıyla bunları koordine eder. Bu salınımlı sistem, transkripsiyonel translasyonel düzenleyici geri besleme döngülerine (TTFL'ler) bağlıdır. Ana döngü, CLOCK, BMAL1 (pozitif kol) ile CRYler, PERler (negatif kol) içerir. Mekanistik olarak, CLOCK ve BMAL1 birbirleriyle sitozolde etkileşime girerler. Ardından bu heterodimer hücre çekirdeğine geçer *ve CRYPTOCHROME* (*CRY*) ve *PERIOD* (*PER*) promotorunda yer alan E-Box elementlerine bağlanır. CRY, PER ve kazein kinaz I izoformu epsilon (CK1ε) sitozolde complex oluşturarak hücre çekirdeğine girer ve CLOCK-BMAL1 tarafından regüle edilen kendi transkripsiyonlarını inhibe eder. Bu mekanizmada bir bozulma olduğunda hastalıklar veya bozukluklar ortaya çıkabilir. Bu bozulmuş mekanizmayı düzeltmek için küçük molekül modifikatörleri önemli birer araçtır.

Bu çalışmamda, CLK8 molekülünün 100 kat daha etkili bir türevi olan ABE2A adlı yeni bir küçük molekülü karakterize ettim. ABE2A, özellikle CLOCK ile etkileşimini

artırarak sirkadiyen ritmin genliğini artırırken CLOCK ile BMAL1 arasındaki etkileşimi azaltmaktadır. Yapılan deneyler, ABE2A'nın CLOCK, BMAL1'ın hücre çekirdeğindeki bulunma miktarını azalttığını ve BMAL1'ın sitoplazmadaki bolluğunu artırdığını ortaya çıkarmıştır. ABE2A, CLOCK protein seviyesini azaltırken PER2 seviyesini arttırmaktadır. Ayrıca *DBP, BMAL1* and *PER2* gibi saat genlerinin ifadesini düşürmektedir. Genel olarak, CLK8'e benzer şekilde, ABE2A hücre çekirdeği içindeki pozitif kol bileşenlerini azaltarak negatif kolun transkripsiyonunu sınırlar. Böylece TTFL'deki baskılama aktivitesi stabilize olur ve sirkadiyen ritmin genliği artar. Bu nedenle ve nanomolar dozlarda etkili olması sebebiyle, ABE2A, metabolik hastalıklar, ruh hali bozuklukları ve hızlanmış yaşlanma gibi azalmış genliğe bağlı sağlık sorunlarına karşı bir ilaç adayı olarak değerlendirilmektedir.

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ABBREVIATIONS

AMPK AMP: Activated Protein Kinase

bHLH: Basic Helix-Loop-Helix

BMAL1/ ARNTL1: Aryl hydrocarbon receptor nuclear translocator-like protein 1

CCGs: Clock-Controlled Genes

CDK5: Cyclin Dependent Kinase 5

CHX: Cycloheximide

CKIε/Iδ: casein kinase 1 epsilon/1 sigma

CLOCK: Circadian Locomotor Output Cycles Caput

Co-IP: Co-Immunoprecipitation

CRY: Cryptochrome

CT: Circadian Time

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

FASPS: Familial Advanced Sleep Phase Syndrome

FBXL3/21: F-box/LRR-repeat protein 3/21

GSK-3β: Glycogen synthase kinase 3 beta

HAT: Histone Acetyltransferase

HEK 293T: Human Embryonic Kidney Cells

ipRGC: Intrinsically Photoreceptive Retinal Ganglion Cells

LUC: Luciferase

MEF: Mouse Embryonic Fibroblast

NEAA: Non-Essential Amino Acids

NES: Nuclear Export Signals

NFIL3: Nuclear Factor Interleukin 3 regulated

NIH 3T3: Mouse fibroblast cells

NLS: Nuclear Localization Signal

NMDA: N-methyl-D-Aspartic Acid

NR1D1/2: Nuclear Receptor Subfamily 1 Group D Member 1/2

PACAP: Pituitary Adenylate Cyclase-Activating Polypeptide

PAS: Per-Arnt-Single-minded

PER: Period

PHR: Photolyase Homology Region

PTM: Posttranslational Modification

REV-ERB: Nuclear receptor subfamily 1, group D

RHT: Retinohypothalamic Tract

ROR: RAR-related orphan receptor

RORE: RAR-related orphan receptor response element

SCN: Suprachiasmatic Nucleus

SIRT1: Sirtuin 1

TAD: Trans-Activating Domains

TTFL: Transcriptional-Translational Feedback Loop

U2OS: Human bone osteosarcoma epithelial cells

ZT: Zeitgeber Time

Chapter 1:
INTRODUCTION

Circadian rhythms are the ~24-h periods of the processes in the body. This autoregulatory system drives many processes in our body. These are mental, physiological, biochemical, biological, and behavioral changes [1]. Circadian rhythms are also a universal mechanism because they can be exhibited by animals, plants, fungi, and cyanobacteria [2]. Additionally, almost every cell in the body contains a circadian clock. This natural mechanism can be synchronized by external clues called zeitgebers, like light, temperature, and nutrition [3]. As this system responds to these environmental cues, it has the ability to adapt to environmental changes. The other important aspect of the circadian clock is its self-sufficiency [4]. That is, it does not require external signals to work. The reason is that circadian-related machinery in our body is regulated by the master biological clock, located in the suprachiasmatic nucleus [5].

There are some positive and negative loops to regulate circadian rhythms in mammals. The molecular mechanism starts with the constitution of heterodimer by CLOCK and BMAL1[6]. This heterodimer binds to E-boxes (CACGTG), and this binding results in the transcription of *CRY1, CRY2, PER1, PER2*, and other clock-controlled genes (CCGs) [6]. The negative loop starts with PER and CRY proteins' going into the nucleus and displacement of CLOCK and BMAL1, which inhibit their transcription [6]. Some post-translational modifications (PTMs) are involved in these processes. For example, PER and CRY heterodimers interact with casein kinase Iε and glycogen synthase kinase-3, which allows them to be translocated into the nucleus and repress the CLOCK-BMAL1 heterodimer [7]. Additionally, PER1/2 proteins undergo ubiquitin-mediated degradation with phosphorylation by the 26S proteasomes [7]. The other feedback loop included nuclear receptor subfamilies: activator RORα and inhibitor REV-ERBβ [7,8]. There is competition between these two. Although REV-ERBβ represses BMAL1 transcription, RORα activates BMAL1 expression [7,8].

As a science field, chronobiology investigates biological rhythms and tries to understand their mechanism [5]. This leads to finding out the relationship between the

diseases and the defects in the system. This further allows new therapeutics to be discovered. Circadian clock dysfunction can be observed because of abnormalities in the internal mechanism or misalignment of external stimuli with the internal clock mechanism [9]. This type of dysfunction disturbs the healthy physiology and behavior of the organism. Thus, disease and disorder risks such as sleep disorders, neurological disorders, mood disorders, metabolic disorders, diabetes, obesity, cancer, cardiovascular diseases, and early aging risk rise [9].

Once the precision of circadian rhythm is perturbed, clock-modifying small molecules is one of the best tools to improve biological timing [10]. These compounds can modify either period, amplitude, or phase and thus have different molecular targets [11]. Additionally, they have some common properties which make them powerful tools. For instance, they have reversible and dose-dependent effects [9]. The phonotype they resulted in does not persist throughout the life of an organism, and their effect can be increased gradually and abolished afterward.

Our group recently discovered a small molecule CLK8 which binds to CLOCK and consequently reduces the interaction between CLOCK and BMAL1. This results in the enhancement of amplitude [9]. It was also found that with aging robustness of the circadian rhythm decreases. This means the amplitude of the circadian rhythm dampens [9]. Thus, this CLOCK-binding small molecule might slow down the aging process. This molecule might also have a role in treating some of the mood, metabolic disorders, and cancer.

In this thesis, I aimed to identify a more effective unique derivative of CLK8 as a drug candidate. For this purpose, some unique analogs of CLK8 were retrosynthesized in Mustafa Guzel's lab, and *in silico* analysis was conducted by Dr. Seref Gul. The ones with the highest score in docking were selected for *in-vitro* and *in-vivo* testing. I have tested seven of these derivatives and selected one, ABE2A, which can be a drug candidate. Even though ABE2A results in a similar phenotype in various genetic backgrounds as CLK8 does, it shows its effect at a dose as small as 0,100 μM, which makes it 100 times more effective than CLK8. This makes it a promising drug candidate.

Chapter 2 of the thesis is a literature review. It explains the circadian rhythm, its genetic and molecular mechanism, the relationship of circadian rhythm with diseases and disorders, and the role of small molecule modifiers.

Chapter 3 of the thesis is materials and methods and provides information about MTT assay, real-time monitoring of circadian rhythm, immunoprecipitation and pull-down assays, lentiviral production and transduction, sub-cellular fractionation, protein, and qPCR analysis.

Chapter 4 of the thesis is the results of the study, which shows the results obtained from several experiments.

Chapter 5 of the thesis is a deep discussion based on the results and conclusions.

Chapter 2:**LITERATURE REVIEW**

2.1 Biological Rhythms

Many species have biological rhythms, which are composed of circadian, ultradian, infradian, circannual, and seasonal rhythms [12]. These are classified based on the cycle lengths. For instance, circadian rhythms can be defined as the ∼24-h periodicity of the processes in the body as they evolved as an adaptation to World's Day and night cycles [13]. The other one, ultradian rhythms, are more frequent than 24h, like hormone secretions, while infradian rhythms are longer than 24h and shorter than one year, like menstrual cycles [12,14]. Circannual rhythms, however, are approximately one-year cycles, generally seen in breeding activities like reproductive endocrinology [12,14]. Seasonal rhythms are similar to circannual rhythms concerning cycle length; however, they depend on the environmental cues to be generated, such as bird migration patterns [12].

Some parameters should be satisfied for any process fit to circadian rhythm. These are the presence of endogenous, self-sustainable, 24h period rhythm; robustness; temperature compensation, and entrainablity [9]. That is, even though there is constant light or darkness, the rhythmicity should persist (free running), and under changing temperatures, period length, and rhythm pattern should not differ [9]. Additionally, circadian rhythm can be synchronized by an external stimulus such as light.

2.2 Circadian Rhythms

Many species have an adaptation system to 24h period of the solar day. This endogenous mechanism is called the circadian clock (Latin: circa = about; dies = day) [15]. Many plants, for instance, depend upon regulating the circadian clock to cope with diurnal changes. This helps plants to achieve fitness, enough growth, and proper development [16]. Another example is cyanobacteria. Many processes in cyanobacterium work in a circadian fashion. For instance, while the photosynthesis rate is maximum during the day, the nitrogen fixation rate is maximum at night [17]. Animals also require a system to cope with different tasks during the day and night. Thus, they have a circadian system that increases their chance of living and reproducing. In animals, many processes follow a circadian pattern. These are the sleep-wake cycle, heartbeat rate, hormone secretion, body temperature, and renal blood flow [18]. When compared to other animals, mammalians have a more complex circadian system. They have tissue-specific individual circadian clocks [23]. Thus, cells in different organs have a specific circadian rhythm. However, circadian rhythm is regulated by the suprachiasmatic nucleus (SCN) in the ventral hypothalamus [19]. The role of SCN is to coordinate the cellular clocks and peripheral tissues [19]. SCN has several types of GABAergic neurons [20]. Even though the exact mechanism is unknown, circadian pacemaking is achieved by intraneuronal communications between these neurons [20]. There are three types of neurons located differently in SCN, which are arginine vasopressin (AVP)- producing, vasoactive intestinal peptide (VIP)- producing neurons in the dorsomedial(shell) part, gastrin-releasing peptide (GRP)- producing neurons in the ventrolateral(core) part [20]. These work in signaling pathways. There are also retinorecipient neurons called photoreceptive retinal ganglion cells (ipRGCs) found in the retinohypothalamic tract (RHT) [21]. These

melanopsin-containing cells are responsible for sensing blue light [21]. Some studies also suggest that rod and cone cells in the retina help ipRGCs for this mission [22].

The master pacemaker SCN working mechanism starts with the sensation of phoetic input by ipRGCs and transfers the light signal to SCN through the hypothalamic track. After that, a temporal information signal in the form of neural or hormonal (sympathetic and parasympathetic) reaches peripheral tissues such as the pancreas, liver, adipose tissue, intestine, and skeletal muscle [23]. This signal can be body temperature change, humoral signals like glucocorticoids, feeding-related cues, and autonomic innervation [23].

Signal transductions start in postsynaptic SCN neurons. Predominantly two signaling pathways which are pituitary adenylate cyclase-activating polypeptide (PACAP) and *N*-methyl-d-aspartic acid (NMDA) glutamatergic signaling pathways in which cAMP and Ca2+ are involved, play a role in this process [24]. This results in some immediate early genes being induced. One of them is a core clock gene: *Period1* (*Per1*), which reset cellular oscillators [24].

A circadian rhythm comprises three parameters: amplitude, phase, and period. Amplitude can be defined as the distance between the peak and through [25]. This parameter defines the robustness of circadian rhythm. The other parameter, period, is the time interval between two peaks [25]. Phase, on the other hand, is a relative parameter. That is, it can be defined as the timing of a reference point (e.g., a peak) in the oscillation/cycle relative to a fixed (e.g., beginning of the day/night phase) event [25]. In order to define circadian rhythms, there are some standardized units of time: circadian time (CT), which can also be defined as intrinsic time, and Zeitgeber time (ZT) (German: Zeit = time; Geber = giver) [9]. While CT0 indicates the beginning of the subjective day, CT12 shows the night [9]. On the other hand, ZT0 implies the beginning of the day, and ZT12 is used for the night in a 12:12 light: dark cycle [9].

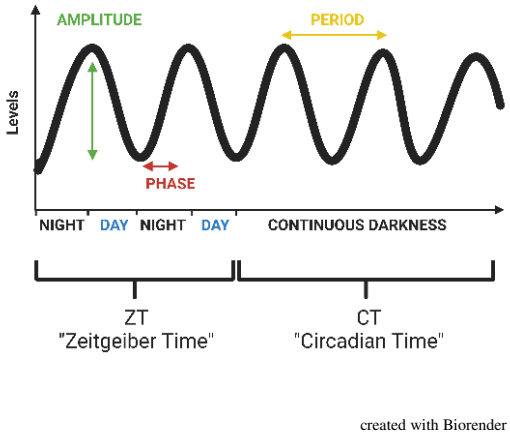


Figure 2.1: Parameters of circadian rhythm [25]

2.3 History of Circadian Clock

The presence of an endogenous rhythm was discovered in 1729 [26]. A French astronomer, Jean Jacques realized the continuous daily movement of leaves, i.e., opening during the day and closing at night; even the plant *Mimosa pudica* was in darkness for days [26]. This showed that there is an internal mechanism generating the rhythm in mimosa. Then, Augustin Pyramus de Candolle, a Swiss botanist, worked on *Mimosa pudica* in 1832 and noticed that the period of leaf folding was shorter than 24h, which was approximately 22h [9]. This showed that not only geophysical cycles but also an endogenous system regulate the circadian rhythm in plants. After these studies, Carl von Linne tried to predict petal opening/closing time and called it a “floral clock” [27]. However, the first genetic evidence showing the heritable period length in beans was suggested by Erwin Bünning [27]. Based on circadian oscillators, he measured seasonal changes and daily cycles by observing plants, which resulted in the origination of the field of circadian rhythms [27]. Then, the endogenous rhythms in bacteria, single-cell eukaryotes, insects, birds, rodents, primates, and humans were discovered. For example, studies on mutant *Drosophila melanogaster* allowed Ronald Konopka and Seymour Benzer to understand the genetic basis of circadian rhythms in 1971 [27]. In this study, gene mutants showed three phenotypes: shortening or lengthening period or arrhythmia. Then, the gene was found to be *Period (Per),* located in a single locus on X-chromosome [27]. Additionally, studies on the fungus Neurospora crassa showed the importance of a

frequency gene for persistent rhythm in conidiation [27]. Furthermore, the heritability of a timing mutation in hamsters was discovered by Martin Ralph and Michael Menaker [27]. These findings pushed scientists to conduct large-scale mutant screens in mice, and dozens of clock-related genes were discovered in prokaryotes and eukaryotes [27]. For instance, the group of Joseph S. Takahashi identified *Clock* (circadian locomotor output cycles kaput) gene; Aziz Sancar group identified *CRY2 (CRYPTOCHROME2)*, and the group of John B. Hogenesch identified BMAL1 (Brain and muscle Arntl-1 like) protein [9]. Now, it is known that all organisms have transcriptional/posttranslational feedback loops for a robust circadian rhythm to be generated, which was discovered by Micheal Young, Michael Rosbash, and Jeffrey Hall, and brought about a Nobel Prize in Physiology/Medicine in 2017 [27].

2.4 Molecular Mechanism

SCN and peripheral cells have autonomous oscillation system which arises from a transcriptional translational regulatory feedback loop. The positive arms initiate the transcription of clock-controlled genes and the core genes in the negative arms [9]. The negative arm components, on the other hand, repress the activity of proteins in the positive arms and thus downregulate their expression [9]. For the maintenance of the circadian clock, many parameters should work together. These are rhythmic expressions of the positive and negative arm components, their amount, stability, activity, and subcellular localizations [9].

2.4.1 Transcriptional-Translational Feedback Loops

In mammalians, primary regulators of circadian rhythm consist of positive and negative arms composed of E-boxes, D-boxes, and ROR-elements (RREs) (**Fig 2.2**). There are some transcriptional activators called CLOCK (Circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like 1/Arntl), which constitute a heterodimer [28]. These two belong to the basic helix-loop-helix; PerArnt-Single-minded (bHLH-PAS) family [29]. This heterodimer binds to E-boxes (CACGTG) [28,29]. The target genes are the ones with E-box cis-regulatory enhancer sequences such as *PER (PERIOD)* and *CRY(CRYPTOCHROME)* [28]. Thus, this binding allows the transcription of *CRY1, CRY2, PER1, PER2, PER3*, and other clock-regulated genes

(CRGs) in mammals [28]. This process is followed by PER and CRY proteins' going into the nucleus and displacing CLOCK and BMAL1, which inhibit their transcription [28].

There is a secondary stabilizing feedback loop including nuclear receptor subfamilies: activator RORα/β/γ, expressed by *ROR*α/β/γ, and inhibitor REVERBα/β, expressed by *NR1D1/2* [30]. There is a shared DNA binding element of REV-ERBβ and RORα called the RORE [7]. Thus, these two compete with each other. While REV-ERBβ represses BMAL1 transcription, RORα leads to BMAL1 transcription being activated [7]. These two regulators have an oscillatory pattern in a circadian fashion, which leads to BMAL1 being expressed in a circadian fashion [30].

There is a third TTFL consisting of the DBP (D site albumin promoter binding protein), which is a bZIP transcriptional activator, and NFIL3 (nuclear factor interleukin 3 protein), which is a bZIP transcriptional repressor [32]. The mission of these two is to regulate D-box-regulated genes. Also, these two are regulated by other genes. For instance, *DBP's* transcription is controlled by the rhythmic binding of CLOCK: BMAL1 to its E-box, while the transcription of *NFIL3* is controlled by RORs and REV-ERBs [32]. NFIL3 and DBP have an anti-phase as they compete for D-box elements and have opposite effects on target genes [32]. That is, *NFIL3* expression peaks when DBP deeps and vice versa. This loop mainly affects period length. For instance, the knockdown of *NFIL3* lengthens the period length, whereas the knockdown of *DBP* shortens the period length [32]. The opposite is observed if they are overexpressed.

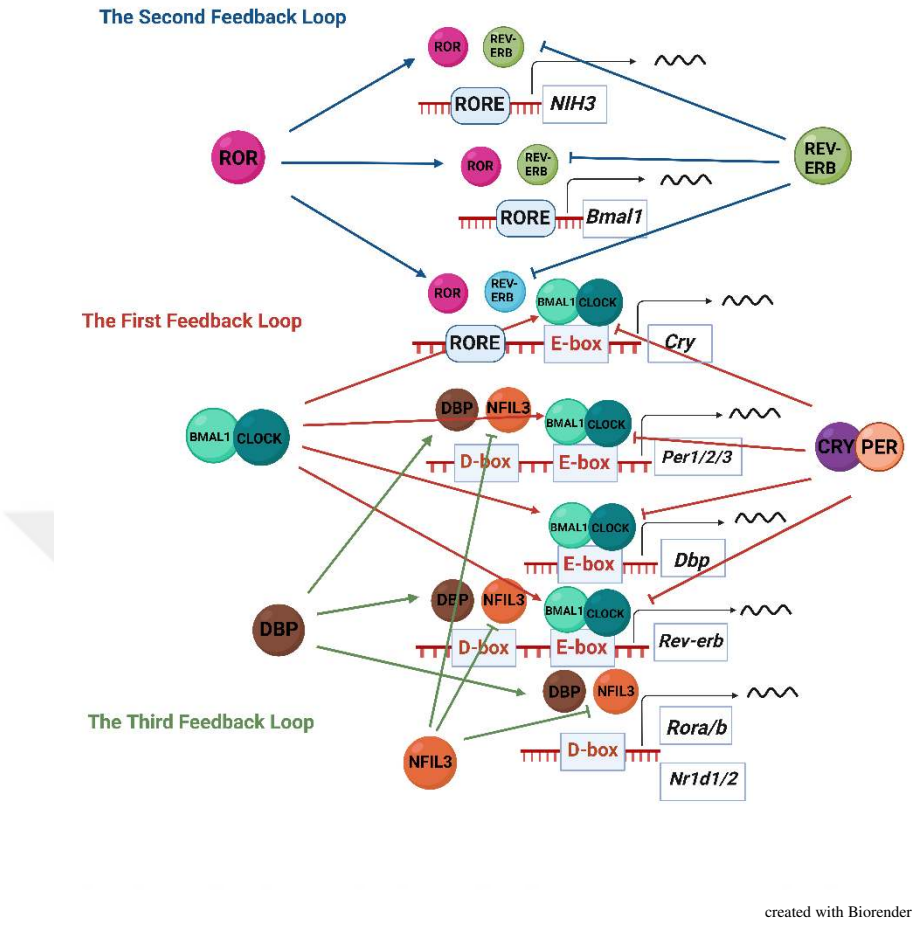


Figure 2.2: Feedback loops of mammalian circadian clock [33]

2.4.2 Post Translational Modifications

Besides these loops, post-translational modifications (PTMs) are involved in circadian mechanisms as regulators: phosphorylation, SUMOylation, methylation, and histone acetylation [7]. These types of regulations affect the proteins' stability, activity, and localization. For instance, PER and CRY heterodimer interacts with casein kinase Iε (CKIε) and glycogen synthase kinase-3 (GSK3) so that they are translocated into the nucleus and repress CLOCK-BMAL1 heterodimer [7].

The stability of PERs is highly affected by PTMs. PER1/2 proteins might have ubiquitin-mediated degradation by the 26S proteasomes due to phosphorylation with casein kinase (CK) Iδ and Iε at their distinct sites [7]. This degradation can reset the clock mechanism and make the CLOCK-BMAL1 complex active again. Furthermore, for

PERs, acetylation brings about an increase in stability. Additionally, a location in human *PER2* has a SNP p.Ser662Gly, which results in familial advanced sleep phase (FASP) syndrome [34]. This FASP site is critical for PER2 stability. Both O-GlcNAcylation and phosphorylation can be seen at this site [34]. Thus, there is a competition between O-GlcNAc transferase (OGT) and CKIs for this site [34]. Here, O-GlcNAcylation results in a drop in stability and a rise in repression, while phosphorylation results in instability.

CRYs are also susceptible to PTMs, which highly affect their stability. For instance, phosphorylation of CRY1 by AMP-activated protein kinase (AMPK) results in destabilization [35]. In addition, CRYs are ubiquitylated by FBXL3 (SCFFbxl3) in the nucleus and subsequently degraded [36]. On the other hand, another ubiquitin ligase FBXL21 has a dual role in CRYs. First, in the nucleus, it protects CRYs from FBXL3 degradation [37]. Second, in the cytoplasm, it leads to CRY degradation [37].

Even though the levels of PER and CRY proteins oscillate, CLOCK and BMAL1 levels do not show that type of oscillation [38]. Still, their phosphorylation pattern shows clear circadian oscillations [38]. Phosphorylation on CLOCK or BMAL1 changes their stability, nuclear localization, and degradation [39]. The main aim of the PTMs is to efficiently turnover CLOCK:BMAL1; otherwise, they need to be degraded for the system to work correctly, known as the transcription-factor cycling phenomenon [40]. Phosphorylation of the complex, for instance, decreases stability and increased transactivation activity [9]. While only GSK-3β is responsible for BMAL1 phosphorylation, in addition to GSK-3β, cyclin-dependent kinase 5 (CDK5) is involved in the phosphorylation of CLOCK [41,42].

There are also many other PTMs on CLOCK and BMAL1. These are ubiquitination, SUMOylation, which enhances the degradation and transactivation, respectively, and O-GlcNAcylation, which increases the stability of the complex [9]. Interestingly, it has been found that CLOCK harbors histone acetyltransferase activity (HAT) and acetylates its partner, BMAL1[43]. This brings about the recruitment of CRY1 to the complex and thus repression of transactivation [43]. On the other hand, deacetylation of BMAL1 is achieved by the NAD+-dependent SIRT1 enzyme so that

CRY1-based repression is removed [44]. The other PTM controlling the stability and transactivation of the complex is the SUMOylation of BMAL1 by SUMO2/3 (small ubiquitin-like modifier) [45]. BMAL1 SUMOylation was mediated by CLOCK [45]. In addition, as the level of SUMOylation increases, so does the phosphorylation level, which leads to transactivation, i.e., induction of circadian-clock genes by the complex [45].

2.4.3 Stochiometric Relationships of Clock Proteins

There should be a stoichiometric ratio between proteins in the positive and negative arms to observe a robust circadian rhythm. If this ratio is disrupted, then circadian oscillations alter. One study showed that, for instance, if CLOCK and BMAL1 are overexpressed in MEF (mouse embryonic fibroblasts) cells, the rhythm is dampened. [46]. It seems that PER:CRY oscillation is vital to persist robust rhythm. Thus, there should be sufficient feedback inhibition. On the other hand, the positive arm contributes to the robustness when the CLOCK:BMAL1 complex is post-translationally modified in a circadian manner[47]. It was found that both positive and negative arm complexes are predominantly located in the nucleus of the mouse [47]. Even though both complexes are equally present in nuclear fractionation, the rate-limiting factor is PER for stable complex formation of CLOCK-BMAL1 and PER-CRY [47]. Still, in a whole cell, for example, in MEFs, the abundance of CLOCK, BMAL1, and CRY1 are approximately equal and twice as PERs [47]. This shows that as a complex, there is a 1:2 ratio between negative and positive complexes.

Some studies showed that regulations on the positive arm affect the circadian rhythm, so over-expression of CLOCK and BMAL1 results in period shortening and lengthening, respectively [12]. In addition, while the overexpression of the complex dampens the amplitude, the knockdown enhances it [9]. For example, *if BMAL1* is knocked down by siRNA, circadian amplitude rises in *Bmal1*: d*Luc* U2OS (human osteosarcoma cell line) cells, probably because of the reduction of CLOCK:BMAL1 complex formation[48]. On the other hand, in the case of the negative arm, the opposite is observed. For instance, overexpression of PER in MEFs increases circadian rhythm amplitude [47]. Additionally, it was shown that downregulation of the *CRY1* genes results in low-amplitude rhythms or arrhythmicity in different cell lines and mice [49].

2.4.4 Nucleocytoplasmic Shuttling of Clock Proteins

In order to maintain the correct pace of the circadian clock, the location(nucleus/cytoplasm) of core clock proteins, their residence time, and the time of entry is critical. Many core clock proteins follow a cyclic pattern to enter the nucleus and export [50]. In order to do that, they should have nuclear import/localization and export signals, or they should bind to a partner who has these signals [50]. This intracellular movement occurs dynamically and affects the interaction partners, protein turnovers, and, thus, the overall function [50].

A study showed that while BMAL1 has a nuclear localization signal (NLS) and nuclear export signals (NES) located in its N-terminal and PAS domains, respectively, CLOCK does not have any of these [51]. Thus, only if CLOCK constitutes a heterodimer with BMAL1 it can go into the nucleus [51]. As mentioned previously, the accumulation of the CLOCK/BMAL1 heterodimer in the nucleus results in the transcription of clock-controlled genes and this heterodimer's degradation. On the other hand, all PER proteins have both NLS and NES sequences [52]. However, it is found that mammalian CRYs, which have NLS and NES, facilitate the nuclear entry of PERs once they form complexes [52]. In addition, phosphorylation by casein kinase Iε affects the nuclear localization of mPER1 and mPER3[52]. Furthermore, once mCRY1 binds to mPER2 from its C-terminal in the nucleus, mPER2 is protected from nuclear export, ubiquitylation, and proteasomal degradation [53].

2.5 The structure of Circadian Core Clock Components

The key components of circadian rhythm CLOCK (855 amino acids) and BMAL1 (626 amino acids) have similar domains [54]. These are Helix-Loop-Helix (bHLH) in the N-terminal and two tandem Per-Arnt-Sim (PAS-A & PAS-B) in the C-terminal and transactivation domains (TADs) [54] (**Fig 2.3**). Once dimerization interactions occur between the corresponding domains, some distinct protein interfaces form, which determine the stability and activity of the complex. These domains have different roles with respect to DNA binding, interaction of these two proteins, and post-translational modifications. For instance, bHLH domains bind the complex to E-box elements (CACGTG); however, PAS-A/B results in interactions between CLOCK and

BMAL1[55]. Thus, while bHLH relates to the stabilization/destabilization of the complex on the DNA, PAS-A/B affects the transcriptional activity of the complex [54]. Despite this similarity in domains, their spatial arrangements, and electrostatic potentials of the domains and, thus, proteins differ [54]. For instance, although CLOCK has mostly an overall negative charge (pI = 5.86 for the bHLH-PAS), BMAL1 has a positive charge or neutral (pI=9.01 for the bHLH-PAS) [54]. Because of that, PER1/2 and CRY1/2 proteins interact differently with CLOCK and BMAL1[54].

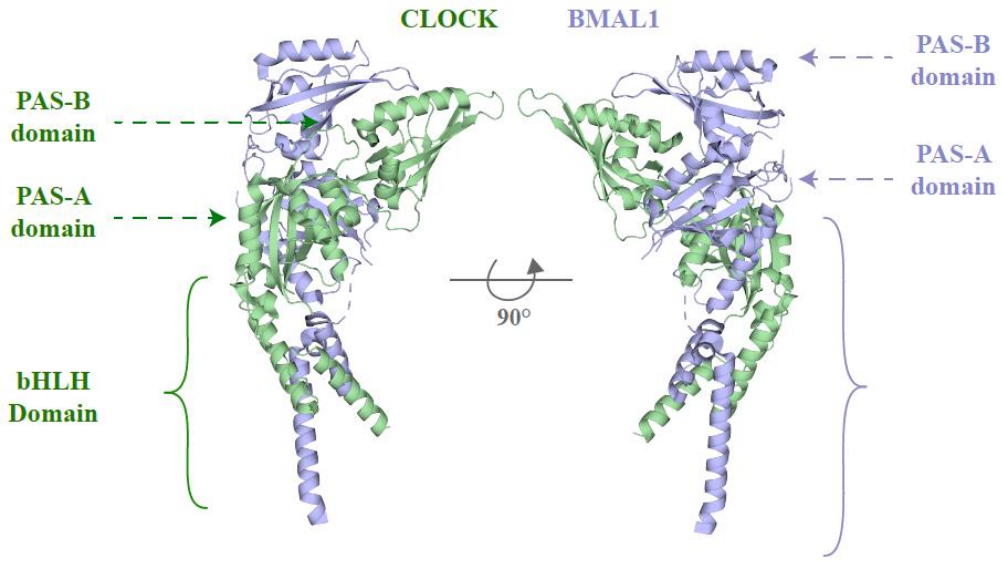


Figure 2.3: The structure of CLOCK:BMAL1 complex.
CLOCK and BMAL1 proteins are composed of N-terminal bHLH and the two tandems in C-terminal PAS-A and PAS-B domains and transactivation domains (TADs).

PER proteins show a highly similar structure to CLOCK and BMAL1. That is, they have bHLH and PASA/B domains [56]. However, CRYs have different structures. CRYs are composed of two domains which are core regions with high similarity to photolyases (photolyase homology region (PHR)) and C-terminal 'tails'[56]. These tails differ in length and sequence between the CRYs, between CRYs and photolyases, and among individual CRYs [56]. In mammalians, CRYs C-termini retain a very conserved helix resembling a coiled coil; that is why it is called CC-helix [57]. CC-helix region is responsible for competitive interaction with PER2 and FBXL3[57]. In this way, the stability of CRYs and subsequent events in circadian clock machinery are regulated.

There are two regions in the PHR domain: an α-helical domain (primary pocket) and an α/β domain (secondary pocket) [58]. The primary role of the secondary pocket is the interaction between the CLOCK PAS B domain [58]. Thus, it is responsible for the repression of BMAL1: CLOCK transactivation. There is also a FAD-binding pocket located in the α-helical domain of PHR, which regulates the interaction with FBXL3/21[58].

2.6 Circadian Rhythm and Disease

Modern life results in disruptions of the circadian clock as humans encounter environmental inputs incompatible with the internal regulation of the circadian clock. Sometimes abrupt changes in environmental conditions might result in these types of outcomes. That is, circadian rhythms can be disturbed via jet lag, night-shift work, or exposure to artificial light at night [59]. For instance, it was shown that shift workers have a greater chance of developing metabolic diseases, cardiovascular diseases, and cancer [60,61].

Whether the problem has a genetic base or not, several types of pathologies or disorders can be observed once the circadian machinery is corrupted. These are metabolic diseases (diabetes, obesity, metabolic syndromes), cardiovascular diseases, Alzheimer's disease, cancer, sleep disorders, brain disorders (autism, depression, and Parkinson's disease), mood disorders, and Jetlag [9,13,62]. The correlation between these diseases and the regulation of circadian genes has already been shown. For instance, abnormalities in the *Clock* gene result in metabolic dysfunction [63]. More specifically, a mutant version of *Clock* gene *ClockΔ19* has been shown to bring about obesity and insulin resistance in mice as there is no transactivation ability even though CLOCK can heterodimerize with BMAL1[63,64]. Another study showed a *Clock* mutant mouse showing hypercholesterolemia, hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and obesity [65]. *Bmal1* mutation, on the other hand, results in different phenotypes. One study suggests that *Bmal1* knockout mice experience premature aging and pancreatic β-cell dysfunction related to impaired insulin secretion [66]. Another study showed that BMAL1 bears a regulatory role in tumor cell apoptosis, cell-cycle progression, DNA damage response, and homeostasis regulation [67]. Thus, once down-regulated, BMAL1

promotes the development of tumors [67]. Another study showed that the primary regulators of circadian rhythm, CLOCK and BMAL, if induced, inhibit the entry from G1 to the S phase and suppress cell growth of SW480 cells (Human colon cancer cell line) [68]. These findings support the relationship between cell rhythm and cell cycle and, thus, cancer. In addition, when there is *Clock* and *Bmal1* simultaneous mutation in mice, glucose tolerance impairment, insulin secretion reduction, and deficiencies in size and proliferation of pancreatic islets were observed [69]. Furthermore, these types of defects worsen with age.

Mutations in *Per*, on the other hand, might have different outcomes. To illustrate, double knockout mPER2 increases plasma insulin levels and results in obesity [70,71]. Cancer is also related to PER proteins in many studies. For instance, the probability of cancer increases in mPer2-deficient mice [72]. Also, in humans, it was found that while the overexpression of *Per1* promotes DNA damage-induced apoptosis in cancer cells, deletion of *Per1* desensitizes cancer cells towards apoptosis [73].

Mutations in *Cry* result in many different phenotypes. For example, some studies showed that *Cry1/2*-double knockout mice have an elevation of blood glucose, reduced glucose clearance, a dramatic decrease in body weight, enhanced cortisol level, and cancer [63].

One of the major defects that might result from the abnormalities in core clock genes are sleep disorders, which can be divided into six categories: delayed sleep phase, advanced sleep phase, irregular sleep-wake phase, free-running, jet lag, and shift work type [74]. It was known that deficiencies in CRY and PER proteins are responsible for these syndromes/disorders. To illustrate, familial delayed sleep phase and attention-deficit/hyperactivity disorders are associated with mutations in *CRY1* [75,76]. On the other hand, polymorphism in *the PER2* gene is found to cause delayed sleep syndrome [77]. Also, the mutation in *PER2* or on *CK1δ* brings about familial advanced sleep phase syndrome (FASPS) [78,79].

2.7 Circadian Modifier – Small Molecules

Modern life results in the impairment of the circadian clock. In addition, there are several diseases resulting from genetic defects of many circadian clock genes. That is why there should be a way to modify the circadian rhythm to treat disorders. One of them is using small molecules targeting core clock proteins. Small molecules have many advantages over other techniques, such as knockout and RNAi-based. For instance, small molecules have a reversible effect, and their effect is time- and dose-tunable [9]. That is, their effect can be increased or decreased by rising or dropping their concentrations, respectively, and it can be done in a time-dependent manner. Additionally, once removed from the environment, their effects are lost.

In order to identify potential small molecules, several methods are used. The first one is high-throughput screening (HTS) [79]. In this method, a reporter cell line expressing endogenous/exogenous luciferase (*Bmal1*-d*Luc* or PER2::LUC) is used, and bioluminescence is monitored after the synchronization with forskolin/dexamethasone in the presence and absence of molecule for several days[79]. In this method, the effect of the related molecule on phenotype can be observed. That is, the effect of the related molecule on period, phase, and amplitude can be detected [80]. However, the exact target of the molecule cannot be determined. Even though many molecules are identified with this technique, only one molecule KL001 (a CRY stabilizer), is found to be targeting core-clock proteins [81]. The second method is the target-directed approach. This screening method requires prior knowledge of known ligands and/or binding cavity structures of targets [80,82]. Molecules discovered using this method are, for instance, IC261, a known CK1 inhibitor, which increases period length [83]. There are also other molecules that were later identified as circadian modifiers due to their known effect on GSK-3β and REV-ERBα (Table 2.1). The third method is developed from HTS, which is in-silico virtual screening (VS) [84]. It is a computational technique to search small molecule libraries and identify molecular structures most likely to bind to a drug target [84]. This screen can be structure-based or ligand base [84]. Virtual screening is conducted so that compound libraries are docked onto the three-dimensional structure of specific targets based on specific binding sites of the molecules on the target [84]. Finally, a binding score is determined, and a subset of compounds is selected for further analysis, such as testing

in luciferase-expressing cell lines to observe phenotypic effect [84]. This method is advantageous because it is more precise, fast (computationally, millions of molecules can be screened simultaneously), and cost-efficient [84]. This method brought about essential molecules to be discovered. For instance, a drug targeting molecule CLK8 was discovered by virtual screening by our group [9]. In addition, virtual screening on the CRY1 FAD binding packet resulted in the discovery of M47 by our group. M47 interacts with the CRY1 from Arg-293 and Trp-399 amino acid residues and lengthens the circadian period [49].

Table 2.1. Small molecule modifiers of circadian clock [13,12]

Name	Target	Phenotype	Reference
CLK8	CLOCK	Amplitude enhancer	21
KL001	CRYs	Period lengthener and amplitude reducer	133
KS15	CRYs	Amplitude enhancer	138
SP600125	CKIδ/ε, JNK	Period lengthener	147
KL101	CRY1	Period lengthener	137
TH301	CRY2	Period lengthener	137
TG003	CKIδ/ε, CLK	Period lengthener	147
SR9011/SR9009	REV-ERBs	Amplitude enhancer	140
PF-4800567	CKIε	Period lengthener	141
LH846	CKIδ	Period lengthener	142
Cmpd-1	CKIε, CKIδ?	Period lengthener	149
CK01	CKIδ/ε	Period lengthener	143
CKI-7	CKIδ/ε	Period lengthener	144
D4476	CKIδ/ε	Period lengthener	145
PF-670462	CKIδ/ε	Period lengthener	146
PD169316	CKIδ/ε, P38	Period lengthener	147
SB202190	CKIδ/ε, P38	Period lengthener	147
Roscovitine	CKIδ/ε, CDK	Period lengthener	147
Longdaysin	CKIδ/ε, CDK7, ERK2	Period lengthener	148
GSK4112	REV-ERBα	Amplitude enhancer	139
Cmpd-2	CKIε, CKIδ?	Period lengthener	149
Cmpd-3	CKIε, CKIδ?	Period lengthener	149
SU5416	CKIδ/ε, VEGFR PTK	Period lengthener	147
IC261	CKIδ/ε	Period lengthener	73
DRB	CKIδ/ε, CK2	Period lengthener	147
PPT	CKIδ/ε, ERα agonist	Period lengthener	147
M47	CRY1	Period lengthener	134
CGS-15943	CKIδ/ε, AR agonist	Period lengthener	147
SR8278	REV-ERBs	Period reducer	61
Nobiletin	ROR agonist	Amplitude enhancer and period lengthener	9
T0901317	ROR agonist	Bmal1 expression reducer	62
Metformin	AMPK activator	Period lengthener and amplitude reducer	63
Lithium	GSK3β inhibitor	Period reducer	64
SRTCD1023	SIRT1 activator	Period lengthener and amplitude reducer	65

Chapter 3:
MATERIALS AND METHODS

3.1 Retrosynthesis, Purification, and In-silico Analysis

This part of the study was conducted by Mustafa Guzel's Lab (Department of Medical Pharmacology, International School of Medicine, Istanbul Medipol University, Turkey).

Firstly, retrosynthetic analysis of CLK8 was conducted to synthesize derivatives of CLK8 by using its own structure. Secondly, some molecules were synthesized. During this retrosynthesis, some side products formed in addition to the main products. Thirdly, dockings and molecular dynamic simulations were performed on these molecular structures. The ones that can bind to the same location of CLOCK and have similar or better binding energy compared to CLK8 were chosen. In addition to this, we also calculated binding energy per heavy atom **(Table 3.1)**. Since larger molecules produce better binding energy values in docking analysis to better understand the affinity of small molecules, we considered the binding energy per atom. Then, these molecules were purified by silica gel chromatography or crystallization. Finally, liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance spectroscopy (NMR) analysis were conducted so that only the purest molecules were selected to be subjected to biological tests.

Table 3.1: Binding energies of CLK8 and derivative molecules.

Molecule	Vina Binding Energy (kcal/mol)	Binding Energy per Atom
ABE-1A	-5.2	-0.35
ABE-1B	-6.2	-0.29
ABE-1C	-6.9	-0.28
ABE-2A	-5.4	-0.34
ABE-2C	-6.4	-0.29
ABE-8A	-6.9	-0.29
ABE-20	-7.2	-0.18
CLK8	-8.2	N/A

3.1.1 Docking

Protein Data Bank (PDB ID 4F3L) was used to obtain the primary structure of the CLOCK protein. NAMD software was used with the CHARMM force field for MD simulations. Crystallographic water molecules were removed. Then, hydrogen atoms were added and converted to PDBQT format using AutoDockTools4. To estimate the protein-ligand affinity and predict the most favorable binding conformations of the compounds AutoDock Vina was employed. Since derivative molecules were designed based on the CLK8 structure, we docked molecules to the binding site of CLK8 that was reported before [9]. The exhaustiveness parameter was set to its default value. We used Pymol 2.4.0 (open source) (http://pymol.sourceforge.net/) to visually examine molecules' binding position and produce a protein-ligand interaction graph.

3.2 Cell culture

The following cell lines were used in this thesis: Human embryonic kidney cells (HEK 293T), human osteosarcoma cell line (U2OS *Bmal1*-d*Luc*), Clock-knockout human osteosarcoma cell line (*CLOCK* knockout U2OS *Bmal1*-d*Luc*), primary mouse skin fibroblast cells (MSF), and mouse fibroblast cell line (NIH 3T3 *Bmal1*-d*Luc*). All these cell lines but HEK and MSF stably express destabilized luciferase under the control of the *Bmal1* promoter. For each cell line, D10 medium (Dulbecco's Modified Eagle Medium) with high glucose supplemented with 10% heat-inactivated Fetal Bovine Serum (Thermo Scientific) was mixed with 100µg/mL Penicillin/Streptomycin cocktail. Incubation conditions were 37°C under 5% CO_2 and 95% humidity in the incubator. When the nutrients were depleted in the medium, the medium was replaced with a fresh one. On the other hand, whenever cells reached 90-95% confluency, subculturing was performed. So that the conditions of cells were kept well. For subculturing, firstly medium was aspirated with a vacuum, and cells were washed with 1X PBS (5mL for 100mm plate) and vacuumed. Secondly, they were trypsinized with Trypsin-EDTA (Multicell, Cat. No 325-043-EL) (1mL for 100cm plate) and incubated for 2 minutes to make cells detached. Thirdly, to inactivate the trypsin, D10 medium was added to cells (3 times of added trypsin), and cells were physically detached from the surface of the plate by pipetting. Finally, cells were split into new 100mm cell culture dishes with a 1:6 ratio.

3.3 Cell Cytotoxicity Assay

In order to measure cell viability, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) calorimetric assay was performed on U2OS *Bmal1*-d*Luc*, NIH 3T3 *Bmal1*-d*Luc*, and MSF cells. Firstly, cells were seeded into a clear 96-well plate ($4x10^3$ cells/well) as 200ul/well as triplicates and incubated at 37°C in a humidified incubator under 5% CO_2 for 48 hours. Secondly, cells were treated with appropriate amounts of ABE2A or 0.25% DMSO (positive control) or 5% DMSO (negative control) in 20 µl/well of DMEM (ThermoFisher Gibco, 11965092) and incubated for another 48 hours in the same conditions. Thirdly, cell media was replaced with a 100 µl/well mixture containing 20% MTT reagent (final concentration: 1mg/mL) and 80% DMEM and incubated for 3-4 hours to let formazan salts form. Fourthly, the media was exchanged with 200ul/well of DMSO: EtOH (1:1) mixture and formazan salts were dissolved by pipetting. Finally, the absorbance (A) of the wells at 600 and 690nm was measured by Synergy H1 Microplate Reader (Bio-Tek Instruments), and the cell viability was calculated according to Equation 3.1.

$$((A600nm - A690nm) \text{ of ABE2A } / (A600nm - A690nm) \text{ of DMSO})) \times 100$$

Equation 3.1. Cell viability calculation formula.

3.4 Luc Degradation Assay

HEK 293T cells were seeded into an opaque 96-well plate ($4x10^4$ cells/well) as triplicates by using D20 medium (Dulbecco's Modified Eagle Medium with 20% FBS), and during this process reverse transfection technique was employed. For this, cells were transfected with PEI (final concentration: 6ng/µL) using 4ng pcDNA4-Luc plasmid and 296ng empty pCMV-Sport6 plasmids in DMEM (equal to D20 amount per well) to complete a total amount to 300ng per well. Total volume should be 100ul/well (50ul DNA/DMEM/PEI mix and 50ul cells in 2X medium). Then, cells were incubated at 37°C under 5% CO_2. Secondly, after at least 19h and at most 24h from reverse transfection, cells were treated with appropriate doses of ABE2A or DMSO (0,25%) in 10 µl DMEM and incubated for 24h at the same condition. The next day, the mixture of HEPES-NaOH (final concentration: 10mM, pH 7.2) and luciferin (final concentration: 0.4mM) was added in the dark into the medium of the cells. Then, cells were incubated at 37°C under

5% CO_2 for an hour. Thirdly, cells were treated with CHX (final concentration: 20μg/ml), which inhibits protein synthesis, and DMEM mixture as 10ul to each well. Finally, the plate was sealed with an optically clear film, and a Synergy H1 Microplate reader was used to record luminescence readings every 15 minutes at 32°C for 24 hours. One-phase exponential decay function was used in order to calculate the protein half-life.

3.5 Real-time Monitoring of Circadian Oscillation

For real-time monitoring, U2OS *Bmal1*-d*Luc*, *CLOCK* knockout U2OS *Bmal1*-d*Luc*, NIH 3T3 *Bmal1*-d*Luc,* and MSF cells were used to see the circadian oscillation profile. LumiCycle 32 (Actimetrics, USA) was used to record luminescence signals.

Cells expressing robust endogenous luciferase, e.g., U2OS *Bmal1*-d*Luc*, NIH 3T3 *Bmal1*-d*Luc*, were plated in a 35mm cell culture dish at a density of 40×10^4 cells/plate, and they were incubated overnight at 37°C under 5% CO_2. The next day, to synchronize cells, the cell medium was replaced with 0.1μM Dexamethasone (Sigma D-8893) containing DMEM and incubated for 2 hours. Then, cell media was exchanged with different doses of ABE2A or DMSO containing recording medium (10g low glucose DMEM powder (Sigma D2902), 3.5mg/mL D(+) glucose powder (Sigma G7021), 0.35mg/mL sodium bicarbonate (Sigma S5761), 10mM HEPES (Gibco 15630106), 5%FBS (Gibco, 16000044), 1% NEAA (Multicell 321-011-EL), 25U/mL streptomycin-penicillin (Gibco 15140-122) supplemented with 0.1mM Luciferin). Finally, silicone grease was used to seal plates to prevent medium evaporation, and plates were placed in LumiCycle. The instrument recorded the luminescence for each plate for 5-7 days with 10 minutes intervals continuously at 37°C. For the analysis of circadian clock parameters (amplitude, period, phase) LumiCycle Analysis software (Actimetrics) was used. The first-day readings were excluded from analysis due to transient deviations caused after applications such as drug treatment and medium change.

Different protocols were applied for the cells that do not express endogenous luciferase or those that lost their ability to express because of a gene knockout, e.g., MSF and *CLOCK* knockout U2OS *Bmal1*-d*Luc*. Cells were plated in a 35mm cell

culture dish at a density of 10×10^4 cells/plate and incubated overnight at 37°C under 5% CO_2. The next day, cells were transduced with lentiviral particles: Cell medium was replaced with a virus-containing master mix including 750µl of virus-containing aliquots 750µL, 250uL DMEM, and 8µg protamine sulfate. Cells were incubated for 16 hours. Finally, cell media was replaced with fresh 1.5mL D10. Two days later (72 hours post-transduction), cells were synchronized with 0.1µM Dexamethasone (Sigma D-8893) containing DMEM and incubated for 2 hours, and the exact protocol above (after synchronization) was followed.

3.6 Lentivirus Production and Transduction

Firstly, HEK 293T cells were seeded in 100mm plates as 2.5×10^6 cells/plate and incubated overnight at 37°C under 5% CO_2 to reach a ~70-80% confluency a day after. Secondly, the next day in the late afternoon, cells were transfected with 10µg pLV6-*Bmal1*-d*Luc*, 9µg pCMV-ΔR8.2dvpr packaging, and 1µg pCMV-VSVG envelope vectors in 400ul of DMEM with 20ul of PEI. This cocktail was incubated at room temperature for 15 minutes, and cells were transfected. Before the transfection, 5 ml of the medium was removed from the cells, and the transfection was applied. Cells were incubated overnight at 37°C under 5% CO_2.

After 16 hours of transduction, 3mL of fresh D10 medium was added to the plates. Then, after 48 hours of transfection, the cell medium was replaced with an 8mL fresh D10 medium. Lentiviral particles were harvested at 72h post-transfection and stored in a 50ml falcon. Then, 8mL of fresh D10 medium was added to the cells. The second harvest was conducted at 96h post-transfection and mixed with the first. Finally, collected particles were filtered through a 0.45µM syringe filter and aliquoted as 750µL for storage at -80°C for further use.

Transduction protocol was applied on MSF and *CLOCK* knockout U2OS *Bmal1*-d*Luc* cells. They were transduced with *Bmal1*-d*Luc* lentiviral particles to make them suitable for luciferin-based assays. Firstly, cells were seeded in a 35mm cell culture dish at a density of 10×10^4 cells/plate, and they were incubated overnight at 37°C under 5% CO_2. The next day, cells were transduced with lentiviral particles: Cell medium was

replaced with a virus-containing master mix including 750ul of virus-containing aliquots, 250uL DMEM, and 8µg protamine sulfate. Cells were incubated for 16 hours. Finally, cell media was replaced with fresh 1.5mL D10. Then luminescence protocol was applied as indicated in section *3.5*.

3.7 Transfection

To produce CLOCK and BMAL1 overexpressed cells, HEK 293T cells were transiently transfected with pCMV-Sport6-*Clock* or 10µg pCMV-Sport6-*Bmal1* or FLAG-tagged pcDNA4a-*Bmal1* or FLAG-tagged pcDNA4a-*Clock* depending on the following protocol applied.

First, cells were seeded to 100mm plates as 2.5×10^6 cells/plate and incubated overnight at 37°C under 5% CO_2 to reach a ~70-80% confluency the next day. Then cells were transfected with 10µg of the appropriate plasmid mixed with 30µL PEI, and the total amount was completed up to 500ul with DMEM. Cells were harvested between 24 to 72 hours post-transfection, with 1mL ice-cold 1X PBS, and centrifuged at 5000xg for 7 minutes at 4°C, and supernatants were discarded. During this harvest, cells from 100mm plates were divided into five 1.5mL Eppendorf tubes so that each tube had cells transfected with 2µg of the plasmid. For storage cells were kept at -20°C.

3.8 Pull Down Assay

Cell pellets previously transfected with FLAG-tagged pcDNA4a-*Clock* were used for pull-down assay. It was important to use fresh cell pellet, i.e., 2 weeks stored at most. Firstly, the pellet was lysed with 500µL lysis buffer (50mM Tris-HCl pH 7.4, 2mM EDTA, 1mM MgCl2, 0.2% NP-40, 1mM Na3VO4, 1mM NaF, 1X PIC) and incubated on ice for 20 minutes and vortexed. Then, the sample was centrifuged for 10 minutes at 7000xg, 4°C. Secondly, 2/3 of the supernatant was taken to a new Eppendorf tube. This dilution was conducted to be able to observe the effect of the molecule. Then, the lysate was mixed with 500ul of 2X binding buffer (100mM Tris-HCl pH 7.4, 300mM NaCl, 0.2% NP-40, 2mM Na3VO4, 2mM NaF, 2X PIC) at 1:1 ratio. Finally, from this 1ml mixture, 60µl of input was taken to a new Eppendorf tube, and the remaining part was divided equally into three separate microcentrifuge tubes for different applications. Each

tube was incubated with DMSO or 40μM biotinylated-CLK8 or 40μM biotinylated-CLK8 and 400μM non-biotinylated ABE2A(competitor) at 4°C for 90 minutes by continuous rotation. Meanwhile, the NeutrAvidin Agarose resin (Thermo Scientific 29201) was equilibrated (30μL settled resin/sample). The manufacturer's protocol was used to equilibrate the resin. Then, lysates were supplemented with equilibrated NeutrAvidin resin and incubated overnight at 4°C with constant rotation. The next day, samples were taken from the rotator and centrifuged at 2500xg for 2 minutes, resin parts were precipitated, and supernatants were discarded. Secondly, the tubes consisting of resin were washed 3 times for 5 minutes with 1X binding buffer with constant rotation at 4°C. After each rotation, tubes were centrifuged at 2500xg for 1 minute. Finally, the resin of each sample was boiled with 1xLaemmli buffer for 7 minutes at 95°C. Samples were stored at -20°C for SDS-PAGE analysis.

Samples subjected to the SDS-PAGE. Then, Western blot analysis was conducted with PVDF membrane (Millipore). Upon completion of the transfer of the proteins, the membrane was washed with 0.15% TBS-T for 5 minutes and then incubated with 5% milk powder prepared in 0.15% TBS-T for an hour. Afterward, the membrane was incubated with anti-FLAG (Sigma, A9469-1MG) antibody diluted in 5% milk powder in 0.15% TBS-T. Finally, the membrane was washed with TBS-T three times and incubated with secondary antibody conjugated with HRP. In order to observe the protein bands, homemade ECL was applied on membrane, and the chemiluminescence images were taken with Bio-Rad.

3.9 Co-immunoprecipitation (Co-IP)

The FLAG-tagged pcDNA4a-*Clock* and pCMV-Sport6-*Bmal1* cell pellets were used in this assay. Firstly, cells were lysed with 130μL Lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% Triton X-100, 1X PIC) and incubated on ice for 30 minutes by vortexing with 15 minutes intervals. Then, lysates were centrifuged at 15000xg for 20 minutes, and supernatants were transferred into new tubes. Thirdly, for each fraction, the total amount of CLOCK was diluted to 1/150, and BMAL1 was diluted to 1/200 with lysis buffer up to 130μl for each. If required, PierceTM 660nm Protein assay (Thermo Scientific, 22660) was used to measure the protein concentration of each lysate. Then,

CLOCK and BMAL1 lysates were mixed. From this mixture, 30 µL of input was taken and boiled with 10µL 4X Laemmli buffer at 95°C for 7 minutes and stored at -20°C. Meanwhile, Anti-Flag M2 Affinity Gel (Sigma-Aldrich, A2220) was equilibrated. First, 10 µL of resin was taken for each sample and centrifuged at 8000g for 1 minute; supernatant glycerol was discarded. Second, 5 times more 1X TBS buffer was added to the resin, and the tube was swirled 10 times and centrifuged at 5000g for 30 seconds (2 times). Third, 5 times more glycine-HCl (0.1M, pH=3.5) buffer was added to the resin, and the tube was swirled 10 times and centrifuged at 5000g for 30 seconds. Fourth, 5 times more 1X TBS buffer was added to the resin, and the tube was swirled 10 times and centrifuged at 5000g for 30 seconds (2 times). Finally, 5 times more 1X TBS buffer was added onto the resin, mixed with pipetting, and divided equally into the new Eppendorf tubes for each application (DMSO/ABE2A) and then centrifuged at 5000g for 1 minute, and the supernatants were discarded. After resin equilibration was completed, the cell lysate mix (CLOCK&BMAL1) was divided into two fractions into these resin tubes. While one fraction is treated with DMSO and the other with 40 µM of ABE2A. Then, these CLOCK-FLAG and BMAL1-S6 containing resin tubes were incubated overnight with constant rotation. The next day these tubes were centrifuged at 5000g for 1 minute, and supernatants were discarded. Resins were washed with 1X TBS buffer and centrifuged at 5000g for 30 seconds (2 times), washed with 1X TBS buffer, and centrifuged at 7000g for 1 minute (1 time). Supernatants were discarded. Then, the bound proteins were eluted with 30µL 4X Laemmli buffer at 95°C for 7 minutes. Finally, samples were quickly vortexed and spun, and loaded into 8% SDS-PAGE, which was followed by Western blot in which Anti-FLAG (Sigma, A9469-1MG) and anti-BMAL1 (Santa Cruz Biotechnology, SC-365645) antibodies and PVDF membrane (Millipore) was used.

3.10 The effect of ABE2A on core clock proteins

U2OS *Bmal1*-d*Luc* cells were used to detect the effect of ABE2A on core-clock proteins. Cells were seeded with a density of 40×10^4 cells/well into 6 well-plates and incubated at 37°C under 5% CO_2 overnight. So, the next day, they reached confluency and were ready to be treated with the molecule. Unsynchronized cells were directly treated with 0,625 µM of ABE2A (final 0.25% DMSO) or 0.25% DMSO and incubated

for 24 hours. Then, cells were harvested once with 1mL ice cold- 1X PBS, and pelleted at 5000xg for 7 minutes at 4°C and stored at -20°C. On the other hand, synchronized cells were synchronized before molecule treatment. First, their medium was replaced with DMEM, including 0.1μM Dexamethasone. Then, cells were subjected to the 0,625 μM of ABE2A (final 0.25% DMSO) or 0.25% DMSO and incubated for 24 hours. Then, cells were harvested at 6 hours intervals for 7 time points (24H-60H) and stored at -20°C.

Harvested cells were lysed with 90μL RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton x-100, 0.1% SDS, 1X PIC) and incubated for 30 minutes on ice by vortexing with 15 minutes intervals. Then, samples were centrifugated at 15000xg for 20 minutes at 4°C, and supernatants were taken to new Eppendorf tubes. Finally, the lysates were eluted with Laemmli buffer at 95°C for 7 minutes. Samples were quickly vortexed and spined and loaded into 10% SDS-PAGE as 15μg which was followed by Western blot in which anti-CLOCK(Bethyl, A302-618A), anti-BMAL1(Santa Cruz Biotech., SC-365645), anti-PER2(Proteintech, 67513-1-Ig), anti-CRY1(Bethyl, A302-614A), and anti-β-Actin(Cell Signaling, 8H10D10) antibodies and PVDF membrane (Millipore) were used.

3.11 Total RNA Isolation, cDNA Conversion and RT-qPCR

The effect of ABE2A on core-clock genes was explored through RT-qPCR. Manual RNA isolation with TRIzol reagent (Invitrogen, Cat. No. 15-596-018) was conducted on ice under the RNA hood for synchronized U2OS *Bmal1*-d*Luc* cells. The synchronization procedure was applied as indicated in section *3.10*. One important difference is that cells were stored at -80°C after harvesting as they were used for RNA isolation.

Before starting RNaseZap (Ambion) was applied in the hood and on any equipment that will be used. In the procedure, cell pellets were resuspended in 4°C cold 1mL TRIzol reagent. Secondly, 200μL chloroform was added to each sample, and the mixture was incubated at room temperature for 5 minutes, and the tubes were swirled 10 times. Thirdly, samples were centrifugated at 14000xg for 20 minutes at 4°C, and only the aqueous transparent phase was transferred to a new microcentrifuge tube. Then, it was

mixed with a 1:1 ratio of isopropanol and 12µg glycogen for each sample. Fourthly, mixtures were incubated at -80°C overnight. The next day, samples were centrifugated at 14000xg for 20 minutes at 4°C, and the pellets formed at the bottom of the tubes. Then, pellets were gently washed with 1mL of 75% ethanol and centrifuged at 14000xg for 5 minutes at 4°C (3 times). After supernatants were discarded, the pellets were left to air-dry at room temperature under the hood without airflow. Then, RNA pellets were dissolved in 30µL nuclease-free water. The quantity of RNAs was analyzed with Nanodrop 2000 (Thermo Scientific), and the quality was controlled with agarose gel electrophoresis. 0.8% agarose gel prepared with DEPC (diethylpyrocarbonate) treated water for agarose gel electrophoresis. It was performed at 100 Volt (V) for 15 minutes. RNA bands and any contamination such as gDNA bands were visualized under UV light. RNA samples were stored at -80°C.

Thermo Scientific's RevertAid First Strand cDNA Synthesis Kit was used as described in the product's manual for cDNA conversion of RNAs. One reaction involves 0.5µg RNA template, 5µM Oligo(dT)18 primer, 1X Reaction Buffer, 1U/µL RiboLock RNase Inhibitor, 1mM dNTP mix, 10U/µL RevertAid M-MuLV RT, and up to 12 µL RNase free water. In the end, cDNAs were diluted with nuclease-free water as 1:5 and stored at -80°C.

For Real-Time quantitative PCR (RT-qPCR) assay, 1:40 diluted cDNAs were used. The reaction mixture containing 8µL 2X SensiFAST SYBR® No-ROX Mix (Bioline), 1µL forward and reverse primer mix (final concentration 0.5µM from each), 8µL nuclease-free water, and 3uL 1:40 diluted cDNA was prepared for each sample. The complete list of the primers is given in Appendix B. As the reference gene RPLP0 (ribosomal protein, large, P0) was used. The cycling protocol was performed using CFX Connect Real-Time PCR detection system (Bio-Rad), which consists of 2 minutes of initial denaturation at 95°C followed by 39 amplification repeats which subsequently include 5 seconds at 95°C, 30 seconds at 60°C and 20 seconds at 72°C. Additionally, fluorescence signals were recorded upon each amplification step. After completing the amplification steps, reaction mixtures were incubated for 5 seconds at 95°C to form a melt curve from 65°C to 95°C with 0.5°C increments for 5 seconds for each temperature. Moreover, in conditions where the melting temperature of the primers highly differ, the

annealing step was conducted as a gradient. Delta-delta Ct (2-ΔΔCt) method was used for the analysis.

3.12 Subcellular Fractionation

Subcellular fractionation was performed to explore the effects of ABE2A on the subcellular localization of core-clock proteins. A day before small molecule treatment, U2OS *Bmal1*-d*Luc* cells were plated as 40×10^4 cells/well in 6 well plates and incubated at 37°C under 5% CO_2 overnight. The next day, cells were treated with 0.1μM Dexamethasone (Sigma D-8893) in DMEM medium for synchronization and incubated for 2h. Then, the medium was replaced with DMSO (0.25%) or 0,625μM ABE2A containing D10. The harvest time of the cells was selected based on the time when the CLOCK amount is the highest after synchronization, which is 30h [13]. Thus, after 30 hours of incubation, cells were harvested with 1mL ice cold- 1X PBS, centrifuged at 5000xg for 7 minutes at 4°C, and supernatants were discarded. These newly pelleted fresh cells were used for fractionation assay. First, pellets were dissolved with 200μL Cytosolic Lysis Buffer (CLB) (10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA pH 8.0, 0.05% NP-40, 1X PIC) by pipetting and for 10 minutes incubated on ice. Then, samples were centrifuged at 660xg for 5 minutes at 4°C. Supernatants were separated as cytosolic fractions, and to obtain nuclear fractions, pellets were kept. Pellets were washed with 200μL CLB buffer and centrifugated at 660xg for 5 minutes at 4°C (3 times) to remove any cytosolic part. To the cell pellets, 100μL Nuclear Lysis Buffer (NLB) (20mM HEPES pH 7.9, 0.4M NaCl, 1mM EDTA, 10% Glycerol, 1X PIC) was added and mixed with pipetting. Then, these were subjected to sonication at 60% power, 10 seconds, 3 cycles (Bandelin Sonopuls HD 2070 homogenizer) for 2 times. Finally, these were centrifugated at 15000xg for 5 minutes, and nuclear fractions were transferred into new tubes. Both of the fractions boiled with 1x Laemmli buffer for 7 minutes at 95°C and 15μg of proteins measured by PierceTM 660nm Protein assay (Thermo Scientific, 22660) were loaded into 10% SDS-PAGE gel. For Western blot analysis, PVDF membrane (Millipore) and the following antibodies were used: anti-PER2(Proteintech, 67513-1-Ig), anti-CLOCK (Bethyl, A302-618A), anti-BMAL1(Santa Cruz Biotech., SC-365645), anti-CRY1(Bethyl, A302-614A), and anti-Histone H3 (Abcam, ab1791) as an internal control

of nuclear fractions; and anti-αTUBULIN (Sigma-Aldrich, T9026) as an internal control of cytosolic fractions. The remaining samples can be stored at -20°C.

3.13 Statistical Analysis

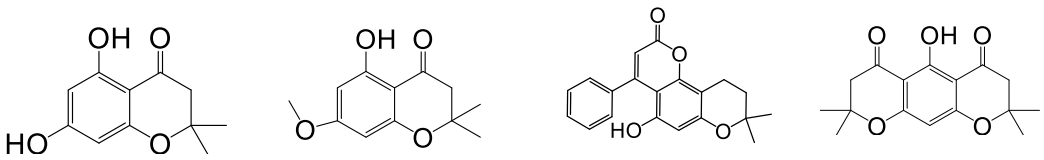
To evaluate the statistical significance of Student's t-test, one-way Anova or two-way ANOVA was applied using GraphPad Prism (GraphPad Software, California, USA). The number of biological replicates for each experimental setup was at least n=3. [*p-value<0.05, **p-value<0.01, ***p-value<0.001.]. Additionally, mean ± SEM (Standard Error of Mean) were represented for all data.

Chapter 4:
RESULTS

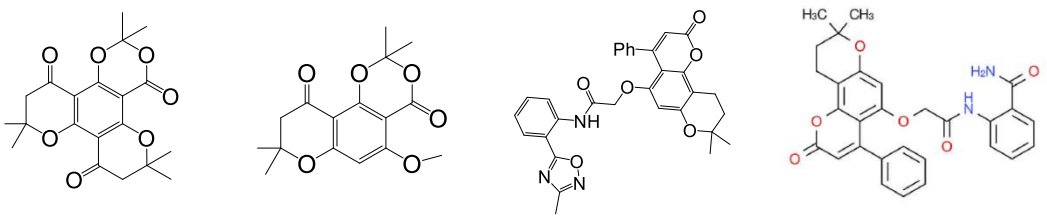
4.1 Retrosynthesis of CLK8 derivatives

In previous studies, the lowest dose of CLK8 showing effect was found to be 10μM [9]. In this study, I searched for a derivate that shows its effect at a dose lower than 10μM, preferably at nanomolar levels. For this purpose, seven derivatives of CLK8 were retrosynthesized by Mustafa Guzel's Lab (Department of Medical Pharmacology, International School of Medicine, Istanbul Medipol University, Turkey) previously (**Fig 4.1**).

ABE1A ABE2A ABE8A ABE1B



ABE1C ABE2C ABE20 CLK8



Small Molecule	Name
CLK8	2-[2-(8,8-Dimethyl-2-oxo-4-phenyl-9,10-dihydropyrano[2,3-*f*]chromen-5-yl)oxyacetamido]benzamide
ABE1A	5,7-dihydroxy-2,2-dimethylchroman-4-one
ABE2A	5-hydroxy-7-methoxy-2,2-dimethylchroman-4-one
ABE8A	5-hydroxy-8,8-dimethyl-4-phenyl-9,10-dihydro-2*H*,8*H*-pyrano[2,3-*f*]chromen-2-one
ABE1B	5-hydroxy-2,2,8,8-tetramethyl-2,3,7,8-tetrahydro-4*H*,6*H*-pyrano[3,2-*g*]chromene-4,6-dione
ABE1C	2,2,6,6,10,10hexamethyl-6,7,10,11-tetrahydro-4*H*,8*H,12H*-[1,3]dioxino[4,5-*f*]pyrano[2,3-*h*]chromene-4,8,12-trione
ABE2C	5-methoxy-2,2,8,8-tetramethyl-8,9-dihydro-4*H*,10*H*-[1,3]dioxino[4,5-*f*]chromene-4,10-dione
ABE20	2-((8,8-dimethyl-2-oxo-4-phenyl-9,10-dihydro-2*H*,8*H*-*pyrano[2,3-f]chromen*-5-yl)oxy)-*N*-(2-(3-methyl-1,2,4-oxadiazol-5-yl)phenyl)acetamide

Figure 4.1: Structures of retrosynthesized molecules

Approximately 20 molecules were retrosynthesized from CLK8 molecule. During this retrosynthesis, in addition to main products some side products formed. The ones used for further analysis in this study can be seen in (**Fig 4.1**).

4.2 Determination of the Most Prominent Molecule

Firstly, lumicyle assay for all these derivatives of CLK8 was conducted at 1,25μM and 2,5μM doses except for ABE20, which was conducted at 5μM, 10μM, and 20 μM (**Fig 4.2A**). It was found that ABE20 did not show any effect at a dose lower than 10μM. That is why it was the first derivative eliminated and not used for further analysis. Secondly, ABE1B, 1C, and 2C did not show an effect at the dose of 1,25μM **(Fig 4.2B)**. As their effect was not at the nanomolar level, they were eliminated and not used for further analysis. On the other hand, the ones that showed their effect (amplitude increase) at 1,25μM (1A,2A,8A) were further tested by lumicyle at lower doses **(Fig 4.2C)**, MTT **(Fig 4.2D)** and luciferin degradation assays **(Fig 4.2E)**. Based on these experiments, it was found that ABE1A, ABE2A, and ABE8A have effects at nanomolar doses. In addition, they are not toxic to the U2OS *Bmal1*-d*Luc* cell line, which was used in the lumicycle assay. Furthermore, neither of them influences luciferin half-life. Thus, any of these molecules seemed to be candidates for this study at first sight. However, it was shown that ABE2A shows its effect at the lowest dose (0,156 μM). In addition, it shows

a dose-dependent amplitude increase and a saturation point **(Fig 4.2C)**. Neither ABE1A nor ABE8A can satisfy these parameters. That is why ABE2A was selected as a candidate molecule for this study.

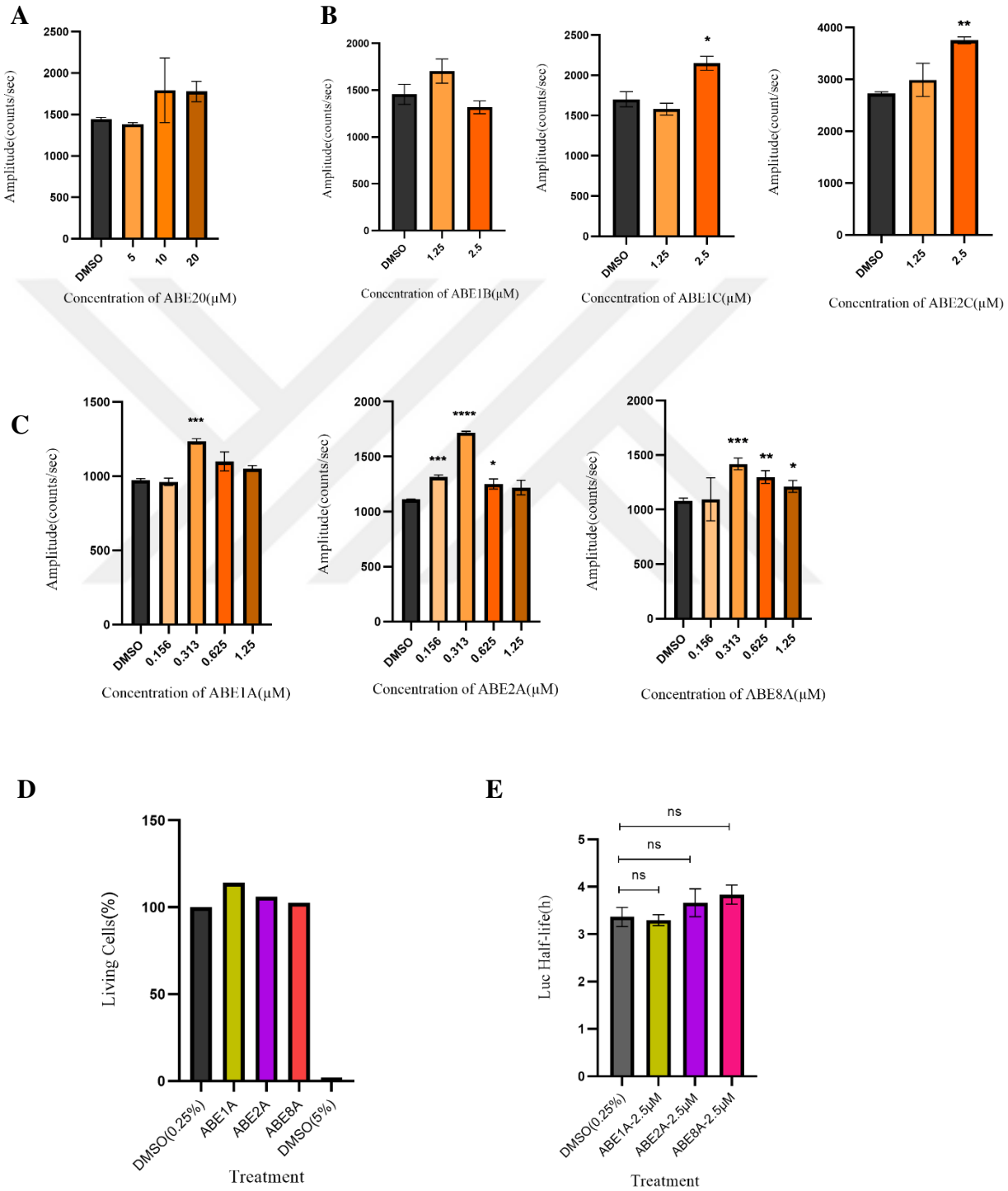


Figure 4.2: Comparison between CLK8 derivatives
Continuous monitoring of circadian oscillations in U2OS *Bmal1*-d*Luc* cells treated with (A) ABE20, (B) ABE1B,1C,2C, (C) ABE1A,2A,8A. (D) Assessment of cell viability in U2OS *Bmal1*-d*Luc* cells. Cells were treated with 20 µM molecule (0.25%DMSO) or DMSO in different concentrations as positive (0.25%) and negative (5%) control. Cell viability was determined using Synergy H1 Microplate Reader (Bio-Tek Instruments). (E) The effect of ABE1A,2A,8A on luciferase half-life was assessed in *pcDNAdLuc* transfected HEK3T3 cells. After 2,5um molecule (0.25%DMSO) or DMSO treatment luciferase signal was recorded until it reaches a plateau. For A, B, C, E the data presented are mean ± SEM from three independent experiments. Statistical analysis was performed using Student's t-test *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001. For D n=1, statistical analysis was not applied.

4.3 Cell Viability Test

The cellular cytotoxicity of ABE2A was assessed using U2OS *Bmal1*-d*Luc*, NIH 3T3 *Bmal1*-d*Luc*, and MSF cells through the MTT assay. The results indicated that ABE2A did not exhibit any toxic effects on these cell lines at a dose of 20 µM (**Fig. 4.1**). This suggests that treatment with ABE2A at or below 20 µM in these cell lines did not lead to cell death. Therefore, ABE2A concentrations lower than 20 µM were used throughout the study.

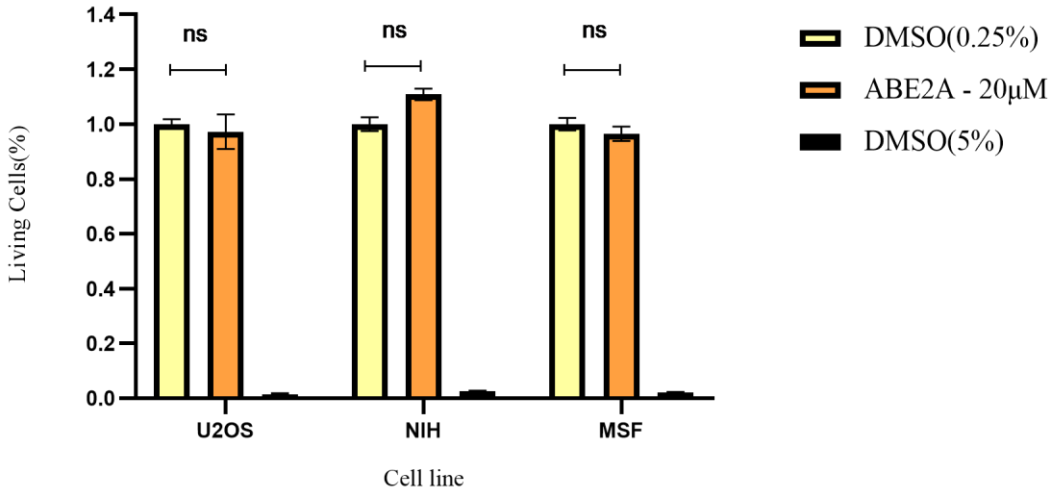


Figure 4.3: Determination of the toxicity of ABE2A by MTT assay
Assessment of cell viability in U2OS *Bmal1*-d*Luc*, NIH 3T3 *Bmal1*-dLuc and MSF cells. Cells were treated with 20 µM ABE2A (0.25%DMSO) or DMSO in different concentrations as positive (0.25%) and negative (5%) control. Cell viability was determined using Synergy H1 Microplate Reader (Bio-Tek Instruments). All measurements were normalized against control (0.25% DMSO). Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

4.4 The effect of ABE2A on circadian rhythm

Most assays I used in this thesis rely on luciferase-based systems. To eliminate the possibility of interference from the ABE2A molecule on luciferase activity, I assessed its effect on the activity and half-life of luciferase. For this purpose, HEK 293T cells were transfected with pcDNA-Luc and then treated with cycloheximide to inhibit protein biosynthesis. Subsequently, the cells were treated with ABE2A to determine its effect on luciferase by measuring its decay rate. As shown in **Fig. 4.2**, ABE2A did not significantly affect luciferase half-life or light intensity at a concentration of 2.5 µM.

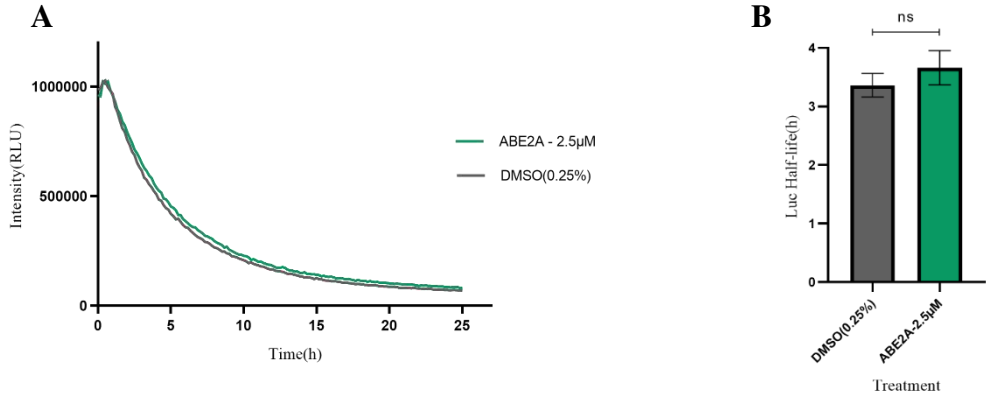
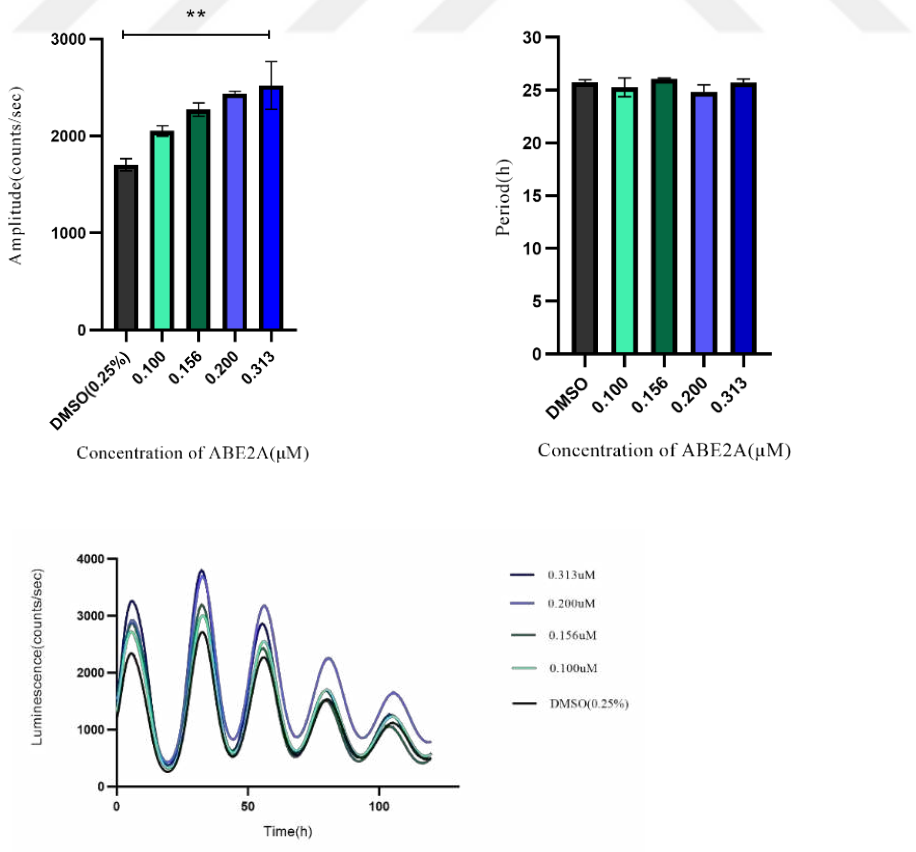


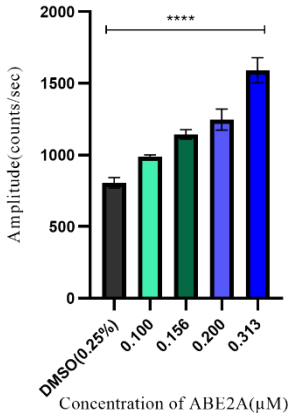
Figure 4.4: The effect of ABE2A on Luciferase
(A) The effect of ABE2A on luciferase half-life was assessed in *pcDNA*d*Luc* transfected HEK3T3 cells. After 2,5µM ABE2A(0.25%DMSO) or DMSO treatment, luciferase signal was recorded until it reaches a plateau. (B) Graphical representation of the quantified LUC half-life. Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

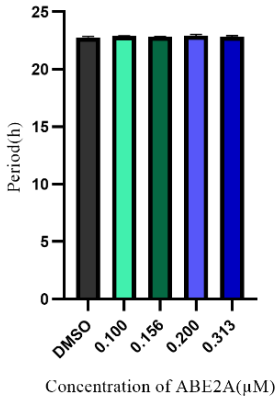
I then deeply investigated the effect of the ABE2A molecule on circadian rhythm using U2OS *Bmal1*-d*Luc* cells. The cells were treated with ABE2A in a dose-dependent manner, and real-time monitoring of the circadian rhythm was measured by Lumicycle. Calculation of circadian rhythm parameters of amplitude and period length showed that ABE2A selectively enhances amplitude (**Fig. 4.5A**). Furthermore, I further assessed the molecule's effect in a different genetic background and primary cell lines NIH3T3 *Bmal1*-d*Luc* and MSF, respectively. Consistently, ABE2A enhanced only the amplitude of circadian rhythm in these cell lines (**Fig. 4.5 B, C**). These data suggested that ABE2A mediates its effect through core clock protein. To confirm that the effect of ABE2A is mediated explicitly by CLOCK proteins, I utilized *CLOCK* knockout U2OS *Bmal1*-d*Luc* cells. Since these cells lack CLOCK protein, I anticipated that the ABE2A molecule would not affect the circadian rhythm in a dose-dependent manner. As expected, the bioluminescence of *CLOCK* knockout U2OS *Bmal1*-d*Luc* cells was not affected by the ABE2A molecule (**Fig. 4.5D**).

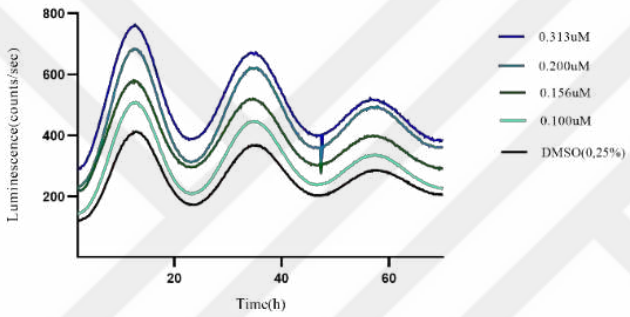
A – U2OS *Bmal1*-d*Luc*



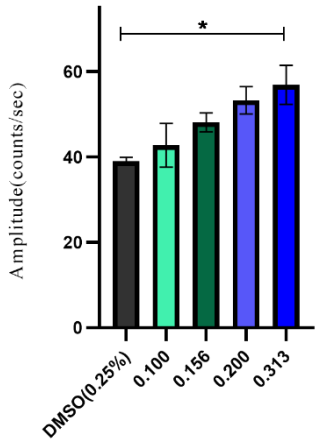
B - NIH3T3 *Bmal1*-d*Luc*

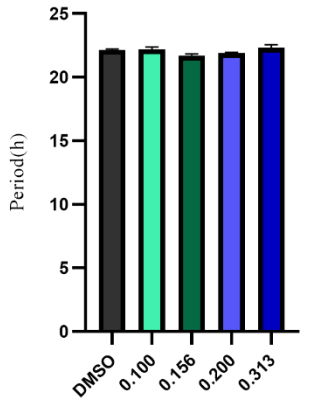


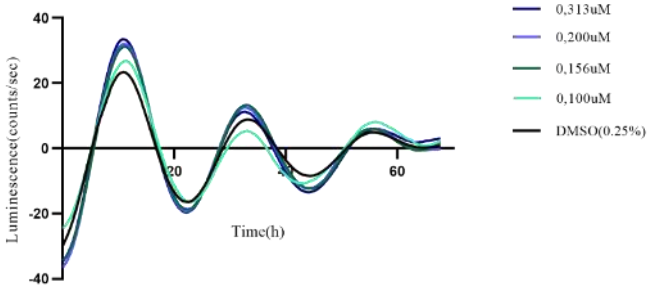




C - MSF







D - *CLOCK* knockout U2OS *Bmal1*-d*Luc*

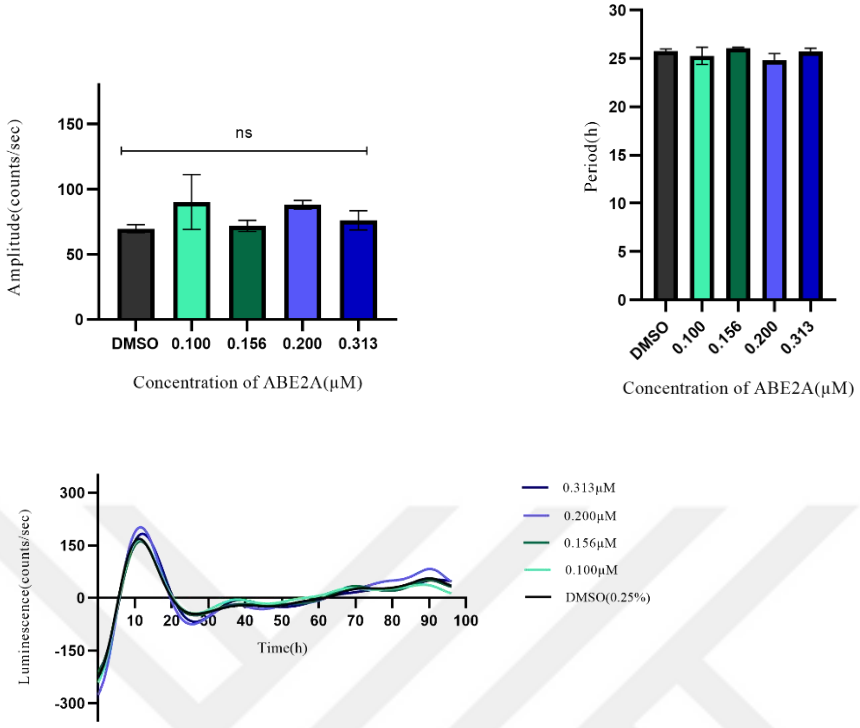


Figure 4.5: ABE2A increases the amplitude of circadian rhythm dose dependently. Continuous monitoring of circadian oscillations (A) in U2OS *Bmal1*-d*Luc* cells (B) in NIH3T3 *Bmal1*-d*Luc* cells (C) in MSF cells (D) in *CLOCK* knockout U2OS *Bmal1*-d*Luc* cells. Amplitude and period parameters are shown in the right panel. Synchronized cells were treated with indicated doses of ABE2A(0.25%DMSO). Then, cells were placed in Lumicycle and luminescence signals were recorded luminescence for 5-7 days with 10 minutes intervals. For the analysis, Lumicycle, Actimetrics was used. The data of the first day was omitted. Statistical analysis was performed using ordinary one-way Anova. The data presented are mean ± SEM from three independent experiments. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

All these studies suggested that ABE2A enhances the amplitude of the circadian rhythm at cellular through the CLOCK. This brings about a possibility that ABE2A might physically interact with CLOCK. Thus, this interaction was investigated as the next step of this study.

4.5 Physical Interaction of ABE2A and CLOCK

Considering ABE2A is a part of an original molecule of CLK8 (**Fig. 4.6A**), I hypothesized that the molecule should compete for the same binding region. To test that biotinylated CLK8 was used to pull down CLOCK protein in the presence and absence of ABE2A (**Fig. 4.6A**). In this assay, I expected that the level of CLOCK would decrease in the presence of ABE2A due to the same binding region when it is pulled down with biotinylated CLK8 (bCLK8).

To test that FLAG-tagged pcDNA4a-*CLOCK* was used for the transfection of HEK 293T cells. Then, CLOCK-FLAG was precipitated with bCLK8, and the amount of the CLOCK was quantified in the presence and the absence of competitor ABE2A.

This experiment revealed that ABE2A significantly decreased the interaction between bCLK8 and CLOCK (**Fig. 4.6C**). Thus, ABE2A and CLK8 share the same binding residues on CLOCK. To verify this observation, one of our group members conducted a docking experiment of ABE2A on CLOCK protein (**Fig. 4.7**). It was found that ABE2A binds to CLOCK and indeed to the exact location where CLK8 binds (**Fig. 4.7**).

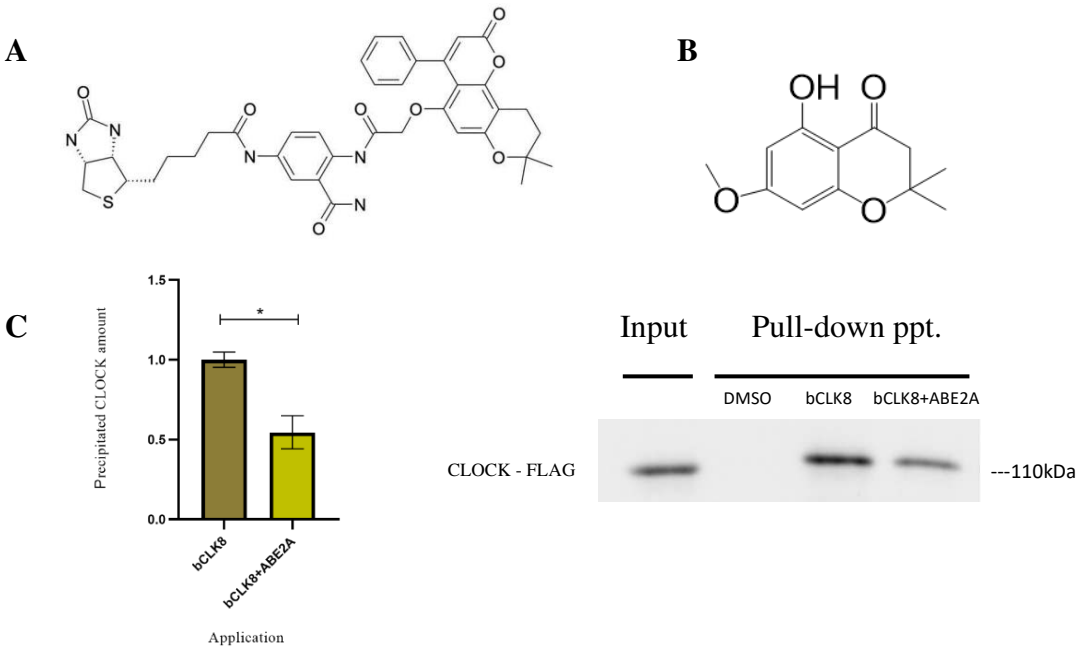
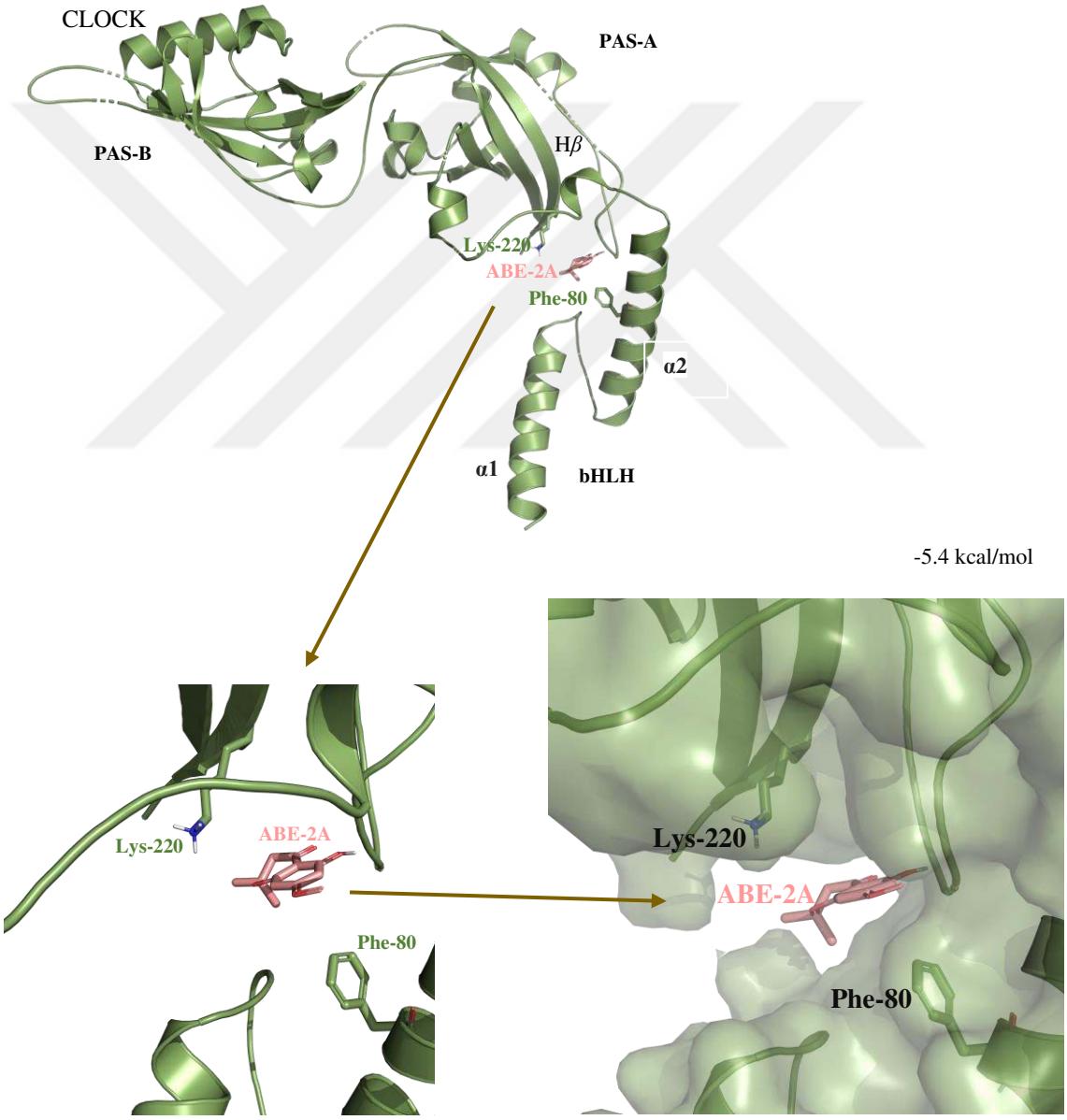


Figure 4.6: Specific binding of ABE2A to CLOCK.

The chemical structure of (A) biotinylated CLK8(bait), (B) non-biotinylated ABE2A(competitor). (C) Pull down assay of cell lysates of CLOCK overexpressed HEK 293T cells. Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

A



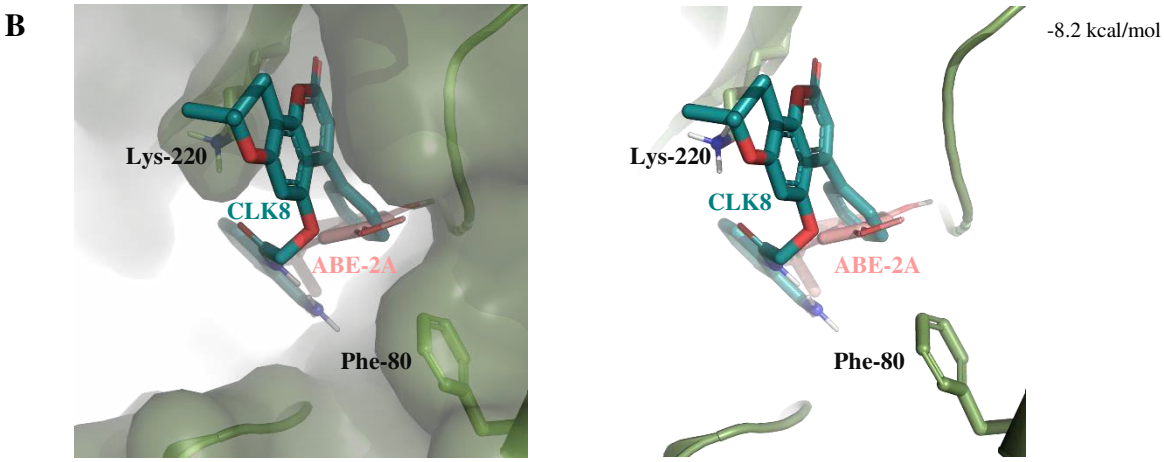


Figure 4.7: The docking results of ABE2A and CLK8.
(A)The best binding mode of ABE2A to CLOCK (green) with a predicted binding energy of -5.4 kcal/mol. ABE2A can enter the hollow between the α2 helix of the bHLH domain and the H*β* strand of the PAS-A domain. The Lys-220 in the PAS-A domain and Phe-80 in the bHLH domain seem to contribute to the interaction between CLOCK and ABE2A. (B)Superimposed binging modes of ABE2A and CLK8 to CLOCK (green). Predicted binding energy of CLK8 is -8.2 kcal/mol.

By interacting with CLOCK at the exact location where CLK8 is, I expected that ABE2A would decrease the interaction between CLOCK and BMAL1. The reason is that it was previously found that the same binding region in the CLOCK structure is shared by CLK8 and Arg-126 residue of BMAL1 so that CLK8's presence decreases the interaction between CLOCK and BMAL1[12]. To test its validity for ABE2A, the following assay focused on the effect of ABE2A on CLOCK and BMAL1 interaction.

4.6 The Effect of ABE2A to CLOCK and BMAL1 Interaction

In the main TFFL (transcription-translation feedback loop), CLOCK forms a dimer with BMAL1 and binds to the E-box within the promoter of clock-controlled genes, initiating their transcription [6]. To investigate the effect of the ABE2A molecule on the interaction between CLOCK and BMAL1, I performed a co-immunoprecipitation assay by precipitating CLOCK and measuring the level of BMAL1 protein in the presence and absence of the ABE2A molecule. For this purpose, cells were transfected with FLAG-tagged pcDNA4a-*Clock* and pCMV-Sport6-*Bmal1*.

After precipitating CLOCK-FLAG using anti-FLAG affinity gel in the presence and absence of ABE2A, the level of CLOCK and BMAL1 was determined using Western blot with anti-FLAG (Sigma, A9469-1MG) and anti-BMAL1 antibodies respectively. Analysis of the results indicated that ABE2A reduced the interaction between CLOCK and BMAL1 at a concentration of 40 μM (**Fig. 4.8**). This result is consistent with a previously published study using parental CLK8, where they also demonstrated that CLK8 reduced the interaction between CLOCK and BMAL1[9].

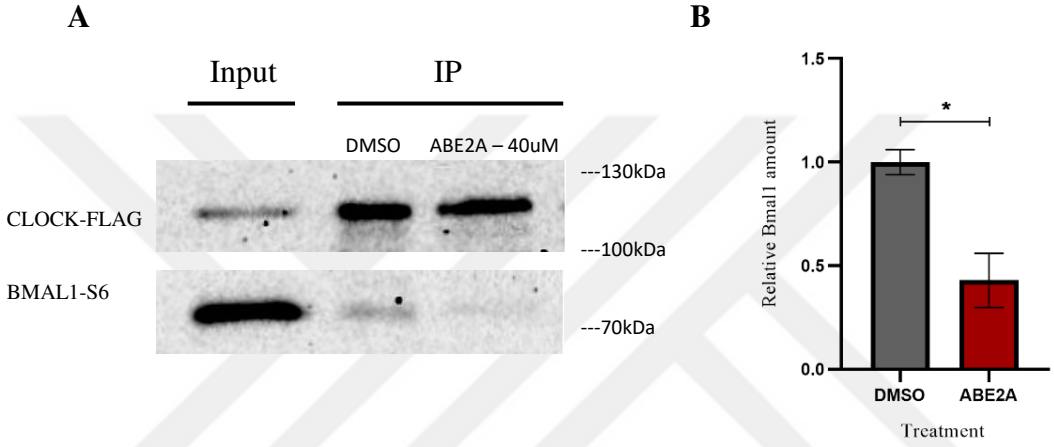


Figure 4.8: ABE2A disrupts the interaction between CLOCK and BMAL1.
Co-IP assay was used to evaluate the effect of ABE2A on CLOCK: BMAL1 interaction. (A)Western blot analysis using anti-FLAG and anti-BMAL1 antibodies. (B) Quantification of Western blotting. The amount of BMAL1 was calculated relative to CLOCK amount and normalized by DMSO control. Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

Another potential impact of ABE2A was its effect on the nucleocytoplasmic shuttling of CLOCK as it decreases the interaction between CLOCK and BMAL1. The reason is that BMAL1 possesses nuclear localization signals (NLS) and nuclear export signals (NES); however, CLOCK does not. Consequently, the nucleocytoplasmic shuttling of CLOCK relies on BMAL1. Thus, this effect was investigated in the following assay.

4.7 The Effect of ABE2A on Subcellular Localization of Clock Proteins

Since the stability of BMAL1 depends on CLOCK [42], I next investigated the cellular distribution of CLOCK and BMAL1 in the presence of ABE2A, reducing the interaction between them. Cellular fractionation was performed on synchronized U2OS *Bmal1*-d*Luc* cells after treatment with ABE2A. The purity of both cytosolic and nuclear fractions was determined with α-TUBULIN protein and HISTONE H3, respectively **(Fig. 4.9A)**. After confirming the clean preparation of the fractions, I assessed the levels of BMAL1 and CLOCK **(Fig. 4.9B)**.

The results indicated a significant reduction in the levels of CLOCK and BMAL1 in the nucleus. In contrast, the level of BMAL1 increased considerably in the cytosol upon treatment with ABE2A (**Fig. 4.9**). Additionally, CRY1 exhibited an increase in the cytosol and a decrease in the nucleus. However, the differences were not statistically significant (**Fig. 4.9**). Similar to the CLK8 molecule, ABE2A also altered the levels of BMAL1 and CLOCK in the nucleus.

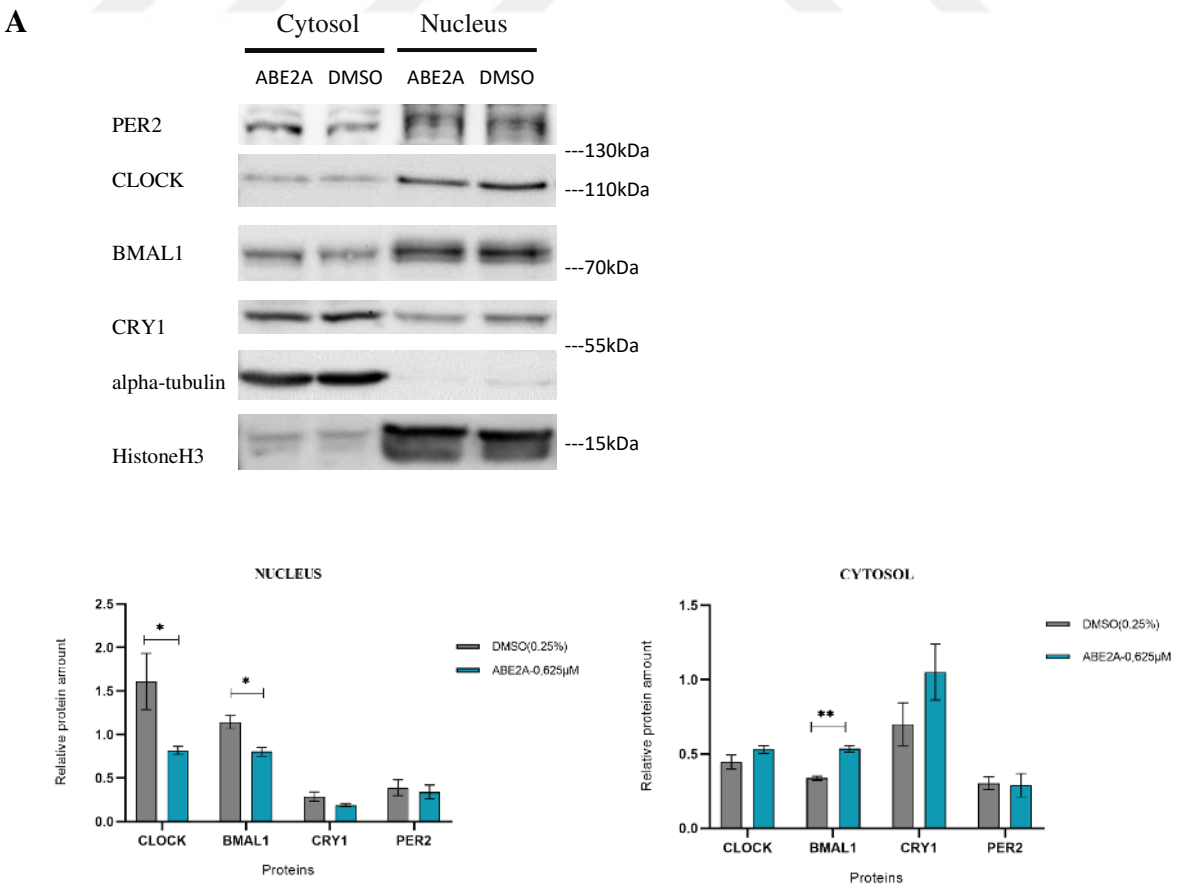


Figure 4.9: ABE2A interferes with the subcellular localization of CLOCK and BMAL1. Synchronized U2OS *Bmal1*-d*Luc* cells were treated with 0,625µM ABE2A or DMSO (0.25%) and after 30h incubation fractionated into cytoplasm and nucleus. (A) Immunoblot of CLOCK, BMAL1, CRY1, and PER2 normalized by HISTONE H3 and α-TUBULIN. (B) Quantification of core-clock protein levels in nucleus and cytoplasm. Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

4.8 The Effect of ABE2A on Core-Clock Components

Several studies have indicated that factors interfering with the transcription-translation feedback loop (TTFL) of the core clock mechanism can impact clock output genes [7]. Considering that ABE2A modulates the interaction between CLOCK and BMAL1 and subsequently affects the circadian clock, I investigated the levels of core clock proteins in both unsynchronized and synchronized U2OS cell lines in the presence of ABE2A. In the unsynchronized system, confluent U2OS cells were treated with 0.625 µM ABE2A or DMSO (0.25%) and harvested after 24 hours. The results revealed a significant decrease only in the level of CLOCK upon exposure to ABE2A (**Fig. 4.10**).

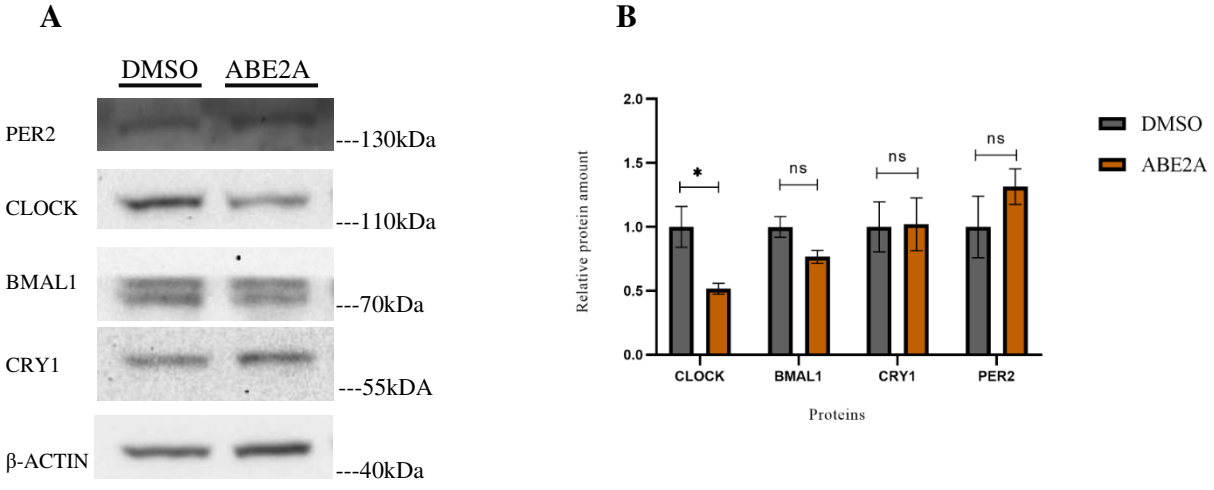


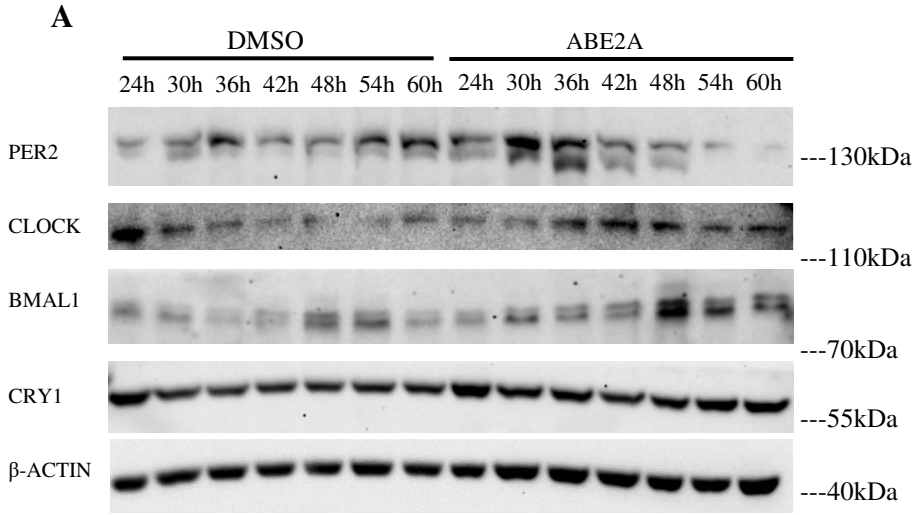
Figure 4.10: The effect of ABE2A on unsynchronized clock proteins

Unsynchronized U2OS *Bmal1*: d*Luc* cells were treated with 0,625 µM ABE2A or DMSO and incubated for 24 hours, harvested and proteins were isolated. (A) Western blot. Protein levels were normalized by β-ACTIN levels. (B) The bar graph indicates quantification of core

clock protein levels. Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

The differences in the levels of core clock proteins depending on the treatment and their rhythmicity can be masked in an unsynchronized system. Thus, to see if there is any hidden effect of ABE2A on these proteins, the same assay was performed on an unsynchronized system. U2OS cells were initially synchronized using 0.1 μM Dexamethasone (Sigma D-8893) in DMEM for 2 hours. Then, the cells were treated with 0.625 μM ABE2A or DMSO (0.25%) and harvested at various time points, ranging from 24 to 60 hours with 6-hour intervals. Consistent with the study mentioned above, the results showed a decrease in the level of CLOCK significantly (**Fig. 4.11**).

If this molecule affects the levels of CLOCK and PER2 proteins, it might also have an impact on transcriptional levels of some circadian genes. Thus, I investigated the transcriptional levels of core-clock genes over time in the presence and absence of ABE2A. Results indicated that the transcriptional levels of *PER2*, *DBP*, and *BMAL1* were reduced while the transcriptional levels of *CLOCK*, *CRY1*, *REV-ERB*, and *RORα* remained unaffected (**Fig. 4.12**).



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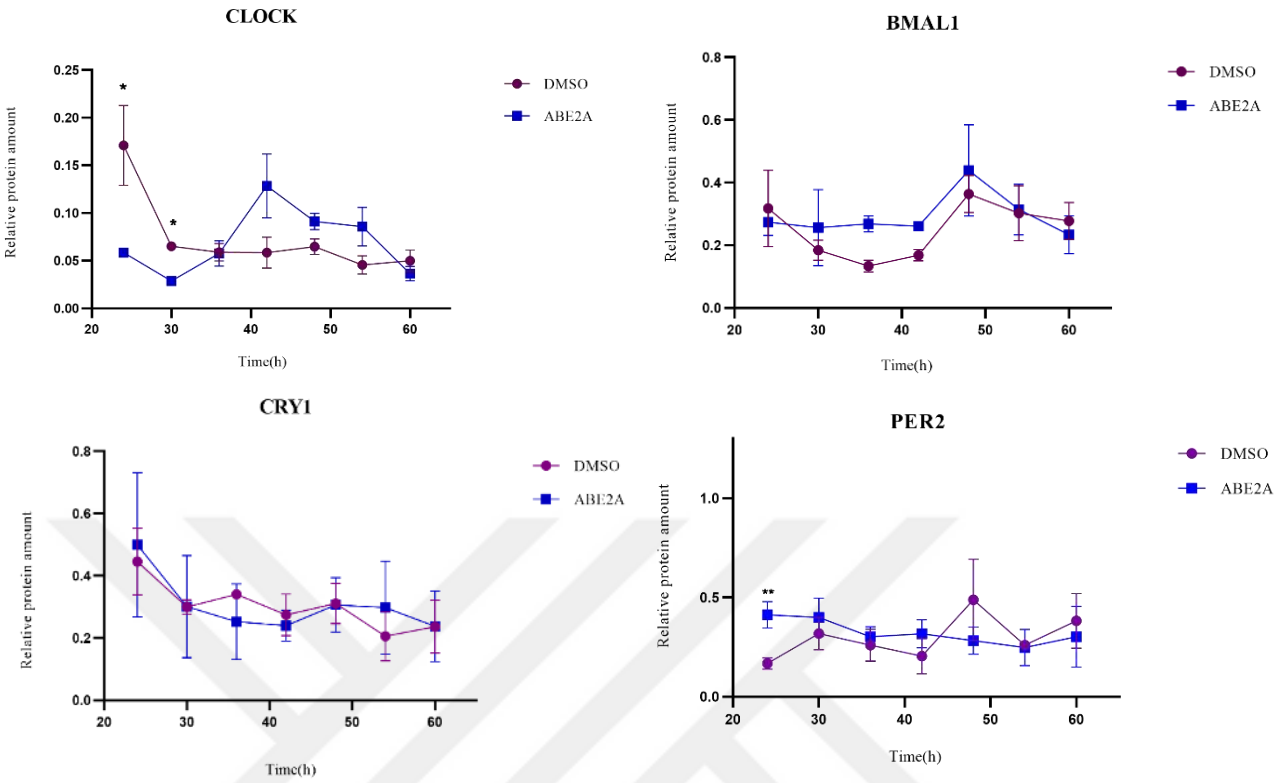
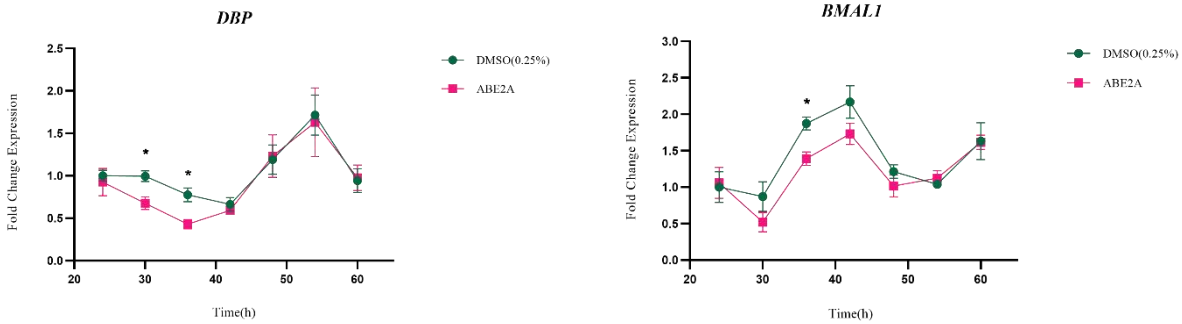


Figure 4.11: The time-dependent effect of ABE2A on clock proteins

Synchronized U2OS *Bmal1*: d*Luc* cells were treated with 0,625 µM ABE2A or DMSO and incubated for 24 hours, harvested with 6h intervals up to 60h and proteins were isolated. (A) Western blot. Protein levels were normalized by β-ACTIN levels. (B) Quantification of protein levels. Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.



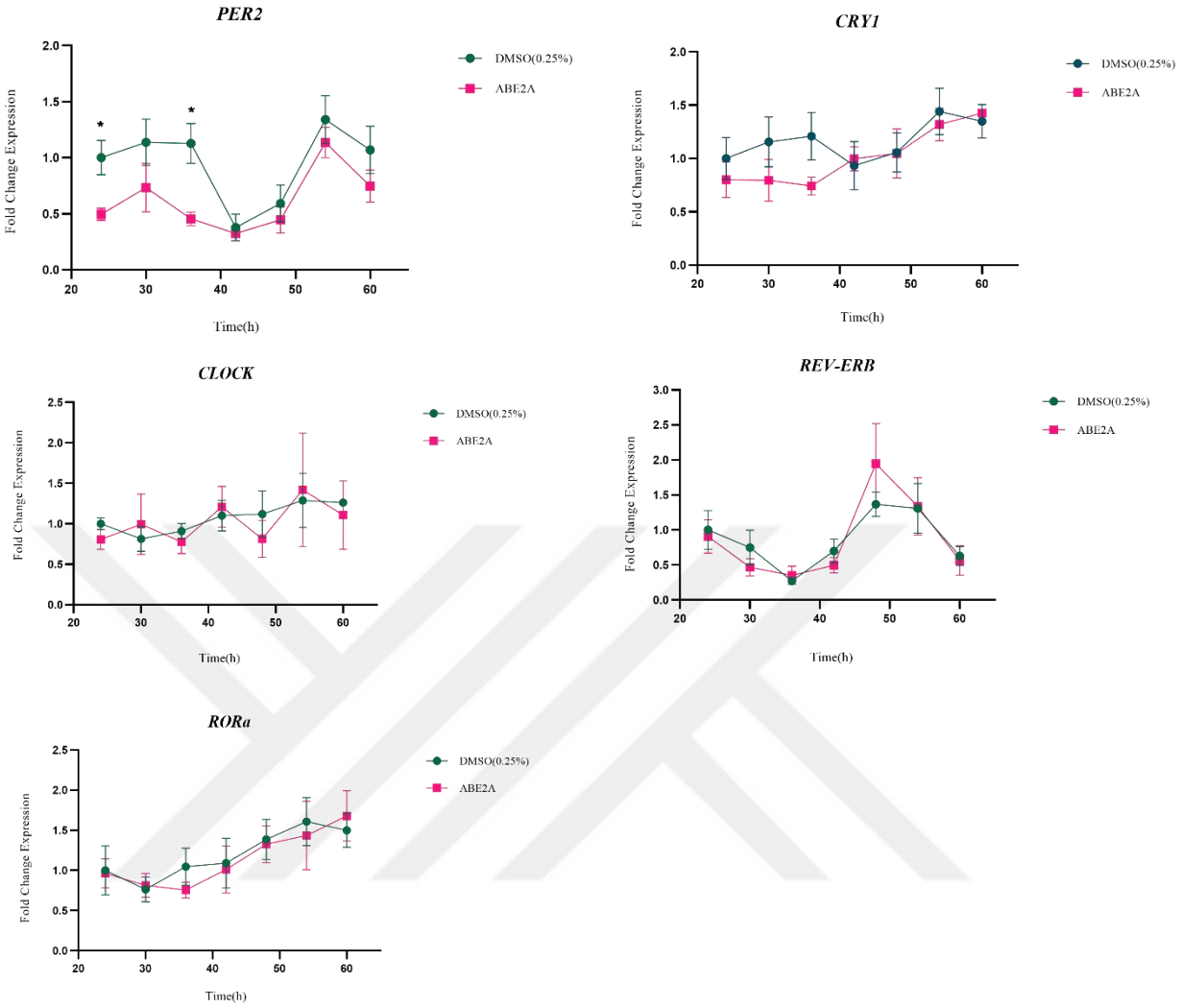


Figure 4.12: The time-dependent effect of ABE2A on transcriptional levels of clock genes

Synchronized U2OS *Bmal1*:d*Luc* cells were treated with 0,625 µM ABE2A or DMSO and incubated for 24 hours, harvested with 6h intervals up to 60h and RNAs isolated which were then converted to cDNAs. RT-qPCR was conducted to measure transcriptional levels. As the reference gene RPLP0 (ribosomal protein, large, P0) was used. Delta-delta Ct (2-ΔΔCt) method was used for quantification. Statistical analysis was performed using Student's t-test. The data presented are mean ± SEM from three independent experiments. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

Chapter 5:
DISCUSSION

In this study, I aimed to discover a circadian amplitude enhancer molecule with a drug-like potency. To achieve that, I characterized seven derivatives of the CLK8 molecule, which was identified as a CLOCK-binding small molecule enhancing the circadian amplitude by our group [9]. These derivatives were retrosynthesized from CLK8 **(Fig. 4.1)**. In the preliminary study these derivatives of CLK8 were used in Lumicycle assay with U2OS *Bmal1*-d*Luc* cells, and the analysis revealed that the derivative ABE2A shows its effect at the lowest dose (0,156 μM) compared to the other derivatives **(Fig4.2C)**. Furthermore, it exhibits a dose-dependent effect and a saturation point, which proves that the amplitude enhancement effect of ABE2A is real and can be regulated **(Fig4.2C)**. That is why ABE2A was selected for further analysis.

Lumicycle analysis with the additional doses of ABE2A also showed that it increases circadian rhythm amplitude at the dose of 0.100 μM. This result demonstrates that ABE2A is a 100 times more efficient amplitude enhancer than CLK8, with 10μM lowest effective dose [9]. Additionally, it was shown that ABE2A did not show any luciferin interaction at the doses of 2,5 μM **(Fig 4.4)**. This shows that ABE2A does not affect the reliability of luciferin-based assays such as lumicyle at the dose of or lower than 2,5 μM. In addition, as it has no toxic effect on U2OS *Bmal1*-d*Luc*, NIH 3T3 *Bmal1*-d*Luc*, and MSF cells at 20 μM, these cell lines were used in the following assays throughout this study (**Fig4.3**).

It was shown that ABE2A demonstrated the same phenotype (enhanced amplitude) in a different genetic background (NIH3T3 *Bmal1*-d*Luc* cells) and primary cell line (MSF) **(Fig. 4.5 B, C)**. These suggest that the ABE2A effect is mediated through core clock proteins. Furthermore, lumicycle assay utilized on CLOCK knockout U2OS *Bmal1*-d*Luc* cells proved that the effect of ABE2A is directly specific to CLOCK protein as ABE2A did not exhibit a dose-dependent amplitude enhancement in this cell line. **(Fig. 4.5 D)**. In order to prove a physical interaction between CLOCK and ABE2A docking and pull-down assays were performed **(Fig. 4.6C,4.7)**. Docking of ABE2A onto CLOCK was critical to set two criteria for the selection of candidate molecules. Firstly, a

candidate molecule should locate a similar binding location with CLK8 when docked onto CLOCK, the cavity covered by the α2-helix of the bHLH domain, and the Hβ strands on the PAS-A domain. Secondly, it should have comparable binding energy with CLK8 towards CLOCK. Even if the second was not satisfied, a high binding energy per heavy atom could compensate for this criterion **(Table 3.1)**. It was found that ABE2A binds to CLOCK and indeed to the exact location where CLK8 binds (**Fig. 4.7**). That is the Lys-220 in the PAS-A domain and Phe-80 in the bHLH domain of CLOCK seem to contribute to the interaction with ABE2A. This binding also confirmed by pull down assay. This experiment revealed that ABE2A significantly decreased the interaction between bCLK8 and CLOCK (**Fig. 4.6C**).

A complex system of circadian rhythm makes the discovery of the exact working principle of ABE2A challenging. Nonetheless, the action mechanism of ABE2A was enlightened in this thesis. It was revealed that ABE2A disrupts the interaction between CLOCK and BMAL1 **(Fig. 4.8)**. The reason is that similar to CLK8, ABE2A targets the same binding region in the CLOCK with the Arg-126 residue of BMAL1 [12]. Thus, binding of ABE2A to CLOCK decreases the CLOCK binding to BMAL1. This reduction has some consequences. For instance, CLOCK, when bound to ABE2A, cannot pass into the nucleus. The reason is that even though BMAL1 has Nuclear Localization Signal (NLS) and Nuclear Export Signal (NES), CLOCK does not have either of them [51]. Thus, only if CLOCK and BMAL1 form a heterodimer they can translocate into the nucleus. Once this interaction is disturbed by ABE2A, the level of CLOCK increases in the cytosol and decreases in the nucleus **(Fig.4.9)**. Additionally, as ABE2A prevents CLOCK binding to BMAL1, it promotes BMAL1 nuclear export, which results in the rise of BMAL1 level in the cytosol **(Fig. 4.9)**. Overall, ABE2A results in inefficient nuclear translocation of CLOCK and BMAL1 without affecting the negative arm of TTFL.

Several studies indicated that factors interfering with the transcription-translation feedback loop (TTFL) of the core clock mechanism can impact clock output genes [7]. Based on that, total cell protein levels were investigated. It was found that ABE2A decreases the level of the CLOCK protein in the unsynchronized and synchronized U2OS cell line, while it increases the level of PER2 in the synchronized system. This might be

because of the decreased interaction between CLOCK and BMAL1 with ABE2A presence and an indirect effect of the loss of stability.

If this molecule affects the levels of CLOCK and PER2 proteins, it might also have an impact on transcriptional levels of some circadian genes. Thus, I investigated the transcriptional levels of core-clock genes over time in the presence and absence of ABE2A. The time-dependent mRNA analysis of U2OS cells revealed that ABE2A significantly mitigates the transcriptional levels of *DBP*, *PER2*, and *BMAL1* genes. This is likely due to the reduction effect of ABE2A on CLOCK and BMAL1 interaction and thus the E-Box driven transcription. These observations suggest that ABE2A behaves as a stabilizer of repression activity of PER2 and CRY1 proteins (the negative arm of TTFL is weakened) by reducing the CLOCK and BMAL1 interaction and overall and nuclear CLOCK amount, which results in the enhancement of circadian amplitude (**Fig. 5.1**). These findings are consistent with previous studies showing that overexpression of CLOCK-BMAL1 decreases amplitude [46].

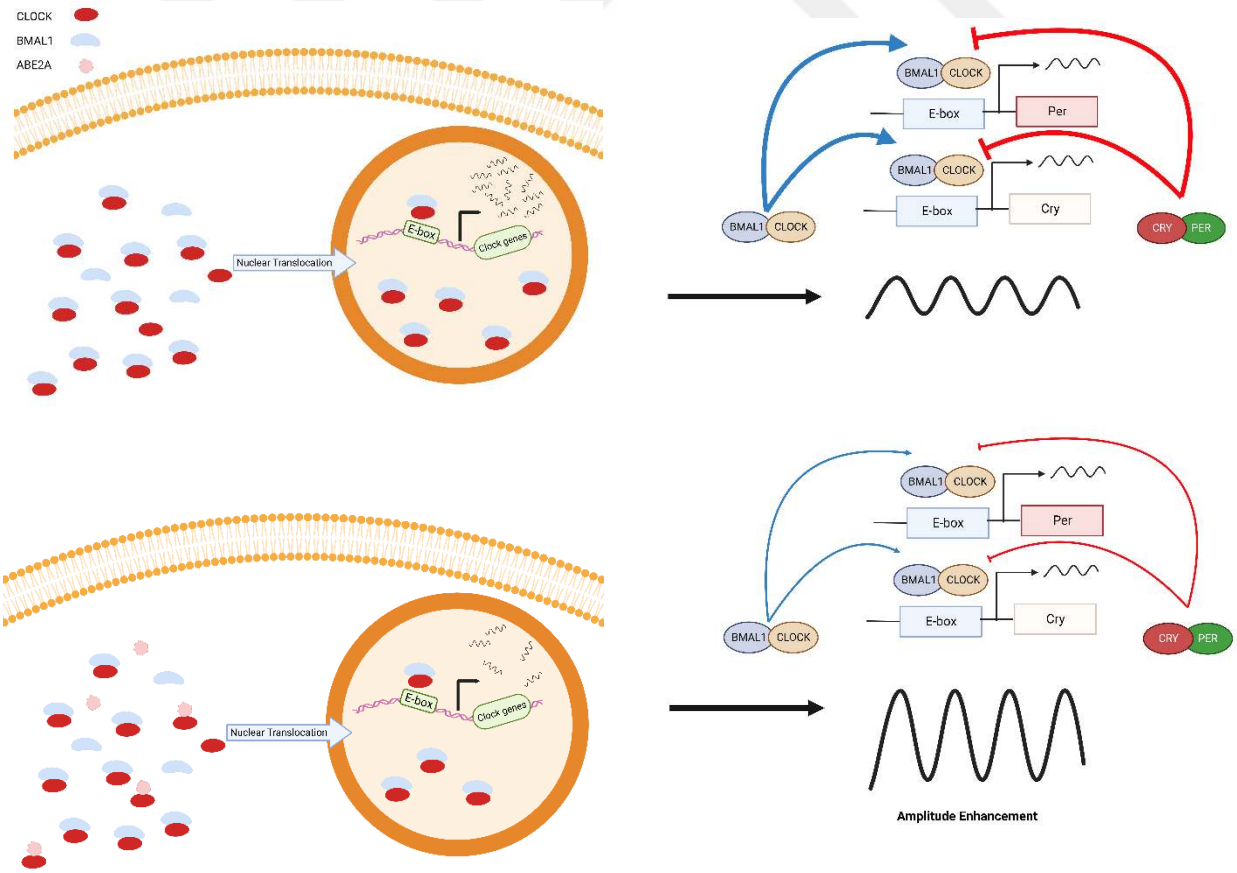


Figure 5.1: The impact of ABE2A on CLOCK and the circadian rhythm.
CLOCK and BMAL1 interact dynamically, but when ABE2A is present, it binds to CLOCK and diminishes the interaction between CLOCK and BMAL1. Moreover, the binding of ABE2A to CLOCK decreases the translocated CLOCK-BMAL1 into the nucleus. At the cellular level, ABE2A enhances the amplitude of the circadian rhythm.

ABE2A, as a clock-enhancing small molecule effective at nanomolar levels, was identified as a molecule with drug-like potency. Additional in-vivo studies by using mouse models and investigations into the pharmacodynamics and pharmacokinetics of this compound will contribute to a better understanding of its precise mode of action and the potential need for chemical alterations. After required modifications and tests are completed, it is highly possible that it can be used to develop novel therapeutics and drugs associated with dampened amplitude, such as metabolic and cardiovascular diseases, sleep and mood disorders, cancer, and accelerated aging [10].

APPENDICES

APPENDIX C: PRIMER SEQUENCES

Table A.1. Primer Sequences

Gene	Forward Sequence	Reverse Sequence
hsRplp0	TGTGGGAGCAGACAATGTGG	CATTCCCCCGGATATGAGGC
hsClock	GGCACCACCCATAATAGGGTA	TGTTGCCCCTTAGTCAGGAAC
hsBmal1	GCCCATTGAACATCACGAGTAC	CCTGAGCCTGGCCTGATAGTAG
hsCry1	ACAGGTGGCGATTTTTGCTTC	TCCAAAGGGCTCAGAATCATACT
hsPer2	AGTTGGCCTGCAAGAACCAG	ACTCGCATTTCCTCTTCAGGG
hsRev-erb	TGGACTCCAACAACAACACAG	GTGGGAAGTAGGTGGGACAG
hsRor-α	ACTACACACCAGCATCAGGC	GCAGGTTTCCAGATGCGATT
hsDbp	GAGGAACTTAAGCCCCAGCC	CTCGTTGTTCTTGTACCGCC

APPENDIX C: PROTEIN MARKER

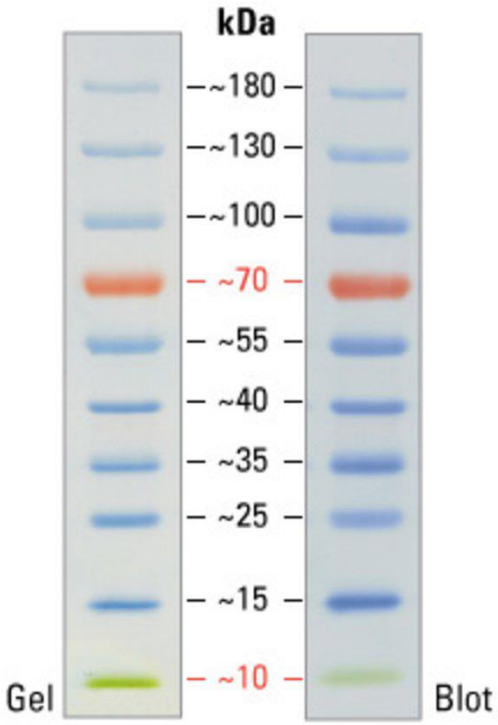


Figure A.1: PageRuler Prestained Protein Ladder within 4-20% Tris-glycine gel (Thermo Scientific)

BIBLIOGRAPHY

[1] G. E. Demas and L. J. Kriegsfeld, "Biological Rhythms," *Encyclopedia of Endocrine Diseases*, pp. 345–351, 2004, doi: 10.1016/B0-12-475570-4/00196-7.

[2] "Circadian Rhythms." https://nigms.nih.gov/education/fact-sheets/Pages/circadian-rhythms.aspx (accessed Jun. 05, 2023).

[3] M. Quante *et al.*, "Zeitgebers and their association with rest-activity patterns," *https://doi.org/10.1080/07420528.2018.1527347*, vol. 36, no. 2, pp. 203–213, Feb. 2018, doi: 10.1080/07420528.2018.1527347.

[4] D. Galimberti and G. Mazzola, "Chronobiology and chrononutrition: Relevance for aging," *Human Aging*, pp. 219–254, Jan. 2021, doi: 10.1016/B978-0-12-822569-1.00006-8.

[5] O. Çalıyurt, "Role of Chronobiology as a Transdisciplinary Field of Research: Its Applications in Treating Mood Disorders," *Balkan Med J*, vol. 34, no. 6, p. 514, Nov. 2017, doi: 10.4274/BALKANMEDJ.2017.1280.

[6] Y. Y. Chiou, Y. Yang, N. Rashid, R. Ye, C. P. Selby, and A. Sancar, "Mammalian period represses and de-represses transcription by displacing CLOCK-BMAL1 from promoters in a cryptochrome-dependent manner," *Proc Natl Acad Sci U S A*, vol. 113, no. 41, pp. E6072–E6079, Oct. 2016, doi: 10.1073/pnas.1612917113.

[7] M. Gallego and D. M. Virshup, "Post-translational modifications regulate the ticking of the circadian clock," *Nat Rev Mol Cell Biol*, vol. 8, no. 2, pp. 139–148, Feb. 2007, doi: 10.1038/NRM2106.

[8] L. A. Solt, D. J. Kojetin, and T. P. Burris, "The REV-ERBs and RORs: molecular links between circadian rhythms and lipid homeostasis," *Future Med Chem*, vol. 3, no. 5, p. 623, Apr. 2011, doi: 10.4155/FMC.11.9.

[9] Y. U. Doruk *et al.*, "A CLOCK-binding small molecule disrupts the interaction between CLOCK and BMAL1 and enhances circadian rhythm amplitude," *J Biol Chem*, vol. 295, no. 11, p. 3518, Mar. 2020, doi: 10.1074/JBC.RA119.011332.

[10] Z. Chen, S. H. Yoo, and J. S. Takahashi, "Development and Therapeutic Potential of Small-Molecule Modulators of Circadian Systems," *Annu Rev Pharmacol Toxicol*, vol. 58, pp. 231–252, Jan. 2018, doi: 10.1146/ANNUREV-PHARMTOX-010617-052645.

[11] Z. Chen, S. H. Yoo, and J. S. Takahashi, “Small Molecule Modifiers of Circadian Clocks,” *Cell Mol Life Sci*, vol. 70, no. 16, p. 2985, Aug. 2013, doi: 10.1007/S00018-012-1207-Y.

[12] Y. U. Doruk, “The effect of small molecule, CLK8, on circadian clock mechanism,” thesis, *Koc University*, Istanbul, 2019

[13] S. Isın, “Stabilization of the CLOCK: BMAL1 Heterodimer Decreases Circadian Rhythm Amplitude,” thesis, *Koc Univ.*, Istanbul, 2021

[14] Biological Rhythms - *Thomas Clarkson Academy*, https://www. thomasclarksonacademy. org/attachments/download.asp?file =517&type=pdf.

[15] J. P. Pett, A. Korenčič, F. Wesener, A. Kramer, and H. Herzel, “Feedback Loops of the Mammalian Circadian Clock Constitute Repressilator,” *PLoS Comput Biol*, vol. 12, no. 12, Dec. 2016, doi: 10.1371/JOURNAL.PCBI.1005266.

[16] A. Venkat and S. Muneer, “Role of Circadian Rhythms in Major Plant Metabolic and Signaling Pathways,” *Frontiers in Plant Science*, vol. 13. Frontiers Media S.A., Apr. 06, 2022. doi: 10.3389/fpls.2022.836244.

[17] C. H. Johnson, T. Mori, and Y. Xu, “A Cyanobacterial Circadian Clockwork,” *Current Biology*, vol. 18, no. 17, pp. R816–R825, Sep. 2008, doi: 10.1016/j.cub.2008.07.012.

[18] N. Kronfeld-Schor, G. Bloch, and W. J. Schwartz, “Animal clocks: when science meets nature,” *Proc Biol Sci*, vol. 280, no. 1765, 2013, doi: 10.1098/RSPB.2013.1354.

[19] U. Albrecht and G. Eichele, “The mammalian circadian clock,” *Curr Opin Genet Dev*, vol. 13, no. 3, pp. 271–277, Jun. 2003, doi: 10.1016/S0959-437X(03)00055-8.

[20] M. Mieda, “The network mechanism of the central circadian pacemaker of the SCN: Do AVP neurons play a more critical role than expected?,” *Front Neurosci*, vol. 13, no. FEB, 2019, doi: 10.3389/FNINS.2019.00139/FULL.

[21] L. S. Mure, “Intrinsically Photosensitive Retinal Ganglion Cells of the Human Retina,” *Front Neurol*, vol. 12, p. 636330, Mar. 2021, doi: 10.3389/FNEUR.2021.636330.

[22] M. M. Schroeder *et al.*, “The Roles of Rods, Cones, and Melanopsin in Photoresponses of M4 Intrinsically Photosensitive Retinal Ganglion Cells (ipRGCs) and Optokinetic Visual Behavior,” *Front Cell Neurosci*, vol. 12, Jul. 2018, doi: 10.3389/FNCEL.2018.00203.

[23] J. A. Mohawk, C. B. Green, and J. S. Takahashi, “Central and peripheral circadian clocks in mammals,” *Annu Rev Neurosci*, vol. 35, pp. 445–462, Jul. 2012, doi: 10.1146/ANNUREV-NEURO-060909-153128.

[24] Z. Chen, S. H. Yoo, and J. S. Takahashi, “Development and Therapeutic Potential of Small-Molecule Modulators of Circadian Systems,” *https://doi.org/10.1146/annurev-pharmtox-010617-052645*, vol. 58, pp. 231–252, Jan. 2018, doi: 10.1146/ANNUREV-PHARMTOX-010617-052645.

[25] M. H. Vitaterna, J. S. Takahashi, and F. W. Turek, “Overview of Circadian Rhythms,” *Alcohol Research & Health*, vol. 25, no. 2, p. 85, 2001, Accessed: Jun. 05, 2023. [Online]. Available: /pmc/articles/PMC6707128/

[26] I. Edery, “Circadian rhythms in a nutshell,” *Physiol Genomics*, vol. 2000, no. 3, pp. 59–74, 2000, doi: 10.1152/PHYSIOLGENOMICS.2000.3.2.59/ASSET/IMAGES/LARGE/H70800018002.JPEG.

[27] S. J. Kuhlman, L. M. Craig, and J. F. Duffy, “Introduction to Chronobiology,” *Cold Spring Harb Perspect Biol*, vol. 10, no. 9, Sep. 2018, doi: 10.1101/CSHPERSPECT.A033613.

[28] Y. Y. Chiou, Y. Yang, N. Rashid, R. Ye, C. P. Selby, and A. Sancar, “Mammalian Period represses and de-represses transcription by displacing CLOCK-BMAL1 from promoters in a Cryptochrome-dependent manner,” *Proc Natl Acad Sci U S A*, vol. 113, no. 41, pp. E6072–E6079, Oct. 2016, doi: 10.1073/PNAS.1612917113.

[29] N. Huang *et al.*, “Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex,” *Science (1979)*, vol. 337, no. 6091, pp. 189–194, Jul. 2012, doi: 10.1126/SCIENCE.1222804.

[30] L. A. Solt, D. J. Kojetin, and T. P. Burris, “The REV-ERBs and RORs: molecular links between circadian rhythms and lipid homeostasis,” *Future Med Chem*, vol. 3, no. 5, p. 623, Apr. 2011, doi: 10.4155/FMC.11.9.

[31] D. J. Kojetin and T. P. Burris, “REV-ERB and ROR nuclear receptors as drug targets,” *Nat Rev Drug Discov*, vol. 13, no. 3, pp. 197–216, 2014, doi: 10.1038/NRD4100.

[32] M. Keniry, R. K. Dearth, M. Persans, and R. Parsons, “New Frontiers for the NFIL3 bZIP Transcription Factor in Cancer, Metabolism and Beyond,” *Discoveries*, vol. 2, no. 2, p. e15, Jun. 2014, doi: 10.15190/D.2014.7.

[33] J. S. Takahashi, “Transcriptional architecture of the mammalian circadian clock,” *Nat Rev Genet*, vol. 18, no. 3, pp. 164–179, Mar. 2017, doi: 10.1038/NRG.2016.150.

[34] K. L. Toh *et al.*, “An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome,” *Science*, vol. 291, no. 5506, pp. 1040–1043, 2001, doi: 10.1126/SCIENCE.1057499.

[35] K. A. Lamia *et al.*, “AMPK Regulates the Circadian Clock by Cryptochrome Phosphorylation and Degradation,” *Science*, vol. 326, no. 5951, p. 437, Oct. 2009, doi: 10.1126/SCIENCE.1172156.

[36] W. Xing *et al.*, “SCFFbxl3 Ubiquitin Ligase Targets Cryptochromes at Their Cofactor Pocket,” *Nature*, vol. 496, no. 7443, p. 64, Apr. 2013, doi: 10.1038/NATURE11964.

[37] S. H. Yoo *et al.*, “Competing E3 Ubiquitin Ligases Determine Circadian Period by Regulated Degradation of CRY in Nucleus and Cytoplasm,” *Cell*, vol. 152, no. 5, p. 1091, Feb. 2013, doi: 10.1016/J.CELL.2013.01.055.

[38] A. Hirano, Y. H. Fu, and L. J. Ptáek, “The intricate dance of post-translational modifications in the rhythm of life,” *Nature Structural & Molecular Biology 2016 23:12*, vol. 23, no. 12, pp. 1053–1060, Dec. 2016, doi: 10.1038/nsmb.3326.

[39] M. L. Spengler, K. K. Kuropatwinski, M. Schumer, and M. P. Antoch, “A serine cluster mediates BMAL-dependent CLOCK phosphorylation and degradation,” *Cell Cycle*, vol. 8, no. 24, p. 4138, Dec. 2009, doi: 10.4161/CC.8.24.10273.

[40] G. Reid *et al.*, “Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling,” *Mol Cell*, vol. 11, no. 3, pp. 695–707, Mar. 2003, doi: 10.1016/S1097-2765(03)00090-X.

[41] Y. Kwak *et al.*, “Cyclin-dependent Kinase 5 (Cdk5) Regulates the Function of CLOCK Protein by Direct Phosphorylation,” *J Biol Chem*, vol. 288, no. 52, p. 36878, Dec. 2013, doi: 10.1074/JBC.M113.494856.

[42] S. Sahar, L. Zocchi, C. Kinoshita, E. Borrelli, and P. Sassone-Corsi, “Regulation of BMAL1 Protein Stability and Circadian Function by GSK3β-Mediated Phosphorylation,” *PLoS One*, vol. 5, no. 1, Jan. 2010, doi: 10.1371/JOURNAL.PONE.0008561.

[43] J. Hirayama *et al.*, “CLOCK-mediated acetylation of BMAL1 controls circadian function,” *Nature 2007 450:7172*, vol. 450, no. 7172, pp. 1086–1090, Dec. 2007, doi: 10.1038/nature06394.

[44] Y. Nakahata *et al.*, "The NAD+-Dependent Deacetylase SIRT1 Modulates CLOCK-Mediated Chromatin Remodeling and Circadian Control," *Cell*, vol. 134, no. 2, p. 329, Jul. 2008, doi: 10.1016/J.CELL.2008.07.002.

[45] L. Cunliffe, "Wrestling with time?," *Nature Reviews Molecular Cell Biology 2005 6:10*, vol. 6, no. 10, pp. 751–751, Sep. 2005, doi: 10.1038/nrm1750.

[46] Y. Lee, R. Chen, H. M. Lee, and C. Lee, "Stoichiometric Relationship among Clock Proteins Determines Robustness of Circadian Rhythms," *J Biol Chem*, vol. 286, no. 9, p. 7033, Mar. 2011, doi: 10.1074/JBC.M110.207217.

[47] M. M. Bellet *et al.*, "Pharmacological modulation of circadian rhythms by synthetic activators of the deacetylase SIRT1," *Proc Natl Acad Sci U S A*, vol. 110, no. 9, pp. 3333–3338, Feb. 2013, doi: 10.1073/PNAS.1214266110.

[48] E. E. Zhang *et al.*, "A genome-wide RNAi screen for modifiers of the circadian clock in human cells," *Cell*, vol. 139, no. 1, pp. 199–210, Oct. 2009, doi: 10.1016/J.CELL.2009.08.031.

[49] S. Gul *et al.*, "Discovery of a small molecule that selectively destabilizes Cryptochrome 1 and enhances life span in p53 knockout mice," *Nature Communications 2022 13:1*, vol. 13, no. 1, pp. 1–17, Nov. 2022, doi: 10.1038/s41467-022-34582-1.

[50] F. Tamanini, K. Yagita, H. Okamura, and G. T. J. Van Der Horst, "Nucleocytoplasmic shuttling of clock proteins," *Methods Enzymol*, vol. 393, pp. 418–435, 2005, doi: 10.1016/S0076-6879(05)93020-6.

[51] I. Kwon *et al.*, "BMAL1 Shuttling Controls Transactivation and Degradation of the CLOCK/BMAL1 Heterodimer," *Mol Cell Biol*, vol. 26, no. 19, p. 7318, Oct. 2006, doi: 10.1128/MCB.00337-06.

[52] K. Miyazaki, M. Mesaki, and N. Ishida, "Nuclear Entry Mechanism of Rat PER2 (rPER2): Role of rPER2 in Nuclear Localization of CRY Protein," *Mol Cell Biol*, vol. 21, no. 19, p. 6651, Oct. 2001, doi: 10.1128/MCB.21.19.6651-6659.2001.

[53] K. Yagita, F. Tamanini, M. Yasuda, J. H. J. Hoeijmakers, G. T. J. Van der Horst, and H. Okamura, "Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein," *EMBO J*, vol. 21, no. 6, p. 1301, Mar. 2002, doi: 10.1093/EMBOJ/21.6.1301.

[54] N. Huang *et al.*, "Crystal Structure of the Heterodimeric CLOCK:BMAL1 Transcriptional Activator Complex," *Science*, vol. 337, no. 6091, p. 189, Jul. 2012, doi: 10.1126/SCIENCE.1222804.

[55] C. Rosensweig and C. B. Green, “Periodicity, repression, and the molecular architecture of the mammalian circadian clock,” *Eur J Neurosci*, vol. 51, no. 1, pp. 139–165, Jan. 2020, doi: 10.1111/EJN.14254.

[56] N. Ozber *et al.*, “Identification of two Amino Acids in the C-terminal Domain of Mouse CRY2 Essential for PER2 Interaction,” *BMC Mol Biol*, vol. 11, no. 1, pp. 1–11, Sep. 2010, doi: 10.1186/1471-2199-11-69/FIGURES/6.

[57] I. H. Kavakli *et al.*, “The Photolyase/Cryptochrome Family of Proteins as DNA Repair Enzymes and Transcriptional Repressors,” *Photochem Photobiol*, vol. 93, no. 1, pp. 93–103, Jan. 2017, doi: 10.1111/PHP.12669.

[58] S. Gul *et al.*, “The Arg-293 of Cryptochrome1 is responsible for the allosteric regulation of CLOCK-CRY1 binding in circadian rhythm,” *J Biol Chem*, vol. 295, no. 50, p. 17187, Dec. 2020, doi: 10.1074/JBC.RA120.014333.

[59] W. H. Walker, J. C. Walton, A. C. DeVries, and R. J. Nelson, “Circadian rhythm disruption and mental health,” *Translational Psychiatry 2020 10:1*, vol. 10, no. 1, pp. 1–13, Jan. 2020, doi: 10.1038/s41398-020-0694-0.

[60] X. Yuan, C. Zhu, M. Wang, F. Mo, W. Du, and X. Ma, “Night Shift Work Increases the Risks of Multiple Primary Cancers in Women: A Systematic Review and Meta-analysis of 61 Articles,” *Cancer Epidemiol Biomarkers Prev*, vol. 27, no. 1, pp. 25–40, Jan. 2018, doi: 10.1158/1055-9965.EPI-17-0221.

[61] J. Arendt, “Shift work: coping with the biological clock,” *Occup Med (Lond)*, vol. 60, no. 1, pp. 10–20, Jan. 2010, doi: 10.1093/OCCMED/KQP162.

[62] R. W. Logan and C. A. McClung, “Rhythms of life: circadian disruption and brain disorders across the lifespan,” *Nature Reviews Neuroscience 2018 20:1*, vol. 20, no. 1, pp. 49–65, Nov. 2018, doi: 10.1038/s41583-018-0088-y.

[63] I. Shimizu, Y. Yoshida, and T. Minamino, “A role for circadian clock in metabolic disease,” *Hypertension Research 2016 39:7*, vol. 39, no. 7, pp. 483–491, Feb. 2016, doi: 10.1038/hr.2016.12.

[64] M. H. Vitaterna *et al.*, “Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior,” *Science*, vol. 264, no. 5159, pp. 719–725, Apr. 1994, doi: 10.1126/SCIENCE.8171325.

[65] F. W. Turek *et al.*, “Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice,” *Science*, vol. 308, no. 5724, p. 1043, May 2005, doi: 10.1126/SCIENCE.1108750.

[66] H. U. Jee *et al.*, “Activation of 5’-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2,” *J Biol Chem*, vol. 282, no. 29, pp. 20794–20798, Jul. 2007, doi: 10.1074/JBC.C700070200.

[67] Z. L. Zeng *et al.*, “Effects of the biological clock gene Bmal1 on tumour growth and anti-cancer drug activity,” *J Biochem*, vol. 148, no. 3, pp. 319–326, Sep. 2010, doi: 10.1093/JB/MVQ069.

[68] W. Sakamoto and S. Takenoshita, “OVEREXPRESSION OF BOTH CLOCK AND BMAL1 INHIBITS ENTRY TO S PHASE IN HUMAN COLON CANCER CELLS,” *Fukushima J Med Sci*, vol. 61, no. 2, p. 111, 2015, doi: 10.5387/FMS.2015-11.

[69] B. Marcheva *et al.*, “Disruption of the Clock Components CLOCK and BMAL1 Leads to Hypoinsulinemia and Diabetes,” *Nature*, vol. 466, no. 7306, p. 627, Jul. 2010, doi: 10.1038/NATURE09253.

[70] S. Yang *et al.*, “The role of mPer2 clock gene in glucocorticoid and feeding rhythms,” *Endocrinology*, vol. 150, no. 5, pp. 2153–2160, May 2009, doi: 10.1210/EN.2008-0705.

[71] Y. Zhao, Y. Zhang, M. Zhou, S. Wang, Z. Hua, and J. Zhang, “Loss of mPer2 increases plasma insulin levels by enhanced glucose-stimulated insulin secretion and impaired insulin clearance in mice,” *FEBS Lett*, vol. 586, no. 9, pp. 1306–1311, May 2012, doi: 10.1016/J.FEBSLET.2012.03.034.

[72] L. Fu, H. Pelicano, J. Liu, P. Huang, and C. C. Lee, “The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo,” *Cell*, vol. 111, no. 1, pp. 41–50, Oct. 2002, doi: 10.1016/S0092-8674(02)00961-3.

[73] S. Gery, N. Komatsu, L. Baldjyan, A. Yu, D. Koo, and H. P. Koeffler, “The Circadian Gene Per1 Plays an Important Role in Cell Growth and DNA Damage Control in Human Cancer Cells,” *Mol Cell*, vol. 22, no. 3, pp. 375–382, May 2006, doi: 10.1016/j.molcel.2006.03.038.

[74] M. J. Kim, J. H. Lee, and J. F. Duffy, “Circadian Rhythm Sleep Disorders,” *J Clin Outcomes Manag*, vol. 20, no. 11, p. 513, Nov. 2013, Accessed: Jun. 05, 2023. [Online]. Available: /pmc/articles/PMC4212693/

[75] O. Emre Onat *et al.*, “Human CRY1 variants associate with attention deficit/hyperactivity disorder,” *J Clin Invest*, vol. 130, no. 7, pp. 3885–3900, Jul. 2020, doi: 10.1172/JCI135500.

[76] A. Patke *et al.*, “Mutation of the Human Circadian Clock Gene CRY1 in Familial Delayed Sleep Phase Disorder,” *Cell*, vol. 169, no. 2, pp. 203-215.e13, Apr. 2017, doi: 10.1016/j.cell.2017.03.027.

[77] S. N. Archer *et al.*, “A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference,” *Sleep*, vol. 26, no. 4, pp. 413–415, Jun. 2003, doi: 10.1093/SLEEP/26.4.413.

[78] K. L. Toh *et al.*, “An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome,” *Science*, vol. 291, no. 5506, pp. 1040–1043, 2001, doi: 10.1126/SCIENCE.1057499.

[79] T. Hirota and S. A. Kay, “High-throughput screening and chemical biology: new approaches for understanding circadian clock mechanisms,” *Chem Biol*, vol. 16, no. 9, pp. 921–927, Sep. 2009, doi: 10.1016/J.CHEMBIOL.2009.09.002.

[80] Z. Chen, S. H. Yoo, and J. S. Takahashi, “Small Molecule Modifiers of Circadian Clocks,” *Cell Mol Life Sci*, vol. 70, no. 16, p. 2985, Aug. 2013, doi: 10.1007/S00018-012-1207-Y.

[81] T. Hirota *et al.*, “Identification of small molecule activators of cryptochrome,” *Science*, vol. 337, no. 6098, pp. 1094–1097, Aug. 2012, doi: 10.1126/SCIENCE.1223710.

[82] J. S. Takahashi, H. K. Hong, C. H. Ko, and E. L. McDearmon, “The Genetics of Mammalian Circadian Order and Disorder: Implications for Physiology and Disease,” *Nat Rev Genet*, vol. 9, no. 10, p. 764, Oct. 2008, doi: 10.1038/NRG2430.

[83] E. J. Eide *et al.*, “Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation,” *Mol Cell Biol*, vol. 25, no. 7, pp. 2795–2807, Apr. 2005, doi: 10.1128/MCB.25.7.2795-2807.2005.

[84] “Virtual Screening-MedChemExpress.” https://www.medchemexpress.com/virtual-screening.html?utm_source=google&utm_medium=CPC&utm_campaign=screening%20libraries&utm_content=Virtual%20Screening&utm_term=virtual%20screening%20in%20drug%20discovery&gclid=Cj0KCQjwgLOiBhC7ARIsAIeetVDE0-nszwmMlkWjI7zoCjxt5qC_-RaB9Betq038wcZLja96D_v6MCsaAjtHEALw_wcB (accessed Jun. 05, 2023).

[85] S. K. Chun *et al.*, “Identification and validation of cryptochrome inhibitors that modulate the molecular circadian clock,” *ACS Chem Biol*, vol. 9, no. 3, pp. 703–710, Mar. 2014, doi: 10.1021/CB400752K.

[86] Y. Isojima *et al.*, “CKIepsilon/delta-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock,” *Proc Natl Acad Sci U S A*, vol. 106, no. 37, pp. 15744–15749, Sep. 2009, doi: 10.1073/PNAS.0908733106.

[87] S. Miller *et al.*, “Isoform-selective regulation of mammalian cryptochromes,” *Nat Chem Biol*, vol. 16, no. 6, pp. 676–685, Jun. 2020, doi: 10.1038/S41589-020-0505-1.

[88] L. A. Solt *et al.*, “Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists,” *Nature*, vol. 485, no. 7396, pp. 62–68, May 2012, doi: 10.1038/NATURE11030.

[89] K. M. Walton *et al.*, “Selective inhibition of casein kinase 1 epsilon minimally alters circadian clock period,” *J Pharmacol Exp Ther*, vol. 330, no. 2, pp. 430–439, Aug. 2009, doi: 10.1124/JPET.109.151415.

[90] J. W. Lee *et al.*, “A small molecule modulates circadian rhythms through phosphorylation of the period protein,” *Angew Chem Int Ed Engl*, vol. 50, no. 45, pp. 10608–10611, Nov. 2011, doi: 10.1002/ANIE.201103915.

[91] Z. Chen *et al.*, “Identification of diverse modulators of central and peripheral circadian clocks by high-throughput chemical screening,” *Proc Natl Acad Sci U S A*, vol. 109, no. 1, pp. 101–106, Jan. 2012, doi: 10.1073/PNAS.1118034108.

[92] R. Arey and C. A. McClung, “An inhibitor of casein kinase 1 ε/δ partially normalizes the manic-like behaviors of the ClockΔ19 mouse,” *Behavioural pharmacology*, vol. 23, no. 4, pp. 392–396, Aug. 2012, doi: 10.1097/FBP.0B013E32835651FD.

[93] K. Vanselow *et al.*, “Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS),” *Genes Dev*, vol. 20, no. 19, p. 2660, Oct. 2006, doi: 10.1101/GAD.397006.

[94] S. Reischl *et al.*, “Beta-TrCP1-mediated degradation of PERIOD2 is essential for circadian dynamics,” *J Biol Rhythms*, vol. 22, no. 5, pp. 375–386, Oct. 2007, doi: 10.1177/0748730407303926.

[95] Q. J. Meng *et al.*, “Setting clock speed in mammals: the CK1ε tau mutation in mice accelerates the circadian pacemaker by selectively destabilizing PERIOD proteins,” *Neuron*, vol. 58, no. 1, p. 78, Apr. 2008, doi: 10.1016/J.NEURON.2008.01.019.

[96] T. Hirota, W. G. Lewis, A. C. Liu, W. L. Jae, P. G. Schultz, and S. A. Kay, “A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3β,” *Proc Natl Acad Sci U S A*, vol. 105, no. 52, p. 20746, Dec. 2008, doi: 10.1073/PNAS.0811410106.

[97] D. Grant *et al.*, “GSK4112, a small molecule chemical probe for the cell biology of the nuclear heme receptor Rev-erbα,” *ACS Chem Biol*, vol. 5, no. 10, pp. 925–932, Oct. 2010, doi: 10.1021/CB100141Y.

[98] D. Kojetin, Y. Wang, T. M. Kamenecka, and T. P. Burris, “Identification of SR8278, a synthetic antagonist of the nuclear heme receptor REV-ERB,” *ACS Chem Biol*, vol. 6, no. 2, pp. 131–134, Feb. 2011, doi: 10.1021/CB1002575.

[99] B. He *et al.*, “The small molecule Nobiletin targets the molecular oscillator to enhance circadian rhythms and protect against metabolic syndrome,” *Cell Metab*, vol. 23, no. 4, p. 610, Apr. 2016, doi: 10.1016/J.CMET.2016.03.007.