The Effect of Small Molecule, CLK8, on Circadian Clock Mechanism

by

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This is to certify that I have examined this copy of a master's thesis by

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To my beloved parents Nazlı and Kemal Doruk
and to my fiancé Alexander Polcyn
for their endless support

ABSTRACT

Circadian rhythms are metabolic, behavioral and physical changes which repeat themselves with about 24-hour period. These rhythms arise from transcriptional-translational regulatory feedback (TTFL) loops at cellular level. In the positive arm of the TTFL, CLOCK and BMAL1 heterodimerize and activate the expression of the Per and Cry genes. In the negative arm of the loop, PER2:CRY1 complex repressed their own transcription by inhibiting transactivation of CLOCK:BMAL1 complex. Properly functional circadian clock is crucial for health as its perturbation leads to severe pathologies including metabolic and cardiovascular diseases, sleep and mood disorders, accelerated aging and cancer progresses. Therefore, discovery of small molecule modifiers of circadian clock is important for the development of potential interventions to repair circadian clock, in turn, to improve health.

In this study, structure-based chemical genetic approach was used to find small molecules that specifically bind to CLOCK. Following the assessment of candidate small molecules by virtual screening according to changes in circadian rhythm, biochemical assays pointed out a compound (CLK8) as a circadian amplitude enhancer which specifically binds to CLOCK protein and modulates the interaction between CLOCK and BMAL1. Both in vitro and in vivo studies revealed that CLK8 reduces the nuclear abundance of CLOCK, overall expression of core clock genes and the protein levels of CLOCK and BMAL1 without altering the protein amount of PER2 and CRY1. These outcomes suggest that CLK8 enhances the amplitude of circadian rhythm by altering the stoichiometry among the core clock components in a way that the repression activity of negative arm of TTFL is stabilized by reducing the positive arm of the TTFL in nucleus. This study identifies CLK8 as a tool to understand the function of CLOCK in the regulation of circadian amplitude and as a potential candidate for therapeutic development for health problems associated with reduced circadian amplitude such as mood disorder, chronic metabolic diseases and accelerated aging.

ÖZETÇE

Sirkadiyen ritimler, yaklaşık 24 saat boyunca kendilerini tekrar eden metabolik, davranışsal ve fiziksel değişikliklerdir. Bu ritimler, hücresel düzeyde transkripsiyonel-translasyonel düzenleyici geri besleme (TTFL) döngülerinden meydana gelirler. TTFL'in pozitif kolunda, CLOCK ve BMAL1 proteinleri heterodimer oluşturup, *Per* ve *Cry* genlerinin anlatımını aktive eder. Döngünün negatif kolunda PER2:CRY1 kompleksi, CLOCK:BMAL1 kompleksinin aktivitesini engelleyerek kendi transkripsiyonlarını baskılar. Düzgün fonksiyonel sirkadiyen saat, sağlık için gerekli olup sirkadiyen salınımındaki bozukluklar metabolik ve kardiyovasküler hastalıklara, uyku ve duygu durumundaki bozukluklara, hızlandırılmış yaşlanmaya ve kanser riskinde artışa sebebiyet verir. Sirkadiyen saate etki eden küçük moleküllerin keşfi, sirkadiyen ritimlerindeki bozuklukları onararak sağlık sorunlarını iyileştirecek ya da tedavi edecek potansiyel müdahalelerin geliştirilmesi için önemlidir.

Bu çalışmada, özellikle CLOCK proteinine bağlanan küçük molekülleri bulmak için yapı-bazlı kimyasal genetik yaklaşım kullanılmıştır. Yaklaşık iki milyon aday küçük molekül bilgisayar ortamında taranıp sonrasında sirkadiyen ritimi üzerinde yarattıkları değişikliklere ve çeşitli biyokimyasal analizlerin sonuçlarına göre CLOCK proteinine spesifik olarak bağlanan ve CLOCK ve BMAL1 proteinleri arasındaki etkileşimi azaltarak sirkadiyen ritiminin genliğini arttırıcı bir molekül (CLK8) bulunmuştur. Hem laboratuvar ortamındaki hem de yaşayan organizma üzerindeki çalışmalar, CLK8'in, PER2 ve CRY1'in protein miktarını değiştirmeden CLOCK proteinin hücre çekirdeğindeki miktarını ve ana saat genlerinin ifadesiyle CLOCK ve BMAL1'in protein seviyelerini azalttığını ortaya koydu. Bu sonuçlar, CLK8'in, ana saat bileşenleri arasındaki stokiyometriyi değiştirerek, TTFL'nin negatif kolunun pozitif kolu üzerindeki baskılama gücünü stabilize ederek sirkadiyen ritimin genliğinin arttırdığını önermektedir. Bu çalışma CLK8'i CLOCK proteinini sirkadiyen ritminin genliğinin düzenlenmesindeki işlevinin anlaşılmasında bir aracı olarak tanımlamanın yanı sıra ve duygu bozukluk durumu, kronik metabolik hastalıklar ve hızlandırılmış yaşlanma gibi azalan sirkadiyen ritiminin genliğinden kaynaklana sağlık sorunlara tedavisel müdehale geliştirilmesine bir aday olarak da tanımlamaktadır.

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NOMENCLATURE

ZT: Zeitgeber time

SCN: Suprachiasmatic nucleus

CLOCK: Circadian Locomotor Output Cycles Kaput

NPAS2: Neuronal PAS domain protein 2

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

CRY: Cryptochrome

PER: Period

ROR: RAR-related orphan receptor

REV-ERB: Nuclear receptor subfamily 1, group D

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

NFIL3: Nuclear factor, interleukin 3 regulated

PACAP: Pituitary adenyl cyclase activation peptide

NMDA: N-methyl-D-aspartate

RORE: RAR-related orphan receptor response element

PARbZip: Proline and acidic amino acid rich basic leucine zipper

HLF: Hepatic leukemia factor

TEF: Thyrotroph embryonic factor

PTM: Post translational modification

CKIε/Iδ: casein kinase 1 epsilon/1 sigma

OGT: O-Linked N-Acetylglucosamine (GlcNAc) Transferase

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

SIRT1: Sirtuin 1

NLS: Nuclear localization signal

NES: Nuclear export signal

KPNB1: Karyopherin Subunit Beta 1

NRON: ncRNA repressor of the nuclear factor of activated T cells

TAD: Transactivating domain

bHLH: Basic helix-loop-helix domain

PAS: Per-arnt-sim domain

MLL1: Myeloid/lymphoid or mixed-lineage leukemia enzyme

HAT: Histone acetyl transferase

GSK-3β: Glycogen synthase kinase 3 beta

AMPK: AMP-activated protein kinase

FASP: Familial advanced sleep phase syndrome

SAD: Seasonal affective disorder

MEF: Mouse embryonic fibroblast cells

U2OS: Human bone osteosarcoma epithelial cells

NIH 3T3: Mouse fibroblast cells

MDA MD 231: Human epithelial breast cancer cell line

HEK 293T: Human embryonic kidney cells

COS7: African green monkey kidney fibroblast-like cells

CHAPTER 1

INTRODUCTION

Circadian rhythms are metabolic, behavioral, biochemical and physical changes which repeat themselves with a period of about 24 hours. In mammals, cell-autonomous oscillators in suprachiasmatic nuclei and peripheral tissues generate these rhythms and synchronize them to daily environmental changes [1]. A number of physiological events including body temperature, hormone secretion, and sleep-awake cycles are regulated by circadian clock [2]. Phase misalignment, dampened amplitude and abnormalities in circadian-related genes result in dysregulated circadian rhythm which leads to multitude of pathologies such as metabolic and cardiovascular diseases, sleep and mood disorders, acceleration in aging and cancer[3,4].

At cellular level, mammalian circadian clock arises from transcriptional translational regulatory feedback loops (TTFLs) (Figure 1). The main TTFL is consist of CLOCK and BMAL1 in the positive arm and PER and CRY in the negative arm; the E-box transcription factors CLOCK and BMAL1 heterodimerize and activate the expression of *Per* and *Cry* genes. Subsequently, PER:CRY complex represses their own expression by inhibiting CLOCK:BMAL1 transactivation. In the second TTFL, the RORE-box transcription factors RORs and REV-ERBs enhances and suppresses the expression of *Bmal1* and *Nfil3* genes, respectively [5]. The third TTFL is made of D-box transcription factors NFIL3 and DBP whose expression is positively controlled by CLOCK:BMAL1 complex [5]. NFIL3 down-regulates the expression of *Ror* and *Rev-erb* genes while DBP up-regulates their expression [5]. These three interlocking loops give rise to cyclic transcription. The phases of expression show variety based on the combination of cis-elements (E- and D-boxes and ROREs) found in promoters and enhancers of clock-controlled genes and the cis-elements are responsible for temporal expression of these target genes [6]. In addition to the expression pattern of core clock genes, post translational regulations which play roles in the stability, sub-cellular localization and transactivation of the clock proteins are also necessary for a robust circadian rhythm [7].

As vigorous clock is required for a healthy body and mind, discovery of small molecule modifies of circadian clock is important to develop potential intervention for disrupted circadian rhythms and related pathologies [4,8]. In fact, small molecule modifiers provide a

useful tool to module circadian system; unlike classical genetic approach and its permanent effects, small molecules have reversible, time-controlled and dose-dependent effects [8]. So far, a number of small molecules targeting the clock components and altering the circadian phenotype has been found. For instance, CRY1/2 stabilizer K001 and ROR agonist Nobiletin lengthen the circadian period and enhances the circadian amplitude, respectively [9,10]. However, none of small molecules targeting CLOCK protein directly and effecting circadian phenotype has been investigated, yet.

Recently, studies on the crystal structure of CLOCK:BMAL1 complex shown that both CLOCK and BMAL1 proteins are composed of C-terminal PAS-A and PAS-B domains with an N-terminal bHLH domain [11,12]. Among the heterodimerization of CLOCK and BMAL1, each of these domains interacts with its corresponding partner. While bHLH domains mediate binding of CLOCK:BMAL1 complex to DNA, PAS-A/B domains primarily provide interaction motifs for CLOCK:BMAL1 heterodimerization [11,12].

Regulations of circadian rhythm tightly depend on the status of CLOCK:BMAL1 heterodimer in terms of phosphorylation, stability, nuclear localization and transactivation activity of the complex [7] meaning that altering the interaction between CLOCK and BMAL1 proteins affects circadian oscillation. Therefore, modulating this interaction by targeting small molecules to the PAS domains of CLOCK protein has a potential for the development of intervention to repair disrupted circadian rhythm. Finding a small molecular modifier of clock acting as an amplitude enhancer might be used to treat pathologies due to the dampened amplitude such as chronic metabolic diseases, mood disorder and aging [13].

In this thesis, structure-based visual screening approach was used to screen approximately 2 million commercially available small molecules for their affinity to the PAS domain of the CLOCK protein and for their effects on circadian phenotype. Further biochemical assays revealed that CLOCK-binding small molecule (CLK8) decreases the interaction between CLOCK and BMAL1 and enhances the circadian rhythm at the cellular level by reducing the translocation of CLOCK in nucleus both *in vivo* and *in vitro*. Moreover, CLK8 down-regulates the transcription of core clock components both in the positive and negative arm of the TTFL while this molecule only changes the amounts of proteins at the positive arm of the TTFL, but not that of the ones in the negative arm of the TTFL. Together with the decrease of CLOCK protein amount in nucleus, the outcomes implied that CLK8 alters the stoichiometry among the clock protein in a way that CLK8 stabilizes the repression

activity of PER2 and CRY1 proteins by reducing the CLOCK and BMAL1 amount which results in the enhancement of circadian amplitude.

Chapter 2 provides a literature review about the molecular mechanism of mammalian circadian rhythm, the structure of CLOCK protein and the importance of small-molecule modifiers to repair disrupted circadian rhythm and their potential as being interventions.

Chapter 3 explains the materials and methods used during this work in detail. Real time monitoring of circadian rhythm, immunoprecipitation assays, lentiviral production and transduction, cloning and transfection, sub-cellular fractionation, protein and mRNA analysis are included in this chapter.

Chapter 4 shows the results obtain during this thesis study that CLK8 enhances the circadian amplitude by reducing the interaction of CLOCK and BMAL1, the abundance of CLOCK in nucleus and the positive arm of the loop at protein level.

Chapter 5 discusses the outcomes obtain from this thesis work and indicates the future perspectives of this study.

CHAPTER 2

LITERATURE REVIEW

2.1. Biological Rhythms

As a consequence of the Earth's rotation on its axis and its orbit around the sun, environmental factors such as light and temperature change periodically. These rotations generate geophysical cycles including daily (app. 24 hours), tidal (12.8 hours), lunar (29.53 days), seasonal (365.24 days) cycles. From cyanobacteria to humans, most species, evolved endogenous biological rhythms for adaptation to temporal changes [14–16].

Biological rhythms are classified as circadian rhythms, ultradian rhythms, and infradian rhythms. Circadian rhythms (Latin: circa = about; dies = day) have period around 24 hours. They are seen in sleep-awake cycles, oscillation of body temperature, and patterns of hormone secretion. Ultradian rhythms including breathing, blinking, and pulse show shorter period than 24 hours, whereas human menstrual cycle, and seasonal migration have infradian rhythms with period longer than 24 hours [2].

2.2. Circadian Rhythms

Circadian rhythms are metabolic, behavioral and physical changes which repeat themselves with a period of about 24 hours. For example, the metabolism of cyanobacteria shows circadian oscillation. Photosynthesis and nitrogen fixation are segregated temporally; photosynthesis happens during day time as it needs for rich oxidative environments [17]. Following the sunset, the level of oxygen decreases, and cyanobacteria starts nitrogen fixation. Another example is the mating behavior of *Spodoptera littoralis*, a moth specie. A pheromone produced by female moths resets the circadian rhythm of male moths and induces male locomotory activity. In this way, mating occurs at night [18]. The plant *Mimosa pudica,* also shows circadian rhythm in the physical movement of its leaves. It opens and closes during a day [19]. In fact, the pattern of the leaf-movement continues under constant dark.

There are three criteria for a biological rhythm to be considered as circadian rhythm. Firstly, circadian rhythms have an endogenous and self-sustaining time-keeping system regulated by genome. This means that even under constant darkness or constant temperature, circadian period is conserved. Temperature compensation is the second characteristic of

circadian rhythms. The period is relatively conserved against a range of changes in temperature. Thirdly, circadian rhythms are entrainable meaning that external stimuli such as light, temperature, social interactions, and feeding patterns called as zeitgebers (German: zeit = time; geber = giver) can reset circadian rhythms and synchronize circadian rhythms with the rhythms of themselves [2].

2.3. The Historical Perspective of Circadian Clock

Jean-Jacques de Marian (1678-1771), a French astronomer, was the first scientist who demonstrated an endogenous daily rhythm in plant. He observed that the leaves of *Mimosa pudica*, open up during a day and fold up among the night. When he placed the plant into constant dark, movement pattern of *Mimosa pudica* was persisted . This indicated that the daily rhythm of the leaves does not need an external stimulus and internal mechanism is responsible for generating such rhythm in mimosa [14].

Augustin Pyramus de Candolle (1778-1841), a Swiss botanist was the first person describing the endogenous circadian clock. Candle noticed that the period of leaf-folding in *Mimosa pudica* was shorter than 24 hours. He concluded that the circadian rhythm is not only regulated by geophysical cycles, but an inner circadian clock of the plant controls the circadian rhythm [2].

The molecular mechanism that generates endogenous circadian clock was unknown for another 100 years. In 1971, Ronald Konopka and Seymour Benzer achieved a breakthrough by identification of the genetic basis of circadian clock. They used forward genetics in *Drosophila melanogaster* and examined two circadian behaviors which are pulp eclosion and locomotor activity. One of three phenotype was observed in mutant flies: arrhythmia, shorter period, or longer period. Following the genetic mapping of the mutants, Konopka and Benzer found that a single locus on X-chromosome, referred as the *Period* gene, was responsible for those phenotypes [20]. Then, three prominent scientist, Micheal Young, Michael Rosbash and Jeffrey Hall won 2017 Nobel Prize in Physiology or Medicine, proposed a transcriptional and translational feedback loop mechanism (section 2.5.1) proposed for *D. melanogaster* [21]. They showed that *Per* encodes a protein that accumulates in the cell during the night and then, is being degraded during the day. Following that, they discovered other core components circadian clock at molecular level which are responsible for governing the self-sustaining clockwork [21].

In 1994, Joseph S. Takahashi and his group discovered another circadian clock gene in *Mus musculus* using forward genetic screening. The gene *Clock* (Circadian Locomotor

Output Cycles Kaput) controls length of the circadian period and maintenance of its rhythmicity [22]. Three years after the discovery of *Clock* gene, John B. Hogenesch characterized ARNTL1 (Aryl hydrocarbon receptor nuclear translocator-like protein 1), known as BMAL1 that it is a component of circadian clock and regulates circadian rhythm at molecular and behavioral levels [23,24].

2.4. Mammalian Circadian Clock

Mammalian circadian clock is composed of multiple oscillators found at cellular, tissue and system levels. At the top of the hierarchy in the oscillators is the suprachiasmatic nuclei (SCN) as master pacemaker [1,5]. It is a group of neural circuits (consist of around 2 thousand neuron cells) located bilaterally in the hypothalamus. Unlike peripheral tissue, SCN oscillators are coupled tightly. Retinal ganglion cells in retina sense blue light and transmit signals to SCN via retinohypothalamic tract. Then, predominantly pituitary adenylate cyclase-activating polypeptide (PACAP) and *N*-methyl-D-aspartic (NMDA) glutamatergic signaling pathways induce *Period 1* gene and reset cellular oscillators [4]. Several other neurotransmitters also play roles in coupling SCN. In turn, SCN synchronizes peripheral tissues and cellular clocks throughout the body via signaling molecules. For instance, the rhythmic release of glucocorticoid hormone from hypothalamus is regulated by SCN [4].

2.5. Molecular Mechanism of Circadian Clock

Even though SCN is the master pacemaker and synchronizes circadian clock throughout the body, the operational unit of the mammalian circadian system is an cell-autonomous oscillator which is not only found in SCN both also found in peripheral cells [25]. This cell-autonomous circadian clock arises from a transcriptional translational regulatory feedback loops where there are positive and negative arms (Figure 1). Basically, the positive arms initiate the transcription of clock-controlled genes including the genes that are core in the negative arms. Then, the negative arms downregulate their own expression by repress the activity of proteins in the positive arms. As much as the rhythmic expression of these elements, their amount, activity, stability and subcellular localization are important for the maintenance of circadian clock [7].

2.5.1. Transcriptional-Translational Regulatory Feedback Loops

In the primary feedback loop, the transcription activators CLOCK and BMAL1 heterodimerize and initiate the expression of target genes which have E-box cis-regulatory enhancer sequences, including *Period 1-3* and *Cryptochrome 1-2* in mammals. At the

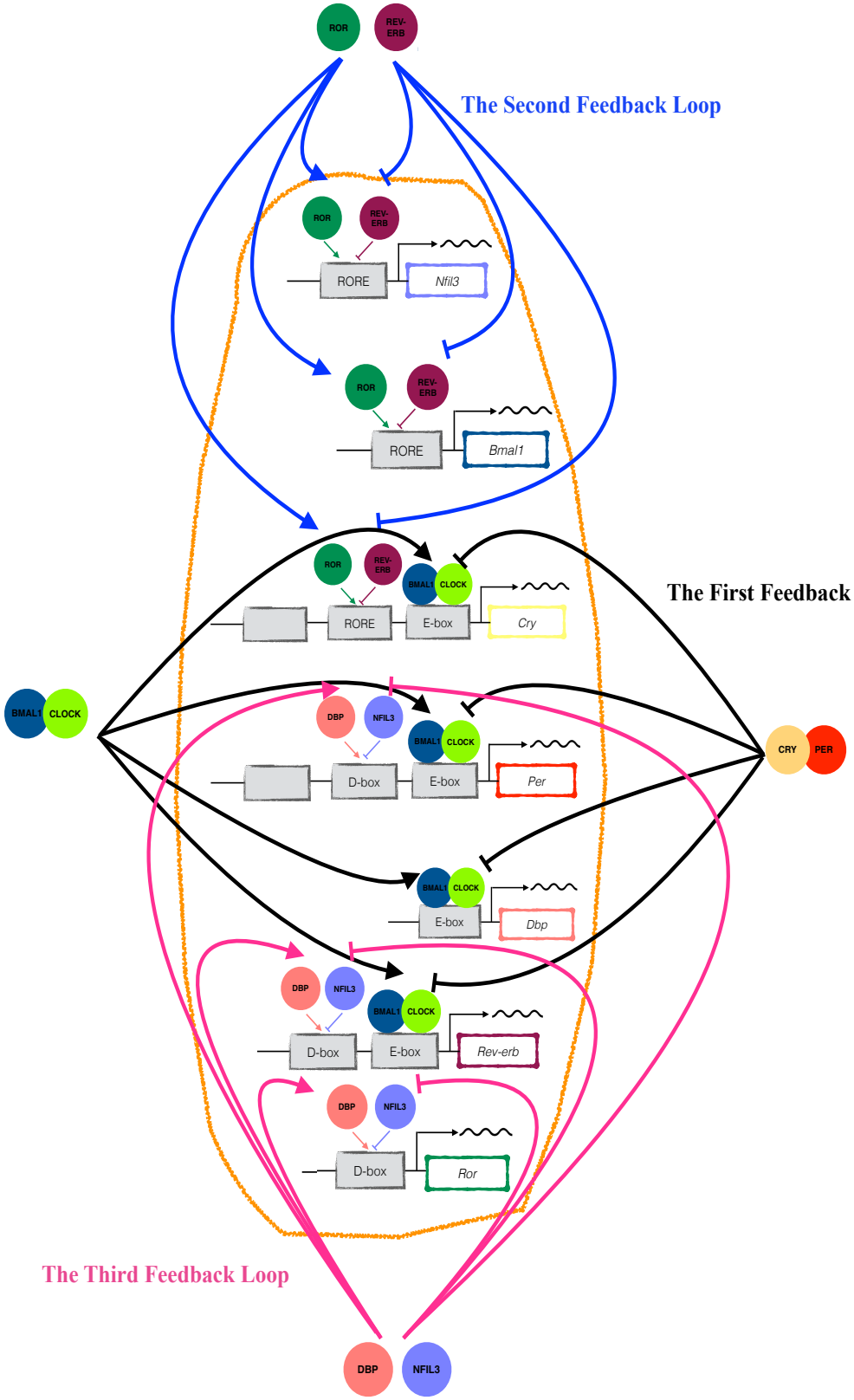

Figure 1. Three interlocked transcriptional-translational regulatory feedback loops. CLOCK and BMAL1 activate *Per* and *Cry* genes. In turn, PER and CRY repress their own transcription. CLOCK and BMAL1 also activate expression of Rev-erb and *Dbp* genes. REV-ERBs repress the transcription of *Bmal1* and *Nfil3* while DBP and NFIL3 are responsible for the activation and repression of *Ror* genes, respectively.

negative arm of the loop, the heterodimeric partners PER and CRY accumulates in cytosol and heterodimerize. Subsequently, the PER:CRY together with casein kinase I epsilon complex translocate into nucleus where they suppress clock-controlled genes and their own transcription via inhibiting the activity of CLOCK:BMAL1 complex [1,6,7]. In mice which are nocturnal animals, CLOCK:BMAL1 activation takes place during the daytime and leads to transcription of Per and Cry by the afternoon. Then, PER and CRY proteins are accumulated by late afternoon or evening and initiate repression [5].

In the second feedback loop, CLOCK:BMAL1 complex activates the transcription of retinoic acid-related orphan nuclear receptors REV-ERBs and RORs which are compete with each other at binding to ROREs (retinoic acid-related orphan receptor response elements) which are found in *Bmal1* promoter. Thus, the circadian oscillation of BMAL1 can be regulated positively and negatively by RORs and REV-ERBs, respectively [1,6,25]. The second loop induces anti-phasic expression rhythms of the *Bmal1* and *Clock* compared to *Per2* expression rhythm [1].

In the third feedback loop, CLOCK:BMAL1 complex activates PARbZip (proline and acidic amino acid rich basic leucine zipper) transcription factors including DBP (D-box binding protein), HLF (hepatic leukaemia factor), and TEF (thyrotroph embryonic factor). They are the activators of genes containing D-box elements. On the order hand, REV-ERB–ROR loop driven repressor NFIL3 (nuclear factor, interleukin-3 regulated, known as E4BP4) competes with these activators for binding to D-boxes [6,25].

It is hypothesized that these three interlocking loops give rise to cycles of transcription. The phases of expression show variety based on the combination of cis-elements (E- and D-boxes and ROREs) found in the promoters and enhancers of target genes [6]. Cis-elements are also responsible for temporal expression of target genes. In this way, E-boxes function in the morning, D-boxes are active during a day, and ROREs take the control on expression profile in the evening [25,26].

2.5.2. Stoichiometric Relationship Among Clock Proteins

The stichometry relationship between proteins in the positive and the negative arms of the regulatory feedback loops are essential for the robustness of the circadian rhythm (Figure 2). Even though CLOCK:BMAL1, components of the positive arm, is more abundant than the negative complex PER:CRY, oscillation of PER:CRY seems more crucial for the maintenance of circadian clock [9,27]. Additionally, post translational modifications of the

CLOCK:BMAL1 are tightly regulated in a circadian manner, which provides robustness of circadian clock.

Gel-filtration chromatography assay with mouse liver shows that CLOCK:BMAL1 and PER:CRY complexes are predominant in nucleus and PER is the rate-limiting factor for the formation of a stable complex between CLOCK:BMAL1 and PER:CRY. In MEFs (mouse embryonic fibroblasts), the level of CRY1 is similar to those of CLOCK and BMAL1 while the abundancy of PERs is approximately half of the abundancy of CLOCK and BMAL1. It suggests that the positive complexes would be twice more than the negative complex [27].

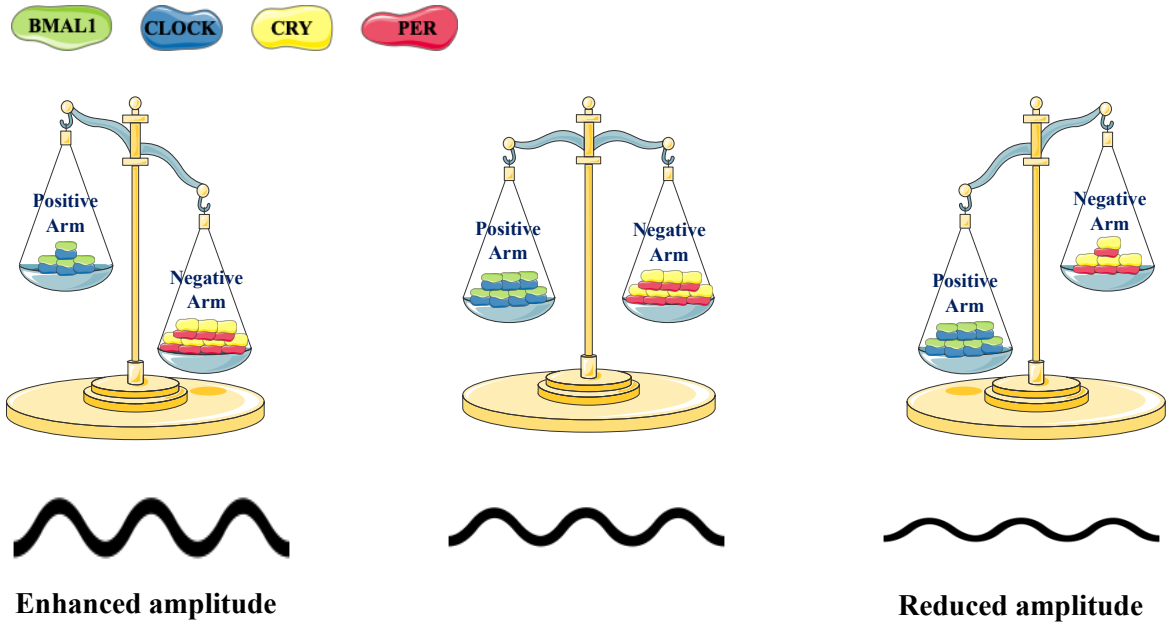

Figure 2. Stoichiometric relationship among clock proteins. CLOCK:BMAL1 heterodimer is in the positive arm of the core regulatory feedback loop while CRY:PER complex is on the negative arm of it. When weight on the positive arm or on the negative arm are elevated, the amplitude of circadian rhythm is reduced or enhanced, respectively. (This figure is created by using Servier Medical Art by Servier liciensed under a Creative Commons Attribution 3.0 Unported License)

CLOCK and BMAL1 are both required for transactivation. Therefore, over-expression of CLOCK or BMAL1 alone does not change the expression profile of E-box genes. However, the circadian period is shortened and lengthened following the over-expression of CLOCK and BMAL1, respectively. When CLOCK and BMAL1 are co-expressed in MEFs, mRNA levels of *Rev-erb* and *Dbp* are increased whereas *Per2* expression is slightly elevated and *Cry1* amount does not changed [27]. Interestingly, CLOCK and BMAL1 co-expression

reduces the amplitude of the circadian rhythm [27]. It is suggested that weaker feedback inhibition due to the higher ration of the positive arm to the negative arm could be the reason of less robust oscillation.

When the negative arm of the feedback loop is elevated by over-expressing PER, the amplitude of the circadian rhythm is increased in MEFs [27]. Another study shows that dose-dependent knockdown of *Bmal1* by siRNA improves circadian amplitude in *Bmal:dLuc* U2OS cells [28]. The possible reason of this phenotype is that reduction of BMAL1 level might attenuate the formation of CLOCK:BMAL1 complex and decrease strength of the positive arm of the regulatory loop. Moreover, Nobiletin, a small molecule modifier of clock, alters the post translational regulations in a way that PER2 and CRY1 proteins are enriched in mouse fibroblast cells after Nobiletin treatment. Consequently, increases the concentration of the proteins participate in negative arm of the regulatory feedback loop and cause increase in the amplitude of the circadian rhythm [9].

2.5.3. Post Translational Modifications of Clock Proteins

Another factor that is important to maintain circadian oscillation is the posttranslational modifications of the core clock proteins (PTM). Phosphorylation, ubiquitination and acetylation are well-characterized PTMs regulating circadian clock [7].

PERs at different region are phosphorylated by casein kinase (CK) Iδ and Iε, which affect stability of PER proteins. An identified rare SNP p.Ser662Gly in humans shown to enhances PER2 degradation and result in shortens circadian period and decreases its suppressor activity, and causes a disease known familial advanced sleep phase (FASP) syndrome [29–31]. Although FASP site is very important to control the stability of PER, it is still not known how this site being phosphorylated. Recently, it has been shown that O-GlcNAc transferase (OGT) competes with CKIs at FASP site for O-GlcNAcylation of PER which accelerates the repression activity of PER while decreasing its stability. The switch between O-GlcNAcylation and phosphorylation depends on glucose accessibility, and it shows the cross-talk between metabolism and circadian clock [32]. PERs are also regulated by acetylation and ubiquitination that stabilizes and degrades PERs, respectively [33].

CRYs repress CLOCK:BMAL1 transactivation strongly, so the PTMs on CRYs are crucial for the maintenance of robust circadian rhythm. FBXL3 (an F-box-type E3 ligase), holding a nuclear localization signal (NLS), promotes CRY degradation via ubiquitinoylation [34]. This enzyme is antagonized by one of its homologs, FBXL21 which ubiquitinylates CRY at different residues, as a consequence, this modification interferes with binding of FBXL3

and stabilizes CRY. PER also competes with FBXL3 for CRY, and the binding affinity of FBXL3 is reduced when CRY is phosphorylated. Altogether, FBXL3 is localized at nucleus, decreases the CRY stability, and regulates circadian period [34,35]. Mutagenesis screen in mice revealed that a mutant known as Overtime caused by loss-of function mutation in FBXL3 lengthens the circadian period for about 2 hours [35].

Unlike PER and CRY, CLOCK does not show significant circadian oscillation at protein level, but PTMs on these proteins show circadian rhythms. Phosphorylation plays a significant role by effecting the transactivation activity of CLOCK:BMAL1 complex via altering their stability, nuclear localization and degradation [7]. Interestingly, chemically inhibition of proteasome leads to the accumulation of CLOCK and BMAL1, but it also decreases E-box driven transcription level. This circumstance is explained by the transcription-factor cycling phenomena which is observed in many transcription factors like estrogen receptor: Following the binding of a transcription factor to DNA and recruitment of a transcriptional machinery subsequently, the transcription factor should be degraded unless it cannot have efficient turnover [36]. Generation of phosphorylation-deficient CLOCK and BMAL1 via site-directed mutagenesis increases the stability of these proteins significantly while their transcriptional activity reduces [37]. These two examples show that instability of CLOCK and BMAL1 improves their transcriptional activity. Addition to phosphorylation, acetylation is the other type of PTMs which is regulates the transcriptional activity of CLOCK:BMAL1. The intrinsic histone acetyltransferase activity of CLOCK is responsible for histone acetylation and acetylation of BMAL1 which enhances binding of CRY1 to the CLOCK:BMAL1 and repression of the complex [38]. One the other hand, binding of SIRT1 to CLOCK:BMAL1 erases acetyl group from BMAL1 that interrupts binding affinity of CRY to the complex, and reinitialize the transactivation [7]. Another PTM that shows circadian fashion and controls transactivation of CLOCK:BMAL1 is SUMOylation of BMAL1 by SUMO2/3 [39]. This modification regulates stability of BMAL1, it turn, affect expression profile of clock-controlled genes [39].

2.5.4. Nucleocytoplasmic Shuttling of Clock Proteins

Unlike the other core components, CLOCK does not have an obvious oscillation at neither transcriptional nor translational levels. However, the subcellular localization of CLOCK is under circadian fashion. When CLOCK level elevates in nucleus, it decreases in cytosol. In fact, studies with MEF cells demonstrated that nuclear localization of CLOCK is BMAL1-dependent. Expression of CLOCK alone results in concentration of CLOCK mainly

in cytoplasm whereas co-expression of CLOCK and BMAL1 leads to formation of CLOCK:BMAL1 complex and nuclear accumulation of CLOCK. Abolishment of nuclear abundance of CLOCK in BMAL1-deficient mice is also in agreement with these findings [40].

A nuclear localization signal (NLS) and nuclear export signals (NES) are found in the N-terminal and PAS domains of BMAL1, respectively. These signals enable BMAL1 to shuttle between cytoplasm and nucleus, in turn, it drives accumulation of CLOCK in nucleus. This dynamic is crucial for the transactivation and degradation of CLOCK:BMAL1 complex. The NLS mutation of BMAL1 abolishes the nuclear accumulation of BMAL1 and so the nuclear translocation of CLOCK. Surprisingly, co-expression of CLOCK carrying an NLS sequence could not rescue this defect. On the other hand, BMAL1 with NES mutation is concentrated in nucleus, predominantly. However, NES mutant BMAL1 fails to mediate nuclear translocation of CLOCK. This reveals that BMAL1 shuttling is necessary for the nuclear accumulation of CLOCK [41].

Studies in COS7s (African green monkey kidney fibroblast-like cells) elucidated that heterodimerization of mPER1:mPER3 and mPER2:mPER3, and nuclear entry of these complexes are induced by serum shock. This still happens in mCry1/mCry2 double-deficient cells. However, mPER3 lacking the NLS sequence cannot promote nuclear translocation of mPER1 and mPER2 [42].

Even though mPER2 can shuttle between the nucleus and the cytoplasm in mCry1/mCry2 double mutant MEF cells, mCRY proteins promotes nuclear accumulation of mPER2 proteins. Once mPER2 is in the nucleus, mCRY1 binds to the C-terminal of mPER2 and prevents both nuclear export and ubiquitylation of mPER2. In this way, mPER2 is protected as it is not degraded by the proteasome system [43].

Later studies elucidated that KPNB1, an importin β component of the ncRNA repressor of nuclear factor of activated T cells (NRON) ribonucleoprotein complex, is important for the nuclear accumulation of PER2:CRY1 complex. Knockdown of KPNB1 blocked nuclear translocation of PER1 and PER2 completely whereas its depletion partly prevents nuclear accumulation of CRY1 [44].

2.6. The Structure of CLOCK:BMAL1 Complex

CLOCK and BMAL1 proteins are made of 855 and 626 amino acids, respectively and both of them contain N-terminal bHLH and the two tandems in C-terminal PAS-A and PAS-B domains with transactivation domains (TADs) [11]. All three domains are responsible for the heterodimerization of CLOCK and BMAL1 proteins, and each domain interacts with the

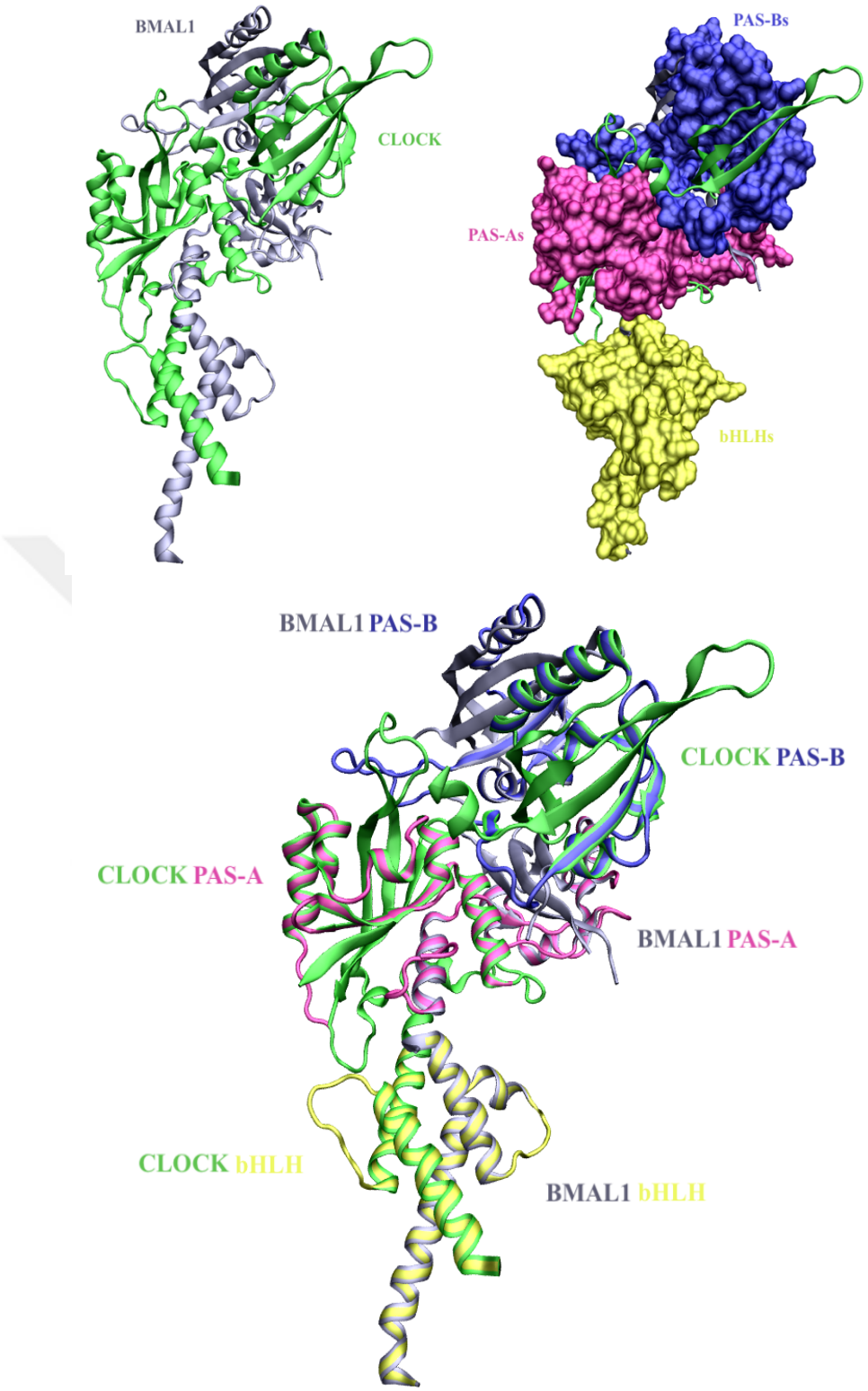

Figure 3. The structure of CLOCK:BMAL1 complex. Both CLOCK and BMAL1 proteins contain N-terminal bHLH and the two tandems in C-terminal PAS-A and PAS-B domains with transactivation domains (TADs). All three domains are responsible for the heterodimerization of CLOCK and BMAL1 proteins, and each domain interacts with the corresponding domain of its partner.

corresponding domain of its partner subunit in a way that CLOCK bHLH is interaction with BMAL1 bHLH, CLOCK PAS-A with BMAL1 PASA, and CLOCK PAS-B with BMAL1 PAS-B [45,46] (Figure 3). bHLH domains mediates binding of CLOCK:BMAL1 complex to E-box DNA sequence (e.g., CACGTG, CCAATG, CATGTG, AACGTG) and PAS-A/B primarily provide interaction motifs for CLOCK:BMAL1 heterodimerization [11]. Although these domains in CLOCK and in BMAL1 are similar to each other, the spatial arrangements of bHLH and PAS-A domains of two proteins are different. The distribution of electrostatic potential of the domains are also dissimilar. CLOCK has an overall negative electrostatic potential (pI of 9.0 for bHLH and 8.5 for PAS-A/B) while BMAL1 has an overall positive electrostatic potential (pI of 5.9 for bHLH and 5.3 for PAS-A/B) [45]. These differences enable interactions of PER and CRY proteins differently with CLOCK and BMAL1.

Mutations at the bHLH domain destabilize CLOCK:BMAL1 heterodimer whereas PAS-A/B mutations reduce transcriptional activity of CLOCK:BMAL1 complex and the affinity of the proteins for each other. When wild-type CLOCK or BMAL1 are overexpressed, the circadian period gets shorter or longer, respectively. However, overexpression of CLOCK bHLH or CLOCK PAS-A/B mutants fail to generate shorter period which indicates that these are loss-of-function mutations. On the other hand, overexpression of BMAL1 bHLH or PAS-A/B mutants resulted in a longer period followed by arrhythmicity or a shorter period which means that such mutations are partial loss-of-function mutations [45].

2.6.1. CLOCK Specific Features

PAS-B domain of CLOCK at C-terminal holds the exon 19 which is one of the critical regions for normal clock function [47]. In fact, it is the only region having a sequence similarity with NPAS2 (a paralog of CLOCK; serves as a binding partner for BMAL1 and plays role in rhythmic activation of clock-controlled genes [48]) [47]. CLK proteins of *Drosophila melanogaster* and *Antheraea pernyi* include homolog sequence to exon 19 at C-terminal. Deletion of these sequences weaken interaction of PERs with CLOCK [11].

CLOCK:BMAL1 transactivation complex recruits several histone modifying enzymes. For instance, H3K4me3 (trimethylation of histone 3 lysine 4) at the promoters of clock-controlled genes has a circadian fashion. MLL1, a histone methyltransferase is important for rhythmic expression of these genes. MLL1 does not show a circadian rhythm in its expression level, but its physically binding to CLOCK:BMAL1 complex does. However,

deletion of exon 19 of CLOCK prevents this interaction so the expression of clock genes is attenuated [49,50].

CLOCK has intrinsic histone acetyltransferases (HAT) activity required for epigenetic regulation H3K9 and H3K14 and for regulation of the expression of *Dbp* and *Per1*. CLOCK also acetylates BMAL1 at residue K537 which is necessary for recruitment of CRY1 to BMAL1. Mutations in the PAS-B domain of CLOCK prevents repressive feedback of CRY1 [38].

2.7. Small-Molecule Modulators of Circadian Clock

Dysregulation of circadian rhythm is characterized by phase misalignment or manipulations in circadian genes and it causes sleep disorders, metabolic and cardiovascular diseases, cancer, and other pathologies [4]. For instance, enforced 28-hour circadian period on humans for 10 days results in glucose tolerance and hyperinsulinemia. Similar outcomes were seen in mouse model for 2-week work shift [51]. Mutations in circadian genes also are followed by a range of physiological deficiency as metabolic syndromes in *Clock*Δ*19/*Δ*19* mutant mice and premature aging in *Bmal1-/-* mutant mice [52,53]. These findings indicate the importance of robust circadian oscillation for heath.

Sequence of a target gene or abundance of a target protein are changed permanently in classical genetic approach whereas small molecule modifiers alter expression profile of a target gene or function of a target protein reversibly in a time-controlled and dose-dependent manner [8]. Therefore, small molecule-based chemical genetic approach provides a useful tool to modulate intracellular complications and there are three methods for identifying small molecule modifiers of circadian clock. The first one is the phenotypic functional assay for measuring effects of modifiers on circadian oscillation where multiple relevant proteins and pathways might be targets of the molecules. Reporter cell lines expressing luciferase form an exogenous clock gene promoter or PER2::luciferase from endogenous Per2 promoter are used to monitor bioluminescence for several days [8]. In this way, circadian rhythm of the model system is visualized to understand how small molecules change period, phase, and amplitude. However, direct targets of novel compounds are left to enlightened. The second method is target-directed screens where effects of compounds on a target are identified, and it requires prior knowledge of known ligands or binding cavity structures of targets [8,54]. For instance, tertiary amines with three lipophilic substituents were discovered as agonists of REV-ERBs and RORs by focus privileged scaffolds and endogenous ligands of these proteins [55]. The third method is structure-based virtual screening that three-dimensional

structure of a target is used to dock compound libraries into a binding site on the target. Then, a subset of these compounds are selected based on their binding scores for further biological evaluation by in vitro and in vivo assays [56]. As millions of small molecules can be screened computationally in this method, it is fast and cost-efficient way to discover lead molecules.

Studies on small molecule modifier of clock reveals potential interventions which are categorized as interventions to train the clock (providing the maintenance of robust circadian rhythm in feeding/fasting, sleep/awake and light/dark loops), interventions to clock the drugs (as known as chronotherapy, providing accurate timing of drug treatment in order to enhance their efficacy and mitigate their side effects), and interventions to drug the clock (providing improved health by targeting circadian clock components directly) [3]. For example, opsinamides has a potential to train the clock by prevents photosensitivity via melanopsin inhibition in rodents [57]. This pharmacological darkness might be used to regulate sleeping pattern during summer months. Another examples comes from chronotherapy that therapeutic outcomes of anticancer drugs significantly improved in ovarian cancer patients by administration of doxorubicin and cisplatin in the evening and in the morning, respectively [58]. The Table 1 below lists same of the examples of small molecules used for drugging the clock.

Table 1. Small molecule modifiers targeting core clock components

Molecule	**Target Activity**	**Circadian Phenoype**	**Reference**
KL001	CRY1/2 stabilizer	Lengthened period and reduced amplitude	[59]
2-ethoxypropanoic acid KS15	CRY1/2 inhibitor	Shortened period and reduced amplitude	[60]
SR9009 and SR0911	REV-ERB agonist	Reduced amplitude	[55]
SR8278	REV-ERB antagonist	Shortened period	[61]
Nobiletin	ROR agonist	Enhanced amplitude, lengthened period	[9]
T0901317	ROR agonist	Reduced Bmal1 expression	[62]
Metformin	AMPK activator	Lengthened period and reduced amplitude	[63]
Lithium	GSK3β inhibitor	Shortened period	[64]
SRTCD1023	SIRT1 activator	Lengthened period and reduced amplitude	[65]

2.7.1. Therapeutic Potential of Small-Molecule Modulators

Metabolism and circadian clock are in close relationship. *Clock*Δ*19/*Δ*19* mutation causes a range of metabolic disorder due to the attenuation of circadian gene expression and the rhythmicity of feeding [53]. On the other hand, high-fat diet disrupt circadian amplitude by suppresses circadian gene oscillation. Nobiletin, clock-enhancing small molecule not only improves circadian functions but also reduce weight gain and enhances metabolic health [9]. Agonist of REV-ERBs and RORs (SR9011 and SR1555, respectively) improve energy homeostasis, reduce weight gain and increases activity by altering circadian behavior in diet-induced obese (DIO) mice [66,67].

Circadian clock controls immune functions such that expression of several inflammatory cytokine genes are regulated by the clock. For example, REV-ERBα takes place in macrophage transcription while RORγt is the key-player in development of T helper cell 17 which is necessary for autoimmune disorder (Chen, Yoo, & Takahashi, 2017). SR1001, a synthetic ligand of RORγ ameliorates symptoms of autoimmune diseases by reducing the expression of downstream cytokine [69].

Studies have elucidated that dysregulated circadian oscillation increases cancer risk and various cancer and the expression of circadian genes is changed in level tumor types [51]. These findings indicate that clock has tumor suppressor functions. In fact, the frequency of breast cancer is higher in women working at night shifts and over-expression of REV-ERBβ in various breast cancer cell lines has been observed [70,71]. Treatment of these cells with SR9011, REV-ERB agonist, decreased cell viability meaning that it can be used as an anti-cancer intervention.

Another state of body regulated by circadian clock is sleep and genetic studies revealed that familial advanced sleep phase syndrome (FASP) and delayed sleep phase syndrome are both clock-related sleep disorders and mutations in PER2 and CSNK1D are responsible for them [4]. PF-670462, an inhibitor of CK1δ persuades behavioral rhythm in arrhythmic mice which means that PF-670462 has a potential of lengthening circadian period in FASP patients [72].

Jet-lag is caused by trans-meridian long-distance flight and it is characterized by failure in entrainment of circadian rhythm. In fact, chronic circadian misalignment is the result of shift-work. Such unsynchronized internal rhythms give rise to serious health problems including gastrointestinal disturbances, cardiovascular disease, cancer and metabolic disorders. Small molecule modifiers with phase-resetting activities might be therapeutic

candidates of such diseases. Mice studies have shown that antagonist mixture of OPC-21268 and SSR 149415 for V1a and V1b (receptors in a signaling pathway for jet-lag), respectively, alleviates phase-shift under jet-lag conditions [4].

Some of psychiatric episodes are affected from circadian clock when clock is unable to adapt to zeitgebers properly. Seasonal affective disorder (SAD) is an example for this. During winter mounts, patients of SAD are suffered from damped rhythms in sleep, body temperature and hormone release as glucocorticoid hormone which regulates stress responses and mood balance. Enhancement of circadian amplitude in glucocorticoid release without changing the overall glucocorticoid level alleviates anxiety in mice [73]. Lithium used as a mood stabilized in bipolar patients also have effects on clock. It enhances the robustness of circadian rhythm by targeting GSK-3β which is a kinase phosphorylating and stabilizing REV-ERBα [74].

Weaker entraining and lower amplitude in hormone secretion, body temperature, sleeping, SCN firing rate, are the circadian marks of aging [75]. In fact, expression of clock genes is dysregulated with age. Sirtuin signaling pathway, one of the well characterized metabolic pathway of aging, plays role in the expression of *Clock* and *Bmal1* gene via PGC-1α. The small molecule resveratrol, activator of SIRT1, has longevity effects while it can restore circadian rhythm in the elderly [76,77]. The correlation between circadian aging and circadian clock is also true for the opposite direction; *Bmal1* knockout mice shows premature aging while and αMUPA transgenic mice with a robust circadian amplitude have longer life span [52,78].

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Culture

The human bone osteosarcoma epithelial cell line (U2OS), mouse fibroblast cell line (NIH 3T3), the human embryonic kidney cell line (HEK 293T) and the human epithelial breast cancer cell line (MDA MB 231) were used. U2OS and NIH 3T3 cells harbor destabilized firefly luciferase whose expression is under the control of mouse Bmal1 and Per2 promoters, respectively. All the cell lines were maintained in Dulbecco's Modified Eagle Medium with high glucose and 2mM L-Glutamine (Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (Thermo Fisher Scientific), 1X Antibiotic-Antimycotic (Thermo Fisher Scientific). They were incubated at 37 °C in a humidified incubator with 5% CO_2, and confluent cells were passaged. For that, the medium was discarded and the cells were wash with 5 ml of 1X phosphate buffered saline buffer. 1 ml of trypsin was added onto the cells and they were incubated at 37 °C for 1 or 2 minutes and resuspended by adding 5 ml of fresh medium. 1 ml of the cell suspension was transferred into a new 10-cm-plates containing 9 ml of fresh medium.

3.2. Real-Time Monitoring of Circadian Oscillation

Bmal1:dluc U2OS and *Per2:dluc* NIH 3T3 cells were seeded in 35 mm-dishes. When the cells became confluent, their media was replaced with replaced 1 ml of DMEM containing 0.1 uM Dexamethasone to synchronize cells. Two hour later, 0.1 mM of Luciferin was freshly added to the recording medium (low glucose DMEM powder (with L-glutamine) supplemented with 10 mM HEPES, 0.35 mg/ml sodium bicarbonate, 3.5 mg/ml D-(+)-glucose powder, 5% FBS, 0.1 mM NEAA, 1X Antibiotic-Antimycotic). Then, the medium was replaced with recording medium containing small molecules at appropriate concentrations while control cells were treated with 0.5% DMSO. The plates were sealed with silicon grease prior to monitoring them on a LumiCycle luminometer (Actimetrics). Bioluminescence was recorded every 10 minutes for 5-7 days. Raw data (counts/sec) were plotted against time. The period and amplitude parameters were obtained using LumiCycle Analysis software (Actimetrics) by fitting the baseline-subtracted data to a sine wave

(damped). The first day's data was excluded from the analysis in order to prevent transient perturbations of luminescence following the medium change.

3.3. Lentivirus Production and Transduction

pLV6-Bmal-luc plasmid (10 μg), pCMV-ΔR8.2dvpr packaging vector (9 μg) and pCMV-VSVG (1 μg) envelope vector were mixed with 20 μl of PEI transfection reagent in 400 μl of DMEM. The mixture was incubated at room temperature for 10 minutes. 75-85% confluent HEK293T cells in 10-cm plate with 5 ml of growth medium were transfected with the mixture. After 16 hour-incubation, 3 ml of fresh growth medium was added, and the cells were incubated for 24 hours. Next day, the medium was replaced with 8 ml of fresh growth medium. As lentiviral particles were released into the culture medium, they were harvested by collecting the medium at 72-hour and 96-hour post-transfection. The virus containing medium was filtered through a 0.45 μm filter and solution was aliquoted for and stored as 750 μl-aliquot of lentiviral particle solution at -80°C.

MDA MD 231 wild-type and Clock-knockout cells were transduced with Bmal1-luc lentiviral particles. MDA MD 231 cells ($30x10^4$ cells/well) were seeded in a 35 mm-dish. Next day, the medium was discarded and a mixture of thawed 750 μl of lentiviral-particle solution, 1 μl of protamine sulfate (8 mg/ml) and 250 μl of DMEM was added into the dish. After 16 hour-incubation, virus-containing media was replaced with 1.5 ml of fresh media. Viral transduction was repeated one more time. At 72-hour post-transfection, media of cells was replaced 1 ml of DMEM containing 0.1 uM Dexamethasone to synchronize cells. Two hour later, 0.1 mM of Luciferin was freshly added to the recording medium (low glucose DMEM powder supplemented with L-glutamine, 10 mM HEPES, 0.35 mg/ml sodium bicarbonate, 3.5 mg/ml D-(+)-glucose powder, 5% FBS, 0.1 mM NEAA, 1X Antibiotic-Antimycotic). Then, the medium was replaced with recording medium containing appropriate amount of CLK8 (10 μM, 20 μM or 40 μM). Control cells were treated with 0.5% DMSO. The plates were sealed with silicon grease prior to monitoring them on a LumiCycle luminometer (Actimetrics). Bioluminescence was recorded every 10 minutes for 3 days. Raw data (counts/sec) were plotted against time. The period and amplitude parameters were obtained using LumiCycle Analysis software (Actimetrics) by fitting the baseline-subtracted data to a sine wave (damped).

3.4. Pull-Down Assay

HEK293T cells were seeded in a 10-cm plate one day prior to transfection in order to obtain confluency of 70-80% on next day. hsClock-pCMVsport6 (8.3 µg) and mBmal1-pCMVsport6 (1.7 µg) plasmids were mixed with 30 µl of PEI reagent and 1 ml DMEM. The mixture was incubated at room temperature for 20 minutes and 1 ml of it was added onto the cells. At 48-hour post-transfection, medium was discarded, and cells were washed with 5 ml of ice-cold 1X PBS buffer. Following the collection of cells in 1 ml of PBS, cells were centrifuged at 5000xg and 4°C for 7 minutes. Then, PBS was aspirated and 1⁄2 of pellet were lysed by resuspending in 500 µl of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM EDTA, 1 mM $MgCl_2$, 0.2% NP-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride and protease inhibitor cocktail). The resuspension was incubated for 20 minutes on ice and centrifuged at 7000xg and 4°C for 10 minutes. The lysate was diluted 1:1 with ice-cold 2X binding buffer (100 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.2% NP-40, 2 mM sodium orthovanadate, 2 mM sodium fluoride, and protease inhibitor cocktail). The lysate was divided into three fractions to be treated with either DMSO, or appropriate concentrations of bitoinylated-CLK8 (bait) with or without CLK8 (competitor). The lysate-compound samples were incubated by mixing continuously at 4°C for 1 hour. Meanwhile, NeutroAvidin Agarose resin (Thermo Scientific) (30 µl settled resin/sample) was equilibrated by washing three times with lysis buffer. The lysate-compound mixture and NeutroAvidin Agarose resin were incubated by mixing continuously at 4°C overnight. Next day, the beads were washed four times with 1X binding buffer, and the bound proteins were eluted by boiling the beads in 30 µl of 4X Laemmli buffer at 95°C for 7 minutes. The beads were collected at the bottom of the tube by quick-spin and 15 µl of supernatant was loaded and run in 10% SDS-PAGE. After transferring proteins to a PVDF membrane (Millipore), the membrane was blocked with 5% milk powder in 0.15% TBS-T for 1 hour and then incubated with anti-CLOCK (Bethyl, 1:1000) and anti-BMAL1 (Santa Cruz Biotechnology, 1:750) antibodies diluted in 5% milk powder in 0.15% TBS-T.

3.5. Co-Immunoprecipitation

HEK293T cells were seeded in a 10-cm plate one day prior to transfection in order to obtain confluency of 70-80% on next day. wild type or F80A-K220A mutant FLAG-tagged hsClock-pCMVsport6 (6.7 µg) and mBmal1-pCMVsport6 (3.3 µg) plasmids were mixed with 30 µl of PEI reagent and 1 ml DMEM. The mixture was incubated at room temperature for 20 minutes and 1 ml of it was added onto the cells. At 48-hour post-transfection, medium

was discarded, and cells were washed with 5 ml of ice-cold 1X PBS buffer. Following the collection of cells in 1 ml of PBS, cells were centrifuged at 5000xg and 4°C for 7 minutes. Then, PBS was aspirated and 1⁄6 of pellet were lysed by resuspending in 500 µl of ice-cold lysis buffer (50 mM Tris-HCl pH 7.7, 150 mM NaCl, 0.1 % Triton X-100, 1mM PMSF and protease inhibitor cocktail) and centrifuged at 15000xg at 4°C for 20 minutes. Meanwhile, anti-FLAG M2 affinity gel (Sigma-Aldrich) (5 µl settled resin/sample) was equilibrated by washing two times with 1X TBS, one time with 0.1 M Glysin-HCl pH 3.5, and then three times 1X TBS. After DMSO and appropriate concentrations of CLK8 were mixed with the whole cell lysate, they were incubated with anti-FLAG M2 affinity gel by mixing continuously at 4°C overnight. Next day, the beads were washed three times with 1X TBS, and the bound proteins were eluted by boiling the beads in 30 µl of 4X Laemmli buffer at 95°C for 7 minutes. The beads were collected at the bottom of the tube by quick-spin and 10 µl of supernatant was loaded and run in 8% SDS-PAGE. After transferring proteins to a PVDF membrane (Millipore), the membrane was blocked with 5% milk powder in 0.15% TBS-T for 1 hour and then incubated overnight with monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, 1:1000) and anti-BMAL1 (Santa Cruz Biotechnology, 1:750) antibodies diluted in 5% milk powder in 0.15% TBS-T.

3.6. Cloning of Clock and Npas2 Genes into Luc-pcDNA4/myc-His A Expression Vector

mClock and hsNpas2 were cloned into Luc-pcDNA4/myc-His A vector. The forward primers of mClock and hsNpas2 contained EcoRV and HindIII while the reverse primers of mClock and hsNpas2 had NotI and XhoI restriction cut sites, respectively. The list of the primers can be found in the table below. Constructs were amplified by polymerase chain reaction that 30ng of full length mClock-pBIND and hsNpas2-pBIND plasmids were used as templates. Reaction mixture with total volume of 20 µl was prepared as follows: 4 µl of 5X GC Buffer (Thermo Fisher Scientific), 0.5 µl of 10mM dNTP mix, 1 µl of 10µM forward primer, 1 µl of 10µM reverse primer, 0.2 µl of Phusion High Fidelity Enzyme (2U/1µL) and 11.9 µl MilliQ water. The PCR conditions were 2 minutes at 98°C, “30 seconds at 98°C, 30 seconds at 68°C and 90 seconds at 72°C for 29 cycles” and 5 minutes at 72°C. The PCR products were run on 0,8% agarose gel at 100 V for 40 minutes, and isolated with NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel). (The list of the primers is in Table 3.)

Both PCR products of mClock and hsNpas2 were sub-cloned into pGEM-T-ccdB vector (Promega) by ligation reaction. The 20 µl-reaction mixture included 2 µl of T4 DNA

Ligase Buffer (Thermo Fisher Scientific), 1 µl of 30 ng/µl pGEM-T-ccdB vector, 90 ng of PCR product, 2 µl of PEG 4000 (Thermo Fisher Scientific) and 0.5 µl of T4 ligase (Thermo Fisher Scientific). The mixture was incubated at room temperature for 1 hour. 5 µl of ligation product was transformed into competent 100 µl DH5α by indicating on ice for 15 minutes, 45 seconds at 42°C and 90 seconds on ice. Then, 1 ml of LB Broth (Miller) was added into the mixture and incubated at 37°C on shaker for 1 hour. Following the centrifugation at 10000xg for 1 min, 950 µl of the supernatant was discarded and the pellet was resuspended with remaining supernatant and spread on LB-agar supplemented 100µg/mL ampicillin and 25 µg/ml chloramphenicol. After 16 hours, the colonies were inoculated into 5 ml of LB Broth supplemented with 100µg/ml ampicillin and 25 µg/ml chloramphenicol and plasmids from those colonies were isolated by using NucleoSpin Mini Plasmid kit (Macherey-Nagel) and their insert were removed for cloning into Luc-pcDNA4/myc-His A vector by restriction reaction. Luc-pcDNA4/myc-His A vector and mClock or hsNpas2-pGEM-T-ccdB plasmids and were restricted with 'EcoRV and NotI' or 'HindIII and XhoI' enzymes, respectively. The reaction mixtures were prepared with 3 µl of FastDigest Green Buffer (Thermo Fisher Scientific), 1 µg of target plasmid, '0.3 µl of FastDigest EcoRV (Thermo Fisher Scientific) and 0.3 µl of FastDigest NotI ((Thermo Fisher Scientific)' or '0.3 µl of FastDigest HindIII (Thermo Fisher Scientific) and 0.3 µl of FastDigest XhoI ((Thermo Fisher Scientific)' and up to 30 µl MilliQ water. The reaction mixtures were incubated at 37°C for 1 hour and the restriction reaction of host plasmid was treated with 2 µl of FastAP Thermosensitive Alkaline Phosphatase Buffer (Thermo Fisher Scientific) and 1 µl of FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) for 10 minuses. The restriction products were run on 0,8% agarose gel at 100 V for 40 minutes, and isolated with NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel). For ligation reaction, 2 µl of T4 DNA Ligase Buffer (Thermo Fisher Scientific), 2 µl of PEG 4000 (Thermo Fisher Scientific) and 0.5 µl of T4 ligase (Thermo Fisher Scientific), host (Luc-pcDNA4/myc-His A vector) and insert (mClock or hsNpas2-pGEM-T-ccdB plasmids) with a molar ratio of 1:3 and up to 20 µl MilliQ water were mixed and it was incubated at room temperature for 1 hour. 5 µl of ligation product was transformed into competent 100 µl DH5α by indicating on ice for 15 minutes, 45 seconds at 42°C and 90 seconds on ice. Then, 1 ml of LB Broth (Miller) was added into the mixture and incubated at 37°C on shaker for 1 hour. Following the centrifugation at 10000xg for 1 min, 950 µl of the supernatant was discarded and the pellet was resuspended with remaining supernatant and spread on LB-agar supplemented 100µg/mL ampicillin. After 16 hours, the colonies were inoculated into 5 ml of LB Broth supplemented with 100µg/ml ampicillin and

plasmids from those colonies were isolated by using NucleoSpin Mini Plasmid kit (Macherey-Nagel).

3.7. Protein degradation assay

HEK293T cells ($4x10^4$ cells/well) were reverse transfected in 96-well white solid-bottom plates by following mixtures:10 ng of mClock-Luc-pcDNA, 140 ng of mBmal1-pCMVsport6 and 150 ng of empty vector (pCMVsport6), 0.6 µl PEI and up to 50 µl DMEM were mixed for degradation of CLOCK,while 10 ng of hsNpas2-Luc-pcDNA, 290 ng of empty vector (pCMVsport6), 0.6 µl PEI and up to 50 µl DMEM were mixed for degradation of NPAS2. After 24 hours, CLK8 at appropriate concentrations or 0.5% DMSO were added into the medium. At 24-hour post-treatment, the medium was supplemented with 1 mM luciferin and 10 mM HEPES, and incubated for 1 hour at 37°C. Then, 20 Pg/ml of cycloheximide was added, the plate was sealed, and the luminescence was recorded every 10 min for 20 hours (Synergy H1 Hybrid Multi-Mode Reader, BioTek). Half-life was obtained by one phase exponential decay fitting with Prism software (GraphPad Software).

3.8. Subcellular Fractionation

U2OS Bmal1-dluc cells ($40x10^4$ cells/well) were seeded in a 35 mm-dish. Next day, the cells were treated with 20µM CLK8 or 0.5% DMSO. After 48 hours, their medium was discarded, and cells were washed with 1 ml of ice-cold 1X PBS buffer. Following the collection of cells with 200 µl of cytosolic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.05% NP-40 and protease inhibitor cocktail), cells were lysed by pipetting gently for 10 times and incubating for 10 minutes on ice. Then, the lysate was centrifuged at 3000 rpm and 4°C for 5 minutes in order to sediment the nuclei and the pellet and the supernatant were used to obtain nuclear and cellular fractions, respectively. The supernatant was centrifuged at 15000xg and 4°C for 5 minutes and 150 µl of the upper phase was used as cytosolic fraction. Meanwhile, the pellet was washed with 200 µl of cytosolic lysis buffer and centrifuged at 3000 rpm and 4°C for 5 minutes. After discarding the liquid phase, 150 µl of nuclear lysis buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol and protease inhibitor cocktail) was added onto the pellet. The mixture was sonicated two times at 60% for 3 cycles and centrifuged at 15000xg and 4°C for 5 minutes and 100 µl of the upper phase was used as nuclear fraction. Then, the fractions were boiled with Laemmli buffer at 95°C for 7 minutes. 10 µl of the samples were loaded and run in 12% SDS-PAGE. After transferring proteins to a PVDF membrane (Millipore), the membrane was blocked with 5%

milk powder in 0.15% TBS-T for 1 hour and then incubated overnight with anti-CLOCK (Bethyl, 1:1000), anti-NPAS2 (Thermo Fisher Scientific, 1:750), anti-BMAL1 (Santa Cruz Biotechnology, 1:750), anti-CRY1 (Bethyl, 1:1000), anti-αTUBULIN (Sigma-Aldrich, 1:5000) and anti-HISTON H3 (Abcam, 1:10000) antibodies diluted in 5% milk powder in 0.15% TBS-T.

3.9. RNA Isolation, cDNA Synthesis and RT-qPCR

U2OS Bmal1-dluc cells ($40x10^4$ cells/well) were seeded in 35 mm-dishes. When the cells became confluent, their media was replaced with replaced 1 ml of DMEM containing 0.1 uM Dexamethasone to synchronize cells. Two hour later, the medium was replaced with fresh medium containing appropriate concentrations of CLK8 or 0.5% DMSO. At 72-hour post-synchronization, cells were harvested every after six hours for 36 hours by washing the cells with 1 ml of ice-cold 1X PBS, collecting in 1 ml of ice-cold 1X PBS and centrifuging at 5000xg and 4°C for 7 minutes. NucleoSpin RNA kit (Macherey-Nagel) was used to mRNAs were isolated from the cell pellets. 500 ng of mRNA was converted to cDNA via First Strand cDNA Synthesis kit (BioLabs). After dilution of cDNA with a rate of 1:25, a reaction mix was prepared as fallows: 12,5 µl of SYBR Green (Bioline, SensiFAST No-ROX kit), 1,25 µl of primer mix (10 pmole of both forward and reverse primers), 2,5 µl of cDNA and 8,75 of µl MilliQ water. List of the primer sequences can be found in table below. The cycling protocol (40 cycles) was performed on LightCycler 2.0 Instrument (Roche). The cycling protocol is performed as denaturation at 95°C for 8 seconds, annealing at 60°C for 10 seconds and elongation at 72°C for 20 seconds. PRLP0 was used as an internal control. (The list of the primers is in Table 4.)

3.10. Protein Isolation and Western Blot Analysis

U2OS Bmal1-dluc cells ($40x10^4$ cells/well) were seeded in 35 mm-dishes. When the cells became confluent, their media was replaced with replaced 1 ml of DMEM containing 0.1 uM Dexamethasone to synchronize cells. Two hour later, the medium was replaced with fresh medium containing appropriate concentrations of CLK8 or 0.5% DMSO. At 72-hour post-synchronization, cells were harvested every after six hours for 36 hours by washing the cells with 1 ml of ice-cold 1X PBS, collecting in 1 ml of ice-cold 1X PBS and centrifuging at 5000xg and 4°C for 7 minutes. Then, PBS was aspirated, and the pellets were lysed by resuspending in 500 µl of ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM PMSF and protease inhibitor cocktail). The suspension was

incubated for 30 minutes on ice and centrifuged at 15000xg at 4°C for 20 minutes. The supernatants containing proteins were collected and protein concentrations were measured by Thermo Fisher Scientific, Pierce 660nm Protein Assay Reagent as stated in product information. 10-15 µg of the samples were loaded and run in 10% SDS-PAGE. After transferring proteins to a PVDF membrane (Millipore), the membrane was blocked with 5% milk powder in 0.15% TBS-T for 1 hour and then incubated overnight with anti-CLOCK (Bethyl, 1:1000), anti-BMAL1 (Santa Cruz Biotechnology, 1:750), anti-PER2 (Bethyl, 1:1000), anti-CRY1 (Bethyl, 1:1000) and anti-βACTIN (Cell Signalling, 1:5000) antibodies diluted in 5% milk powder in 0.15% TBS-T.

3.11. Subcellular Fractionation of Mouse Liver

Mice were sacrificed 6 hours after CLK8 or DMSO treatment (25mg/kg). For western blot analysis, 10 mg of liver tissue from each mouse was lysed in 500 µl of cytosolic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.05% NP-40 and protease inhibitor cocktail) by pipetting gently for 30 times and incubating for 10 minutes on ice. Then, the lysate was centrifuged at 3000 rpm and 4°C for 5 minutes in order to sediment the nuclei and the pellet and the supernatant were used to obtain nuclear and cellular fractions, respectively. The supernatant was centrifuged at 15000xg and 4°C for 5 minutes and 250 µl of the upper phase was used as cytosolic fraction. Meanwhile, the pellet was washed with 1000 µl of cytosolic lysis buffer and centrifuged at 3000 rpm and 4°C for 5 minutes. After discarding the liquid phase, 300 µl of nuclear lysis buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol and protease inhibitor cocktail) was added onto the pellet. The mixture was sonicated two times at 60% for 3 cycles and centrifuged at 15000xg and 4°C for 5 minutes and 250 µl of the upper phase was used as nuclear fraction. Then, the fractions were boiled with Laemmli buffer at 95°C for 7 minutes. 10 µl of the samples were loaded and run in 12% SDS-PAGE. After transferring proteins to a PVDF membrane (Millipore), the membrane was blocked with 5% milk powder in 0.15% TBS-T for 1 hour and then incubated overnight with anti-CLOCK (Bethyl, 1:1000), , anti-BMAL1 (Santa Cruz Biotechnology, 1:750), anti-PER2 (Bethyl, 1:1000), anti-CRY1 (Bethyl, 1:1000), anti-αTUBULIN (Sigma-Aldrich, 1:5000) and anti-HISTON H3 (Abcam, 1:10000) antibodies diluted in 5% milk powder in 0.15% TBS-T.

3.12. Protein and mRNA Analysis of Mouse Liver

Mice were sacrificed 6 hours after CLK8 or DMSO treatment (25mg/kg). For western blot analysis, 10 mg of liver tissue from each mouse was lysed in 500 µl of ice-cold RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM PMSF and protease inhibitor cocktail) by homogenizing for 2 minutes on ice, and vortex vigorously. Following 20 minutes incubation on ice, samples were centrifuged at 10000xg and 4°C for 30 minutes. The supernatants containing proteins were collected and protein concentrations were measured by Pierce 660nm Protein Assay Reagent (Thermo Fisher Scientific) as stated in product information. 10-15 µg of the samples were loaded and run in 10% SDS-PAGE. After transferring proteins to a PVDF membrane (Millipore), the membrane was blocked with 5% milk powder in 0.15% TBS-T for 1 hour and then incubated overnight with anti-CLOCK (Bethyl, 1:1000), anti-BMAL1 (Santa Cruz Biotechnology, 1:750), anti-CRY1 (Bethyl, 1:1000) and anti-βACTIN (Cell Signalling, 1:5000) antibodies diluted in 5% milk powder in 0.15% TBS-T. For RT-qPCR analysis, NucleoSpin RNA kit (Macherey-Nagel) was used to mRNAs were isolated from 15 mg of liver tissue from each mouse. 500 ng of mRNA was converted to cDNA via First Strand cDNA Synthesis kit (BioLabs). After dilution of cDNA with a rate of 1:25, a reaction mix was prepared as fallows: 12,5 µl of SYBR Green (Bioline, SensiFAST No-ROX kit), 1,25 µl of primer mix (10 pmole of both forward and reverse primers), 2,5 µl of cDNA and 8,75 of µl MilliQ water. List of the primer sequences can be found in table below. The cycling protocol (40 cycles) was performed on LightCycler 2.0 Instrument (Roche). The cycling protocol is performed as denaturation at 95°C for 8 seconds, annealing at 60°C for 10 seconds and elongation at 72°C for 20 seconds. GAPDH was used as an internal control. (The list of the primers is in Table 4.)

CHAPTER 4

RESULTS

4.1. Molecular Dynamic Simulation and Virtual Screening

Circadian rhythms are metabolic, behavioral and physical changes which repeat themselves with a period of about 24 hours. In mammals, cell-autonomous oscillators in SCN as master pacemaker and in peripheral tissues generate these rhythms and synchronize them to daily changes in the environment [1]. This cellular clock arises from a transcriptional translational regulatory feedback loop where CLOCK:BMAL1 complex is found at the positive arm of the loop while CRY:PER complex is at the negative arm of the loop. The weigh on these arms (Figure 2) is essential for a robust circadian clock. Studies on PER and CRY proteins have shown that stronger feedback inhibition via a higher ratio of the negative arm to the positive arm of the loop enhances amplitude of the circadian oscillation [27]. Therefore, altering the positive loop by modulating CLOCK and BMAL1 interaction would be a novel approach to train circadian system.

As a robust circadian clock is necessary for health, any dysregulation of circadian rhythm which is characterized by phase misalignment or manipulations in circadian genes, causes several pathologies including sleep disorders, metabolic and cardiovascular diseases, increase in the risk of cancer. To re-regulate disrupted circadian rhythms, targeting CLOCK:BMAL1 complex with small molecules can provide a use tool because of their reversible, time-controlled and dose-dependent effects [8].

The crystal structure studies on CLOCK:BMAL1 complex have revealed that both the proteins are composed of C-terminal PAS-A and PAS-B domains with an N-terminal bHLH domain. Among the heterodimerization of CLOCK and BMAL1 proteins, each of these domains interacts with its corresponding partner. While bHLH domains mediate binding of CLOCK:BMAL1 complex to DNA, PAS-A/B domains primarily provide interaction motifs for CLOCK:BMAL1 heterodimerization [11,45].

Since CLOCK and BMAL1 firstly heterodimerize and then their phosphorylation, stability, nuclear localization and transactivation activity regulate circadian rhythm tightly [7,41], modulating this interaction via targeting small molecules to the PAS domains of CLOCK

protein can alter the circadian functions, in turn, provide potential intervention to treat circadian-related disorders with an enhanced circadian amplitude.

With this purpose, Darya Yarparvar (former PhD candidate from Kavaklı Lab) performed all computational studies and briefly she carried out molecular dynamic simulations and virtual screening to identify molecules that bind to CLOCK. As it summarized in Figure 4A energy minimization simulation was applied to CLOCK (PDB ID: 4F3L) [12] for refinement of atom coordinates. Following to increasement of the system temperature up to physiological temperature (310 K) and subjecting to Langevin piston method for 10 ns, the root mean square deviation (RMSD) analysis revealed that the simulation reached to equilibration after 3-4 ns (Figure 4B). This final structure was used for docking approximately 2 million commercially available small molecules to the PAS domain of the CLOCK protein by using AutoDock Vina [79]. After filtering the results based on "Lipinski's Rule of Five" [80], the top 100 compounds according to their affinity to CLOCK (-7 to -10 kcal/mol) were listed and used in vitro biochemical studies for further characterization.

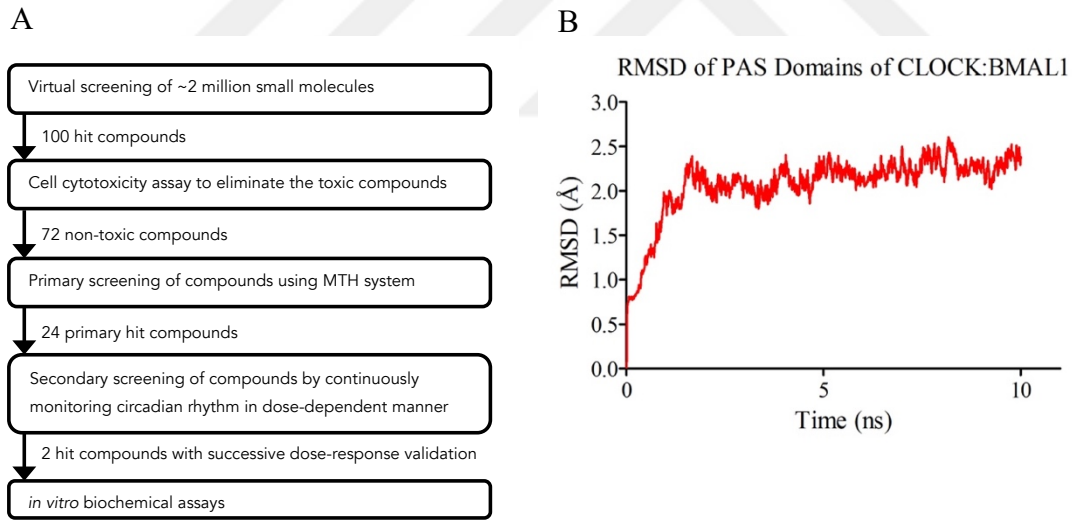

Figure 4. *(A)*Flowchart of the screening and characterization of small molecule modifiers *(B)* The RMSD of the PAS domains of the CLOCK backbone with respect to initial structure molecular dynamic simulation. Following an initial rapidly rising trend, the structure reach to equilibrium after 3-4 ns. (Darya Yarpavar, unpublished data)

4.2. Cell Viability Assessment

Cellular cytotoxicity of the hit 100 compounds was assessed using MTT assay where U2OS cells treated with different compounds at various concentrations (Figure 5). Compounds which failed to yield more than 80% cell viability (relative to 0.5% DMSO control) at 1.25 µM were eliminated. The remaining 72 compounds were further characterized for their effect on CLOCK:BMAL1 interaction and on circadian rhythm.

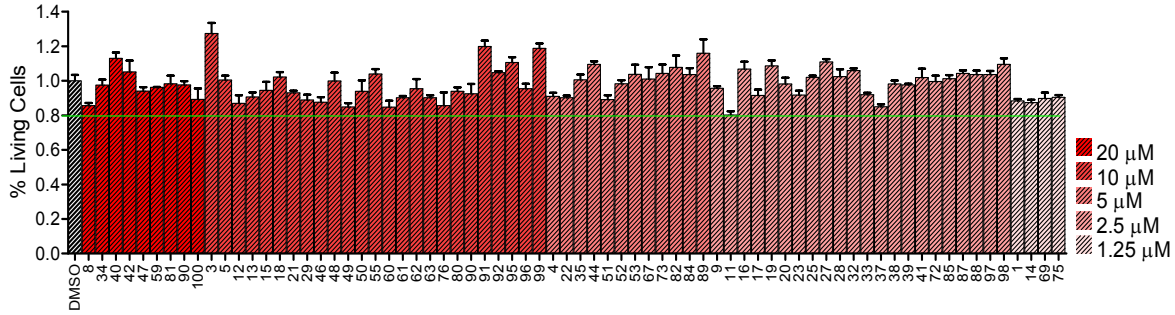

Figure 5. Cell viability assessment of the hit 100 compounds. Following the treatment of U2OS cells with different doses of the compounds, MTT-based cytotoxicity assay revealed that 72 of the compounds were not toxic. (data are mean ± s.e.m.; n=3 independent experiments.) (Darya Yarpavar, unpublished data)

4.3. The Effect of Hit Compounds on CLOCK:BMAL1 Interaction and Circadian Rhythm

As PAS domains of CLOCK is important for CLOCK and BMAL1 interaction and our compounds targeted these domains, we hypothesized that interaction of these proteins would be altered by the compounds. To test that, mammalian two hybrid system (MTHS) [81] was used where CLOCK was fused to Gal4 DBD and bond to GAL4 DNA sequence without activating transcription the lucifer promoter. Meanwhile, BMAL1 was fused to VP16 TAD which could bind to DNA but has transactivation activity. Following the dimerization of CLOCK-GAL4 DBD with BMAL1-VP16 TAD, DBD was tethered to the transactivation domain and lead to the transcription of luciferase gene. Even though MTHS is not sufficient for quantitative analysis, it is strong enough to reveal the degree of changes in protein interaction. When MRHS was performed for 72 non-toxic compounds, it was found that 24 of them significantly changed the interaction between CLOCK and BMAL1 without

effecting CLOCK and BMAL1 protein levels. It was suggested that these compounds might affect circadian rhythm by modulating the positive arm of the transcriptional translational regulatory feedback loop.

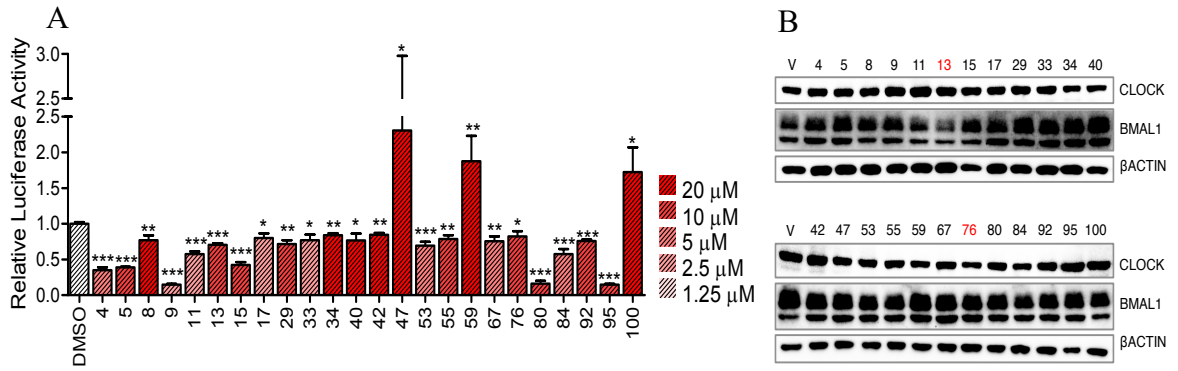

Figure 6. Mammalian Two-Hybrid Assay in transfected HEK 293T cells. (A) The effects of compounds on CLOCK and BMAL1 interaction at their highest non-toxic concentrations. The luciferase activities of samples are relative to that of 0.5% DMSO control. 24 out of 72 non-toxic compounds altered CLOCK and BMAL1 interaction significantly. (B) CLOCK and BMAL1 protein levels in the samples of MTHS were analyzed by western blot analysis. Compounds reducing the CLOCK or BMAL1 protein levels were eliminated from further characterizations. V: vehicle (0.5% DMSO) (Data are mean ± s.e.m.; n=3 independent experiments); [*p-value<0.05; **p-value<0.01; ***p-value<0.0001] (Darya Yarpavar, unpublished data)

Bmal1:dluc U2OS and Per2:dluc NIH 3T3 cell lines have a functional circadian clock and express a destabilized firefly luciferase which is under the control of mouse *Bmal1* promoter and *Per2* promoters, respectively. Therefore, they can be used to real-time monitoring of circadian oscillation. In order to determine the effects of 24 non-toxic and interaction altering compounds, *Bmal1:dluc* U2OS cells were synchronized by dexamethasone and treated with these small molecules. Then, their circadian rhythm was monitored with LumiCycle luminometer (Actimetrics) by recording their bioluminescence. It was founded that the compounds generated one of three circadian phenotype; amplitude enhancers (9 compounds), period-lengtheners (2 compounds), and amplitude-reducers (2 compounds) (Figure 7A). However, only the four of them (CLK8, CLK59, CLK95, CLK100) could successfully pass the dose-response validation as a secondary screening step (Figure 7B). To determine whether these four small molecules have the same effects in different

genetic background or not, their circadian oscillation in *Per2:dluc* NIH 3T3 were monitored fallowing dose-dependent treatment of these molecules. The results revealed that only CLK8 and CLK95 could display the circadian phenotype in *Per2:dluc* NIH 3T3 that they had shown in *Bmal1:dluc* U2OS (Figure 8).

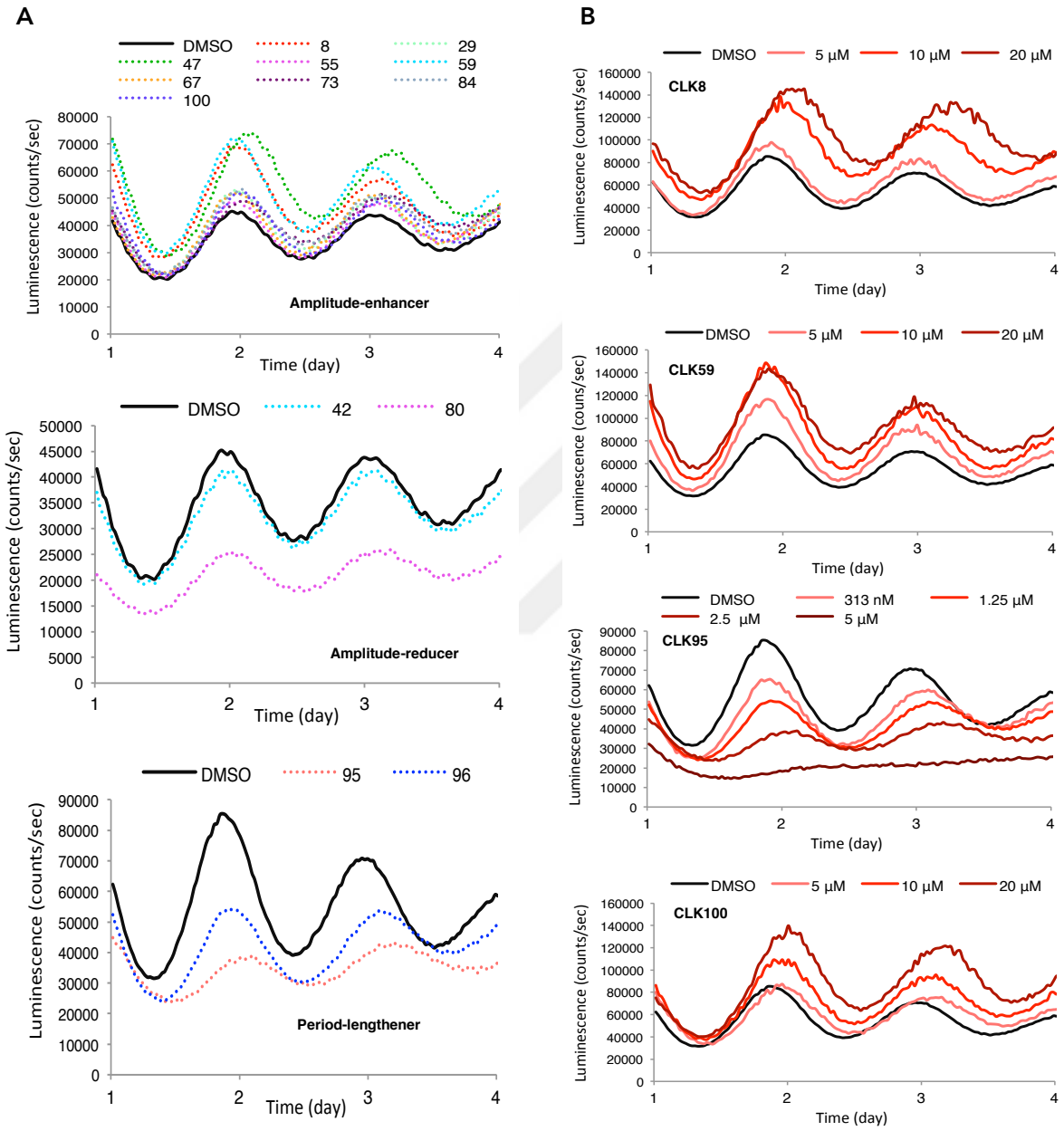

Figure 7. The effect of non-toxic interaction altering compounds on circadian rhythm. *(A)* Continuous monitoring of luminescence rhythms of *Bmal1:dLuc* U2OS cells treated with the non-toxic interaction altering compounds revealed 3 categories of compounds based on circadian phenotype: 9 amplitude-enhancer compounds, 2 amplitude-reducer compounds, and 2 period-lengthener compounds. *(B)* CLK8, CLK59, CLK95, and CLK100 have dose-dependent effect on circadian rhythm. (Darya Yarpavar, unpublished data)

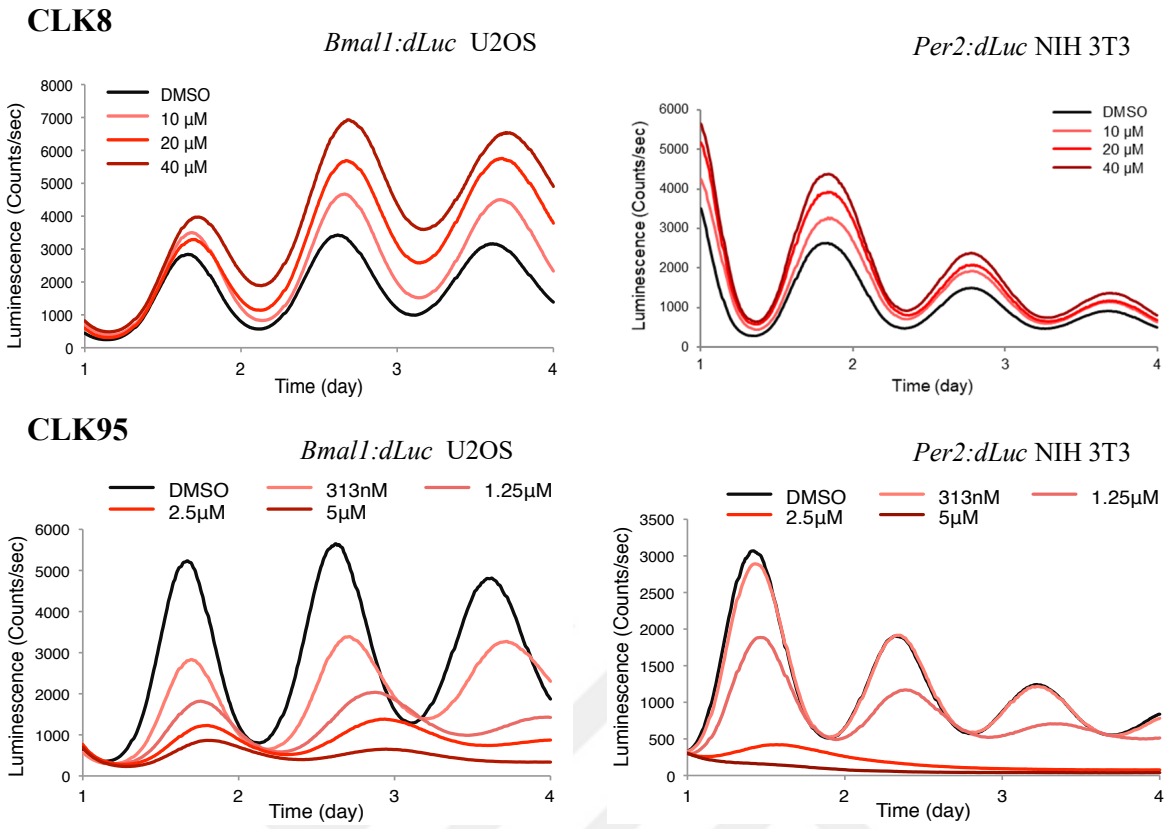

Figure 8. The effects of CLK8 and CLK95 on circadian rhythm at different genetic backgrounds. CLK8 and CLK95 exhibited same phenotypic changes in *Bmal1:dluc* U2OS and *Per2:dLuc* NIH 3T3 cell lines. (data are mean of 3 independent experiments).

4.4. The Effect of CLK8 on Circadian Rhythm

As dampened circadian amplitude causes health problems including metabolic diseases, mood disorders and aging, finding a small molecule modifier acting as an amplitude enhancer might treat circadian-related disorders by improving the amplitude of circadian oscillation. Therefore, we decided to continue to this study with further characterization of CLK8 as it is an amplitude-enhancing compound. In fact, previous studies have shown the importance of the balance between the negative and the positive arms of the TTFL for circadian amplitude enhancement. None of the amplitude-enhancing small molecules have been identified yet which modulate the positive arm of the TTFL whereas there are some modifiers, like Nobiletin, altering the negative arm of the loop. As CLK8 targets PAS domain on CLOCK and changes the interaction of CLOCK and BMAL1 protein, it could provide a novel approach for circadian amplitude enhancement.

Before going into detail characterization of CLK8, the effect of CLK8 on the half-life of luciferase was determined to assure that CLK8 did not interfere with the luminescence signal itself. After transfecting HEK 293T cells with *pcDNA-dluc* and dose-dependent CLK8 treatment, luminescence signals were recorder for 20 hours. When the results were normalized and then fitted to one-phase decay, it was found that CLK8 did not changed the half-life of luciferase protein (Figure 9). It was suggested that CLK8 likely alters the activity of CLOCK:BMAL1 complex on the *Per2* reporter in a E-box-dependent manner which gave rise to the changes in circadian rhythm.

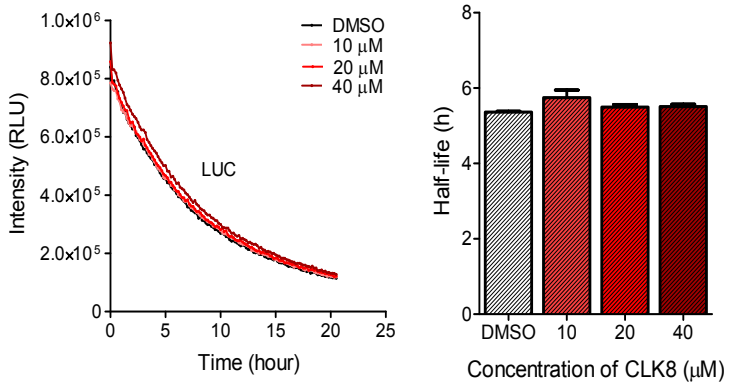

Figure 9. The effect of CLK8 on Luciferase. HEK 293T cells were transfected with *pcDNA-dLuc*. A day after, CLK8 were added at indicated concentrations. The luciferase signal was recorder following CHX treatment until the luciferase signal reached to a plateau. (data are mean ± s.e.m.; n=3 independent experiments) (Darya Yarpavar, unpublished data)

After setting the maximum non-toxic concentration of CLK8 at 40 μM for *in vitro* experiments (Figure 10A), the effect of CLK8 on the circadian rhythm was investigated in detail. It was found that CLK8 enhanced the circadian amplitude in both *Bmal1:dLuc* U2OS and *Per2:dLuc* NIH 3T3 cell lines in a dose dependent manner (Figure 10B, C). Interestingly, the period of circadian in none of these cells has changed following the CLK8 treatment (Figure 10B, C). Effects of CLK8 on the *Bmal1-dluc* knock-in reporter in *Clock*-deficient and wild type MDA MB231 cells were analyzed in order to confirm that amplitude enhancement was not a result of experimental artifact, but it is specific to interaction between CLK8 and CLOCK protein. While CLK8 was able to enhance the amplitude in wild type MDA MB231 cells at different concentration of the CLK8 (Figure 11A), such effect was abolished in the *Clock* knockout cells (Figure 11B). Those results suggested that CLK8 specifically affects CLOCK that results in amplitude enhancement.

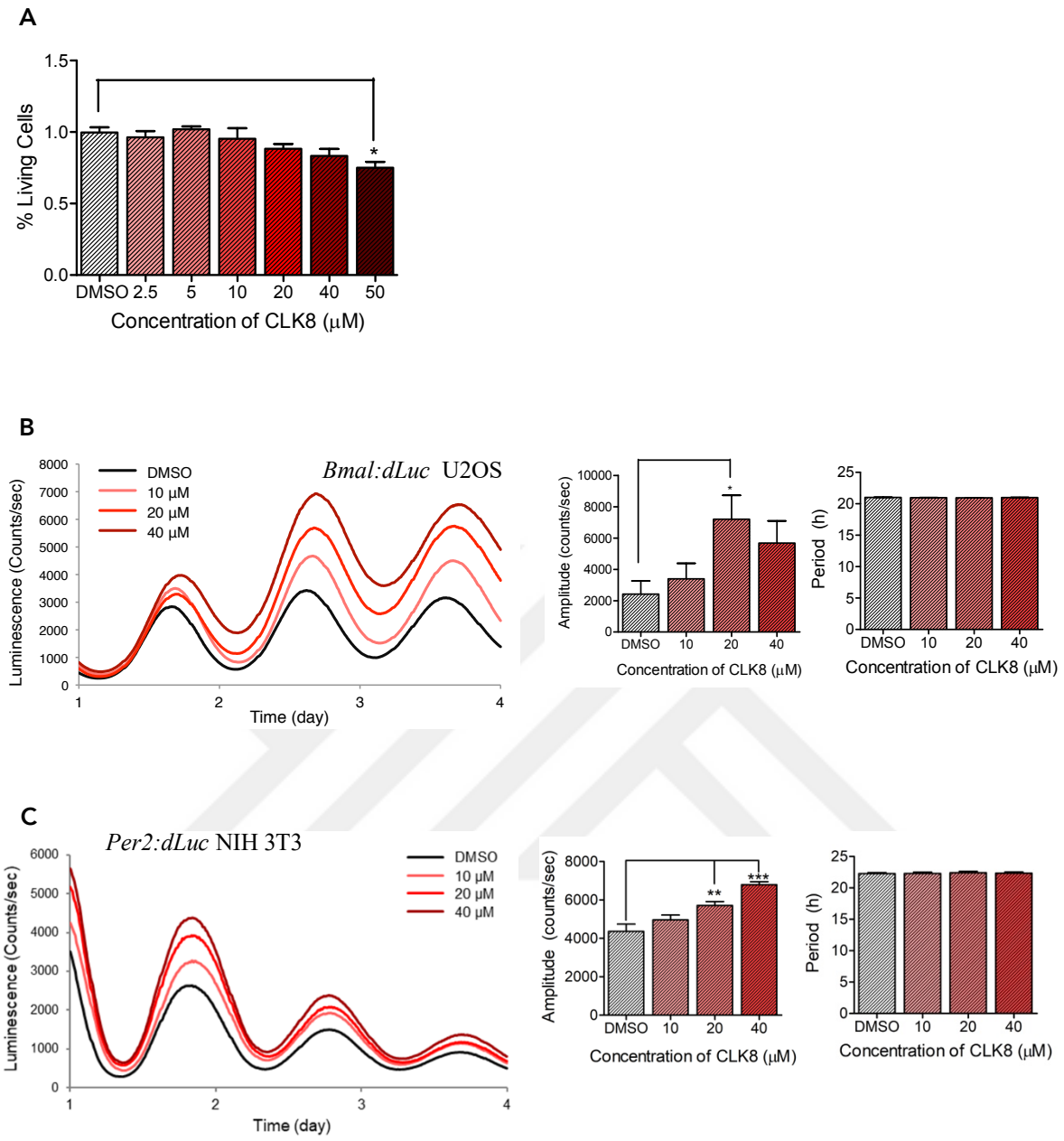

Figure 10. Initial screens for identification of CLK8. (A) Dose-dependent cytotoxicity of CLK8 in U-2 OS cell line revealed that 40 μM was the highest non-toxic concentration of CLK8. Luminescence rhythms of (B) *Bmal1:dLuc* U2OS and (C) *Per2:dLuc* NIH 3T3 cells were continuously monitored to determine dose-dependent effect of CLK8 on circadian rhythm. Amplitude and period parameters (shown in right panel) were obtained by fitting first-order polynomial baseline-subtracted data with sin (damped). The first day was not included in analysis. The results showed that CLK8 enhanced amplitude and did not change period in both cell lines. (luminescence profiles are means of 3 independent experiments. data are mean ± s.e.m.; n=3 independent experiments.). [* p-value<0.05; ** p-value<0.01; ***p-value<0.0001]

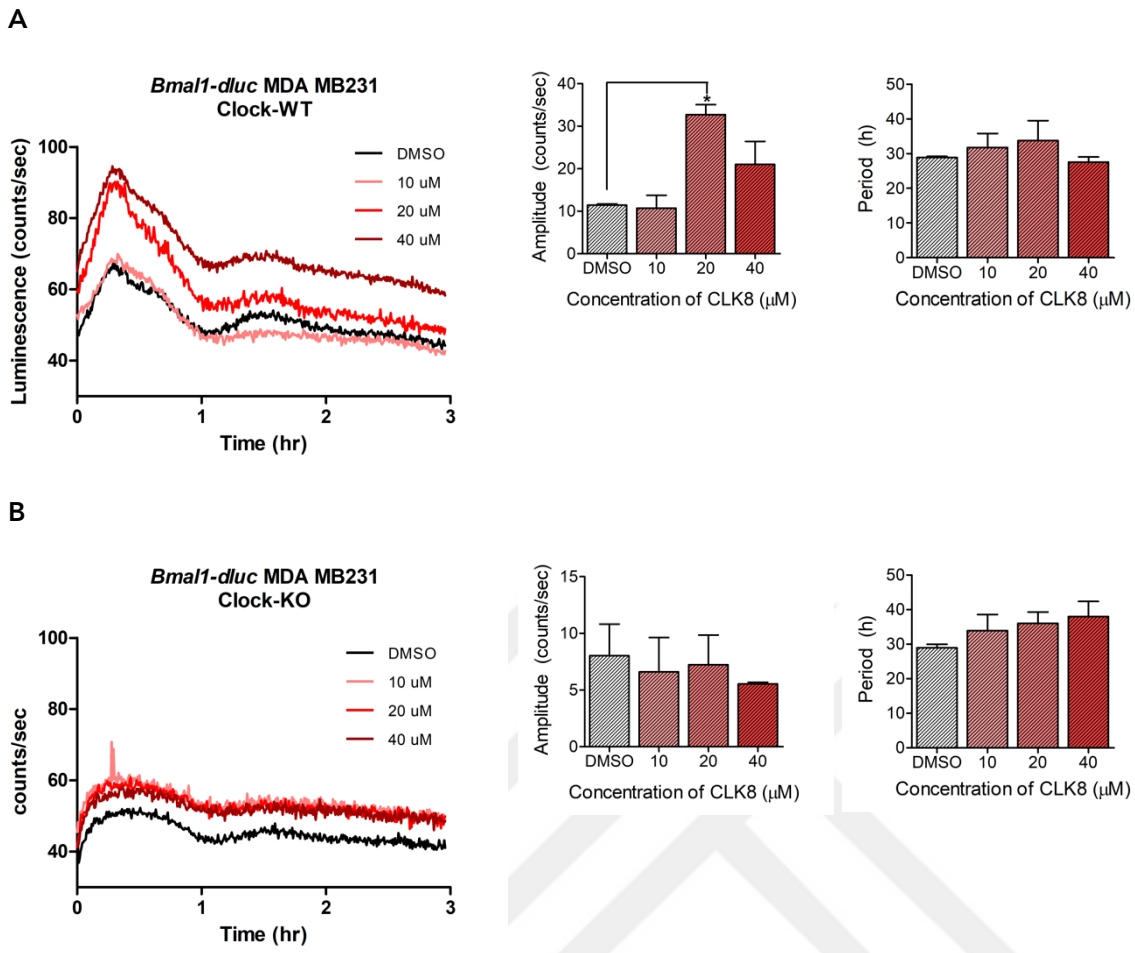

Figure 11. Effects of CLK8 on the *Bmal1-dluc* knock-in reporter in wild type and *Clock*-deficient MDA MB231. MDA MB231 cells were transduced with *Bmal1:dluc* lentiviral particles. After 72-hour post-transduction, the luminescence rhythms of the cells were monitored continuously to determine dose-dependent effect of CLK8 on circadian rhythm. Amplitude and period parameters (shown in right panel) were obtained by fitting first-order polynomial baseline-subtracted data with sin (damped). (A) CLK8 enhanced the amplitude in wild type MDA MB231 cells at different concentration of the CLK8. (B) Such effect was abolished in the *Clock* knockout cells. (Luminescence profiles are means of 3 independent experiments; data are mean ± s.e.m.; n=3 independent experiments.). [* p-value<0.05; ** p-value<0.01;]

4.5. Specific Binding of CLK8 to CLOCK

Biotinylated CLK8 (Figure 12A) was synthesized by Enamine, which is a chemical supplier in Ukraine, and used in pull-down assays to demonstrate physical binding of CLK8 to CLOCK. To do that, HEK 293T cells over-expressing CLOCK and BMAL1 were lysed and the resulting whole cell lysate was incubated with biotinylated CLK8in the presence or

absence of the competitor (CLK8). The data shown that CLOCK was precipitated with biotinylated CLK8 when there was not competitor in the environment whereas biotinylated CLK8 failed to precipitate CLOCK protein in the presence of competitor (Figure 12B). That result indicated the specific binding of CLK8 to CLOCK. To assure that such result was not a false-positive outcome of the over-expression system, binding of CLK8 to CLOCK protein at endogenous level was controlled by using whole cell lysate of U2OS cells expressing in pull-down assay. After western blot analysis, it was clearly seen that biotinylated CLK8 specifically binds to CLOCK protein but not to other core clock component including the ones with PAS domain (PER2 and NPAS2) or to the prologue of CLOCK (NPAS2) (Figure 12C). In fact, the difference between the input and the sample precipitated with biotinylated CLK8 in terms of CLOCK amount indicated the high affinity of CLK8 for CLOCK (Figure 12C).

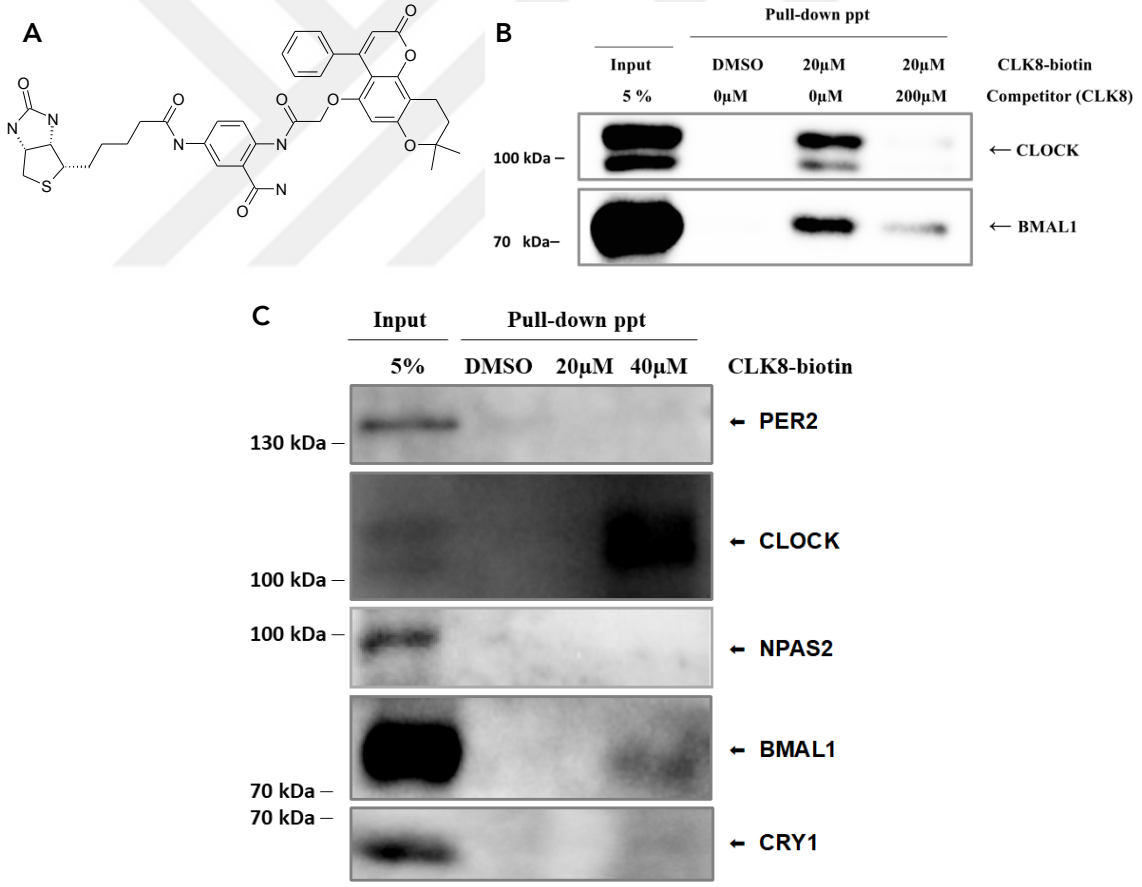

Figure 12. Specific binding of CLK8 to CLOCK. (A) The chemical structure of biotinylated CLK8. Pull down assay using whole cell lysates of (B) pSport6-*Clock* and pSport6-*Bmal1* over-expressing HEK 293T cells, (C) U2OS cells expressing endogenous levels of core clock proteins. CLK8 was used as a competitor when it was needed. (data are representative of 3 independent experiments) (Darya Yarpavar, unpublished data)

The off-targets of CLK8 was investigated by LC tandem mass spectrometry. The amount of precipitated endogenous CLOCK protein (from U2OS cells) was not adequate for the detection of CLOCK in LC-MS/MS. For that reason, whole cell lysate of HEK 293T cells overexpressing CLOCK and BMAL1 were used to precipitate CLOCK protein by biotinylated CLK8 in the absence or presence of free CLK8 as competitor. For the elution, LC-MS/MS was performed. The proteins potentially binding to CLK8 were determined based on a minimum threshold for peptide spectrum that matches and coverage equal to those of the main target, CLOCK. Proteins that had been detected in the negative controls of the CRAPome database [82] were excluded since they are concerned as abundant sticky proteins. No CLOCK detection in LC-MS/MS in the presence of a competitor proved the binding of CLK8 to CLOCK specifically (Table 2) Meanwhile, presence of competitor leaded to reduction of BMAL1 protein amount (Table 2). Other proteins whose amounts were reduced following CLK8 administration were listed (Table 2) as being the possible off-targets of CLK8. However, none of them play roles in circadian clock based on literature.

Table 2. Potential off-targets of CLK8. Whole cell lysates of HEK293T cells overexpressing *Clock* and *Bmal1* were incubated with 20 μM of biotinylated CLK8. Free CLK8 as a competitor was added when it was needed. Precipitated proteins by pull down assay were subjected to LC-MS/MS. The peptide spectrum matches and coverage of the main target, CLOCK, were considered as the threshold in data analysis. Proteins with PSM and coverage lower than threshold were eliminated.

Protein		Peptide spectrum matches	
		Competitor (μM)	
		0	200
CLOCK	Circadian locomoter output cycles protein kaput	42	-
PLIN4	Perilipin-4	395	-
IFT81	Intraflagellar transport protein 81 homolog	52	-
COL28A1	Collagen alpha-1(XXVIII) chain	104	-
CCDC146	Coiled-coil domain-containing protein 146	120	-
SPAST	Spastin	74	-
FSIP1	Fibrous sheath-interacting protein 1	57	-
CCDC144CP	Putative coiled-coil domain-containing protein 144C	88	-
FAN1	Fanconi-associated nuclease 1	79	-
FER	Tyrosine-protein kinase Fe	72	-
BMAL1	Aryl hydrocarbon receptor nuclear translocator-like protein 1	56	46
ZGRF1	Protein ZGRF1	114	105
NAV2	Neuron navigator 2	263	188
FBXO15	F-box only protein 15	73	37

CLK8 binds to the cavity surrounded by the α2-helix of the bHLH domain and the Hβ strands within the PAS-A domain of CLOCK protein as docking results shown. In fact, this cavity allows entering of BMAL1 Arg 126 and its interaction with CLOCK Phe80 on the α2-helix of the bHLH domain. The docking results also showed that in addition to Phe80, Lys220 of CLOCK plays important role in the binding of CLK8 to CLOCK by providing cation-pi interaction.

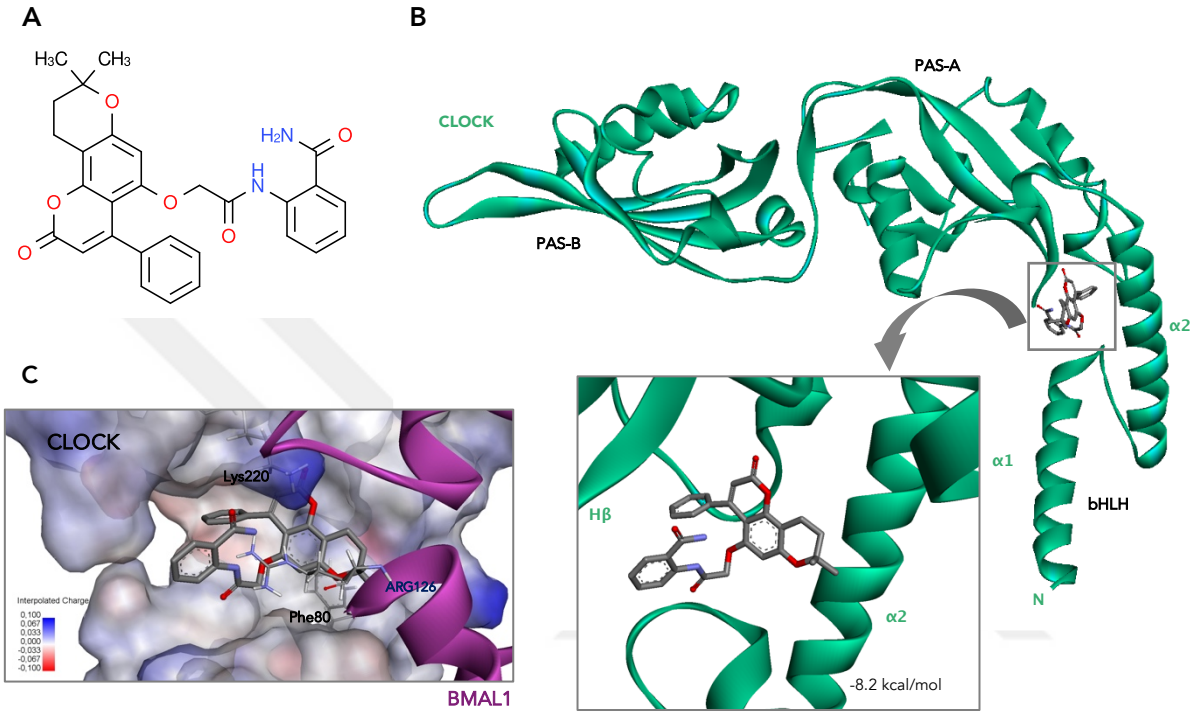

Figure 13. The docking results of CLK8. (A) The chemical structure of CLK8. The best binding mode of CLK8 to CLOCK (green) with predicted binding energy of -8.2 kcal/mol (C) Superposition of CLK8 and ARG126 of BMAL1 (magenta). (Darya Yarpavar, unpublished data)

In order to validate this binding pocket, the Phe80 and Lys220 residues of CLOCK were exchanged with Ala by site-directed mutagenesis (The list of the primes are in Table 3). Firstly, the functionality of double CLOCK mutant (CLOCK-F80A-K220A) was validated by monitoring the activity of *Per1:luciferase.* HEK 293T cells were transfected with *Clock* or *Clock-F80A-K220A, mPer1:luc* and transactivation partner *Bmal1*. Luminescence measurements of those cells revealed that wild type CLOCK and CLOCK-F80A-K220A mutant provided same level of the activation on m*Per1:luc* reporter (Figure 14A). After being sure that F80A and K220A mutations did not alter CLOCK function, it was determined whether the predicted binding site of CLK8 was true by pull-down assay with biotinylated CLK8 (in the absence and presence of the CLK8 as a competitor) in the whole cell lysate of HEK 293T cells over-expressing *Clock-F80A-K220A* and *Bmal1*. The

western blot analysis of the samples verified that Phe80 and Lys220 residues of CLOCK were responsible for binding of CLK8 since mutant CLOCK did not precipitate with biotinylated CLK8 as biotinylated CLK8 had precipitated wild-type CLOCK (Figure 14B). Moreover, the trace amount precipitated mutant CLOCK did not disappear in the presence of the competitor which also confirmed the binding of CLK8 to the Phe80 and Lys220 residues (Figure 14B). Interestingly, BMAL1 did not appeared in the blot (Figure 14B) as it had done following the pull-down assay of biotinylated CLK8 with the whole cell lysate of HEK 293T cells over-expressing wild type *Clock* (Figure 12B, C). This states that BMAL1 was co-precipitated with wild type CLOCK rather than binding to CLK8 non-specifically.

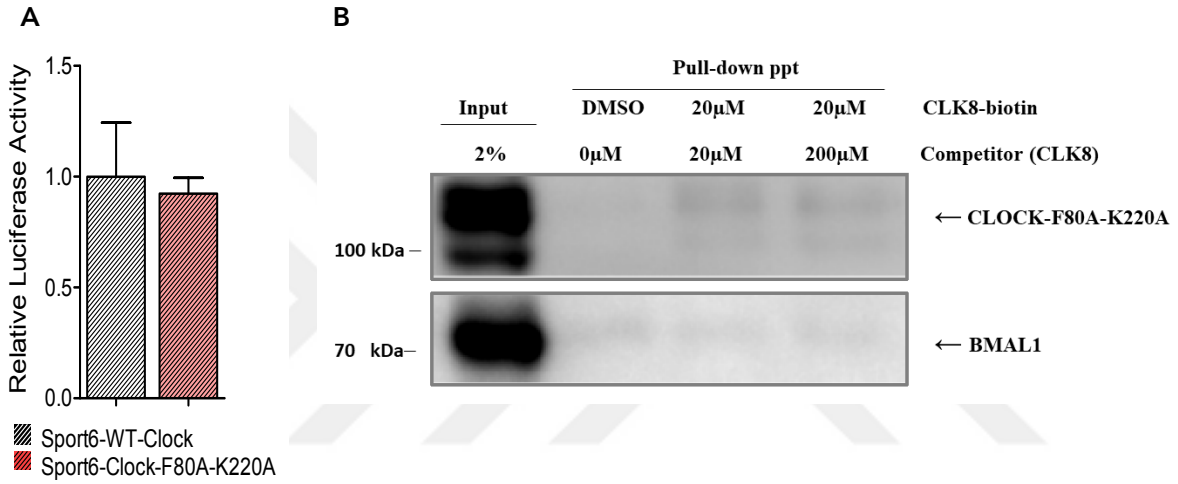

Figure 14. Confirmation of the computationally predicted binding site of CLK8. (A)The transcriptional function of wild type and mutant *Clock* were compared using a *Per1*-Luc assay in HEK 293T cells. (data are mean ± s.e.m.; n=3 independent experiments) (B) Pull down assay using whole cell lysate of HEK 293T cells overexpressing CLOCK-F80A-K220A and BMAL1. CLK8 was used as a competitor when it was needed. (data are representative of 3 independent experiments) (Darya Yarpavar, unpublished data)

4.6. The Effect of CLK8 to the Interaction between CLOCK and BMAL1

MTHS results suggested that there was a CLK8-dependent decrease between CLOCK and BMAL1 interaction. In order to quantitatively analyze this effect, co-immunoprecipitation assay was conducted. Anti-FLAG resin was used in the presence of CLK8 in order to precipitate FLAG-CLOCK from whole cell lysate of wild type or F80A-K220A mutant FLAG-tagged *hsClock* and tag-free *mBmal1* over-expressing HEK 293T cells. While FLAG-tagged wild type or F80A-K220A mutants CLOCK protein amount

appeared in the same extent within all samples, the amount of BMAL1 reduced with respect to increase in the CLK8 concentration when BMAL1 was co-immunoprecipitated with wild type CLOCK (Figure 15). The results implied that CLK8 modulates the interaction between CLOCK and BMAL1 proteins. If that was true, it was hypothesized that the amount of BMAL1 would not change in the presence of CLK8 following the co-immunoprecipitation of BMAL1 with F80A-K220A mutant CLOCK. In fact, the results of the assay with mutant CLOCK revealed that BMAL1 amount remained unaltered in the presence of CLK8 (Figure 15). Those outcomes indicated that CLK8 interferes the interaction between CLOCK and BMAL1.

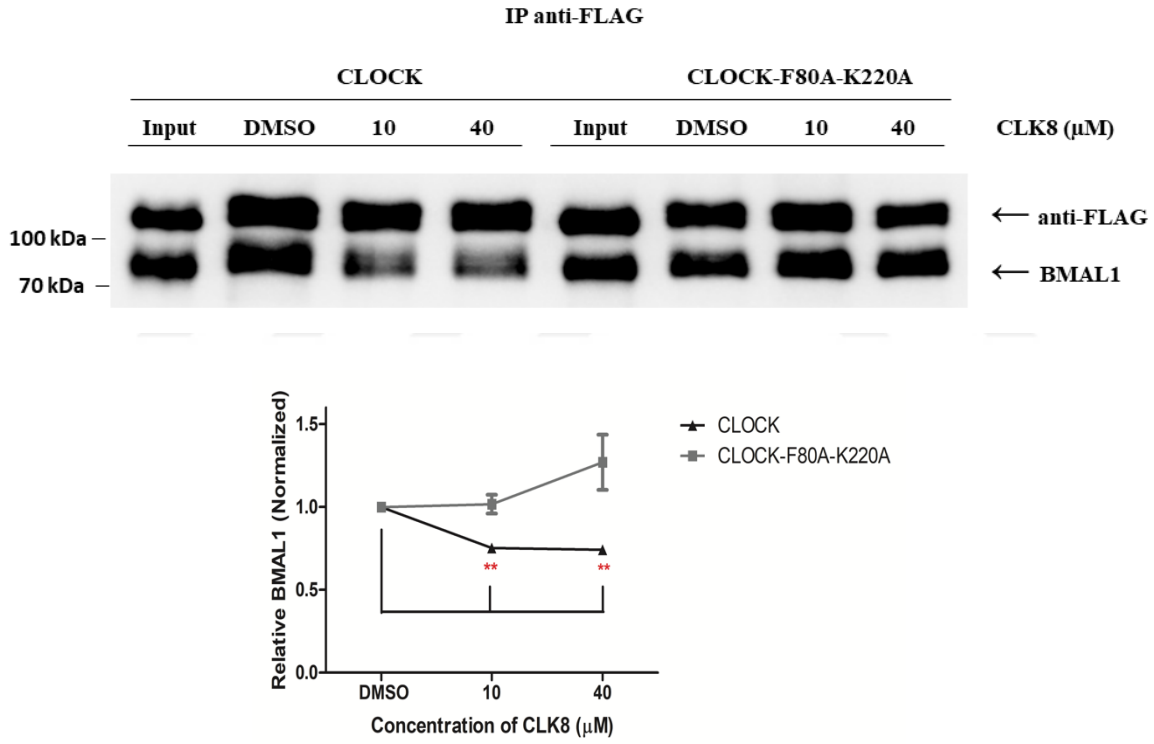

Figure 15. The effect of CLK8 on CLOCK and BMAL1 interaction. Whole cell lysate of HEK 293T cells over-expression wild type or F80A-K220A mutant Clock and Bmal1 was used in co-immunoprecipitation assay with CLK8 as bait. The quantification is the panel below. (data are representative of 3 independent experiments) (data are mean ± s.e.m.; n=3 independent experiments; ** p-value<0.01)

4.7. The Effect of CLK8 to the CLOCK and NPAS2 Protein Stability

Unlike the rest of the core clock components, CLOCK protein does not show significant circadian oscillation, but the post translational modifications on CLOCK following its dimerization with BMAL1 show circadian fashion which alter the stability, sub-cellular localization and transactivation activity of CLOCK [7,40,41]. As the CLK8 reduces CLOCK and BMAL1 interaction, it was hypothesized that the stability, sub-cellular localization and transactivation activity of CLOCK might be modulated by CLK8.

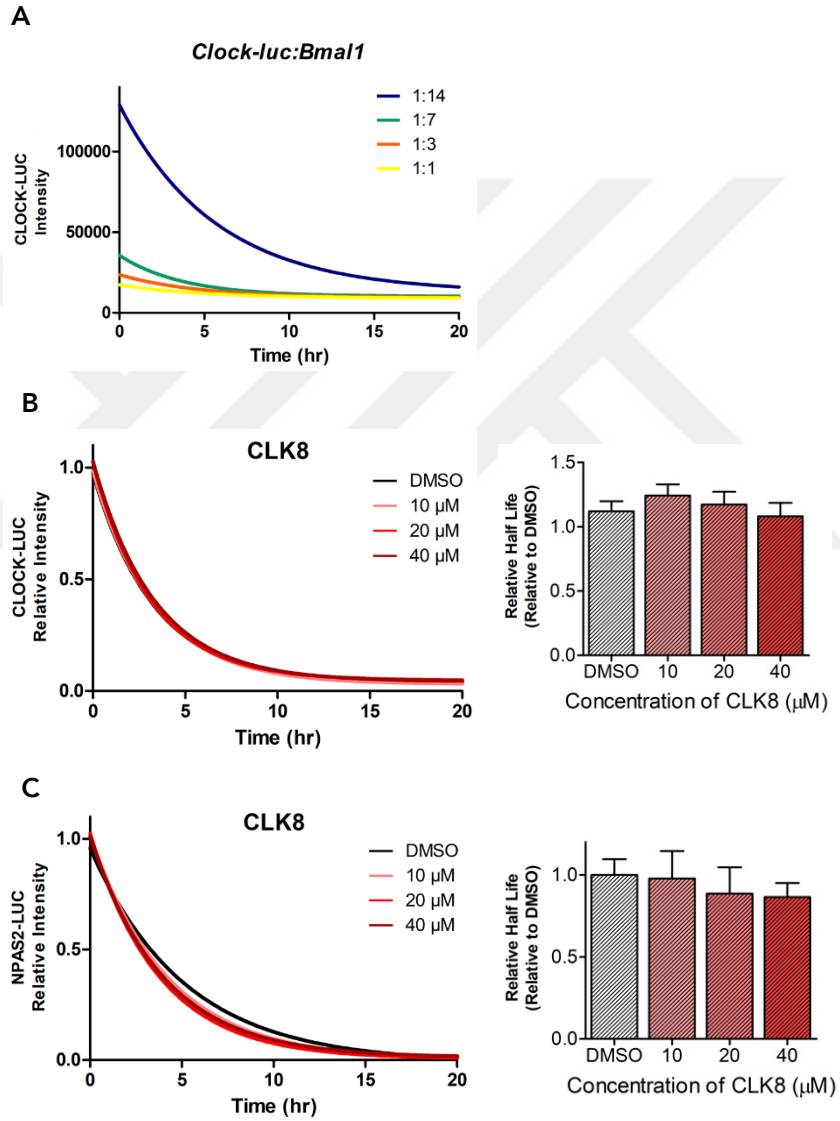

Figure 16. The effect of CLK8 on CLOCK and NPAS2. (A)HEK 293T cells were transfected with *Clock-Luc* and *Bmal1* in different ratios to determine required BMAL1 required amount for the degradation of CLOCK. (B) HEK 293T cells were transfected with *Clock-Luc* and *Bmal1* or (C) *Npas2-Luc*. A day after, 10, 20 and 40 μM of CLK8 were added. The luciferase signal was monitored after CHX treatment until the luciferase signal reached a plateau. Half-life of CLOCK-LUC or NPAS2-LUC are shown in right panel. (data are mean ± s.e.m.; n=3 independent experiments)

In order to analyze such effects, any changes in the stability of CLOCK was determined firstly by protein degradation assay based on CHX (cycloheximide) treatment[83] which prevents protein translation by inhibiting ribosome activity. As the CLOCK degrades in a BMAL1-dependent manner, HEK 293T cells were transfected *pcDNA:Clock:dluc* and pSport6-*Bmal1* at different rations to find out the required amount of BMAL1 for CLOCK degradation. The results suggested that the 1:7 ration between Clock:Bmal1 was sufficient for the aim of the experiment (Figure 16A). Next step was measuring the degradation rate of the CLOCK protein by recording the luminescence signals of HEK 293T cells over-expressing *Clock:dluc* and *Bmal1* (1:7) after the dose-dependent CLK8 treatment. The result revealed that CLK8 did not change protein stability of CLOCK (Figure 16B). The possible off-target effect of CLK8 on the stability of NPAS2, paralog of CLOCK, was analyzed in HEK 293T cells over-expressing *pcDNA:NPAS2:dluc*. It was shown that CLK8 did not change the stability of NPAS2 (Figure 16C).

4.8. The Effect of CLK8 on Subcellular Localization of Core Clock Proteins

After protein degradation assay indicated that CLK8 does not affect the stability of CLOCK protein (Figure 16B), the second possible effect of CLK8 on CLOCK was investigated. BMAL1 has NLS and NES sequences [41] but CLOCK does not. Thus, nucleocytoplasmic shuttling of CLOCK depends on BMAL1. Reduction in the interaction between CLOCK and BMAL1 by CLK8 treatment (Figure 15) brought a question to the mind if CLK8 changes the nuclear localization of CLOCK protein. To test that, U2OS cells were fractionated into cytoplasm and nucleus. α-TUBULIN and HISTON H3 proteins are specifically localized in cytoplasm and nucleus, respectively, meaning that they can be used to evaluate the purity of the fractionation (Figure 17A). The results shown that CLK8 significantly decreased the translocation of CLOCK protein into the nucleus while there was no change of CLOCK amount in cytoplasm (Figure 17B, C). Sub-cellular localization of other clock proteins were also analyzed and it revealed that CLK8 changed the amount of BMAL1, PER2, CRY1 and NPA2 neither in nucleus nor in cytoplasm (Figure 17B, C). Those findings suggested that reduction of CLOCK in nucleus may alter stoichiometry between CLOCK:BMAL1 and PER2:CRY1 complexes.

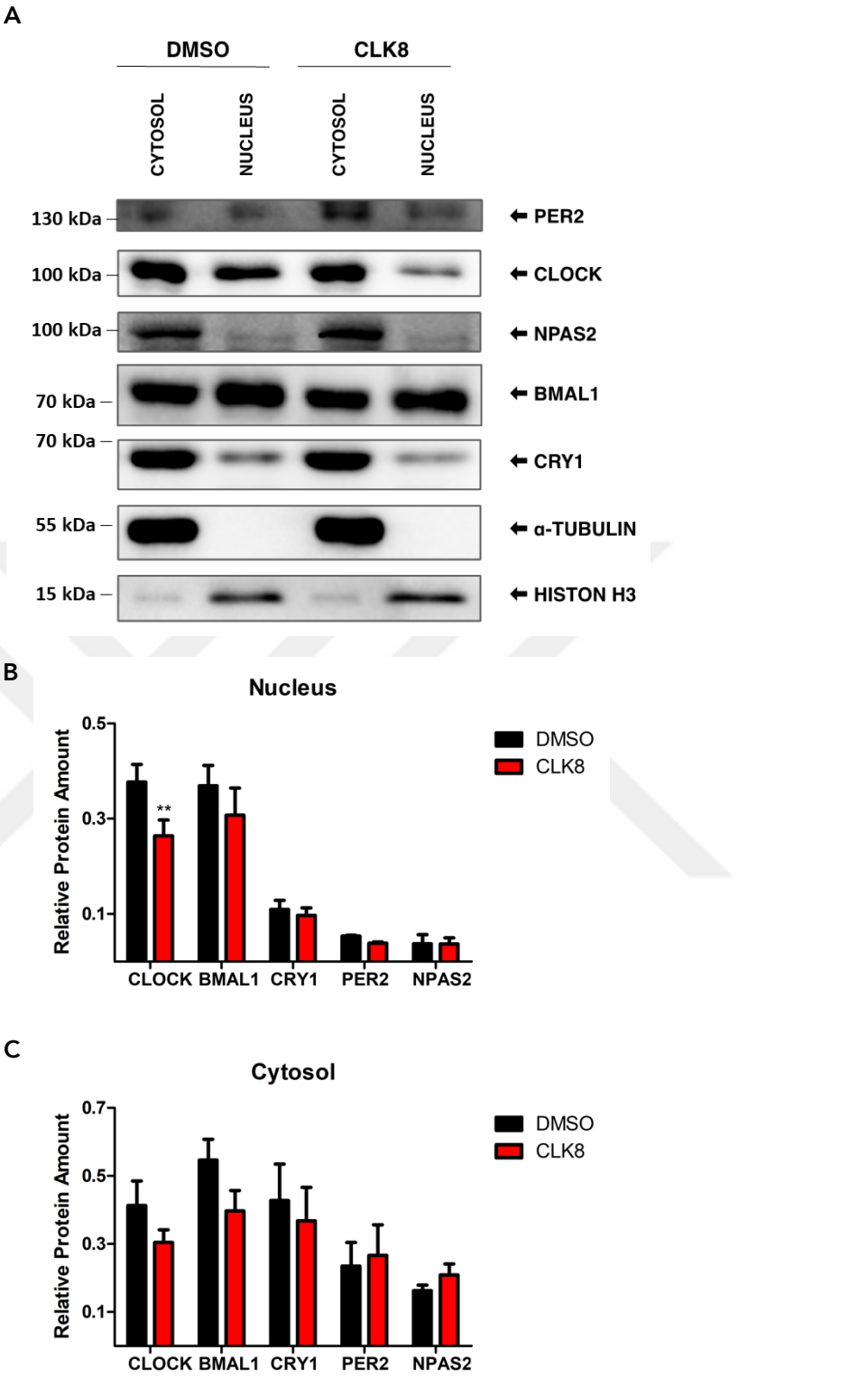

Figure 17. The effect of CLK8 on sub-cellular localization of clock components. Unsynchronized U-2 OS cells were treated with CLK8 and fractionated into cytoplasm and nucleus. (A) Western blot analysis, where CLOCK, NPAS2, BMAL1 and CRY1 were normalized by (B) HISTON H3 for nuclear fraction and by (C) α-TUBULIN for cytosolic fraction. (data are mean ± s.e.m.; n=5 independent experiments; western blot data is representative of 5 independent experiments) (One-way ANOVA with Tukey's test [** p-value<0.01]

4.9. Time-Dependent Effect of CLK8 on Core Clock Components

In addition to stability and nucleocytoplasmic shuttling, transactivation activity of CLOCK depends on its dimerization with BMAL1. As CLK8 directly modulates CLOCK and BMAL1 heterodimerization (Figure 15) and reduces the amount of CLOCK protein in nucleus (Figure 17B), CLK8 might affect the transcription profile of the core clock genes. To test that, unsynchronized U2OS cells treated with CLK8 were assayed to analyze any changes at gene expression level. However, CLK8 did not cause any significant differences in the core clock components at mRNA level compared to DMSO control (data not shown). Then, it was hypothesized that unsynchronized cells may have masked their own rhythmicity which made the effect of CLK8 modulation on CLOCK and BMAL1 transactivation complex undetectable. Therefore, the experimental design switched to the synchronization of U2OS cells by dexamethasone treatment and harvesting the cells at indicated time points after replacing the medium of the cells with fresh medium containing CLK8 or DMSO. The results indicated that CLK8 led to general down regulation in the expression of core clock genes (Figure 18). Such effect suggested that CLK8 reduced the transactivation activity of CLOCK by decreasing both the dimerization of CLOCK with BMAL1 and the amount of CLOCK in the nucleus. Then, the time-depended U2OS samples were used for western blot assay to measure alteration of core clock component at protein level due to CLK8 treatment. Interestingly, it was seen that the overall trend in the protein level of CLOCK and BMAL1, which are in the positive arm of the transcriptional-translational regulatory feedback loop, reduced by CLK8 treatment (Figure 19). That suggested slight increase observed in *Clock* expression at some time points via CLK8 treatment was to compensate the reduction in the CLOCK protein. On the other hand, the protein amount of PER2 and CRY1, which are found in the negative arm of the TTFL, did not altered in CLK8 treated samples compared to DMSO control (Figure 19) in spite of notable reduction of *Per2* and *Cry1* by CLK8 at transcription level (Figure 18). Those results shown that CLK8 alters the transcription of core clock components both in the positive and negative arm of the TTFL while CLK8 only changes the amounts of proteins at the positive arm of the TTFL, but not that of the ones in the negative arm of the TTFL. Together with the reduction of CLOCK in nucleus, the outcomes implied that CLK8 stabilizes the repression activity of PER2 and CRY1 proteins by reducing the CLOCK and BMAL1 amount which results in the enhancement of circadian amplitude. In fact, these findings are in agreement with the studies on stoichiometry between the positive

and the negative arms of TTFL indicating that elevation of negative arm improves the circadian amplitude[9,27].

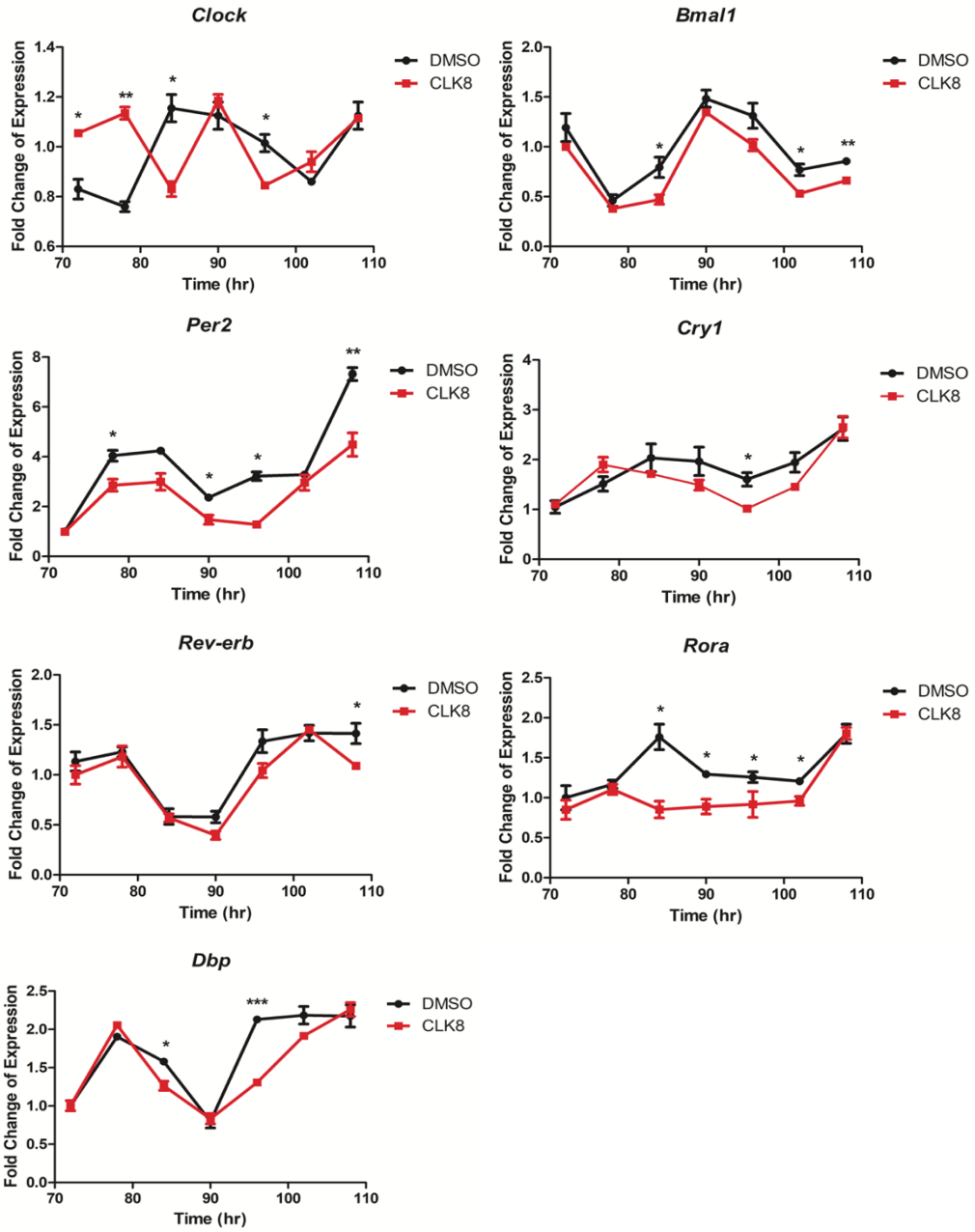

Figure 18. The effect of CLK8 on transcriptional level of clock genes. Following dexamethasone treatment of confluent U2OS cells for synchronization, medium was replaced with fresh medium including CLK8 or DMSO and cells were harvested at indicated time points. RT-qPCR was performed to measure the fold change in the expression level of core clock genes where RPLP0 was used for normalization. (data are mean ± s.e.m.; n=3 independent experiments; western blot data is representative of 3 independent experiments); (One-way ANOVA with Tukey's test); [* p-value < 0.05; ** p-value < 0.01; ***p-value <0.001]

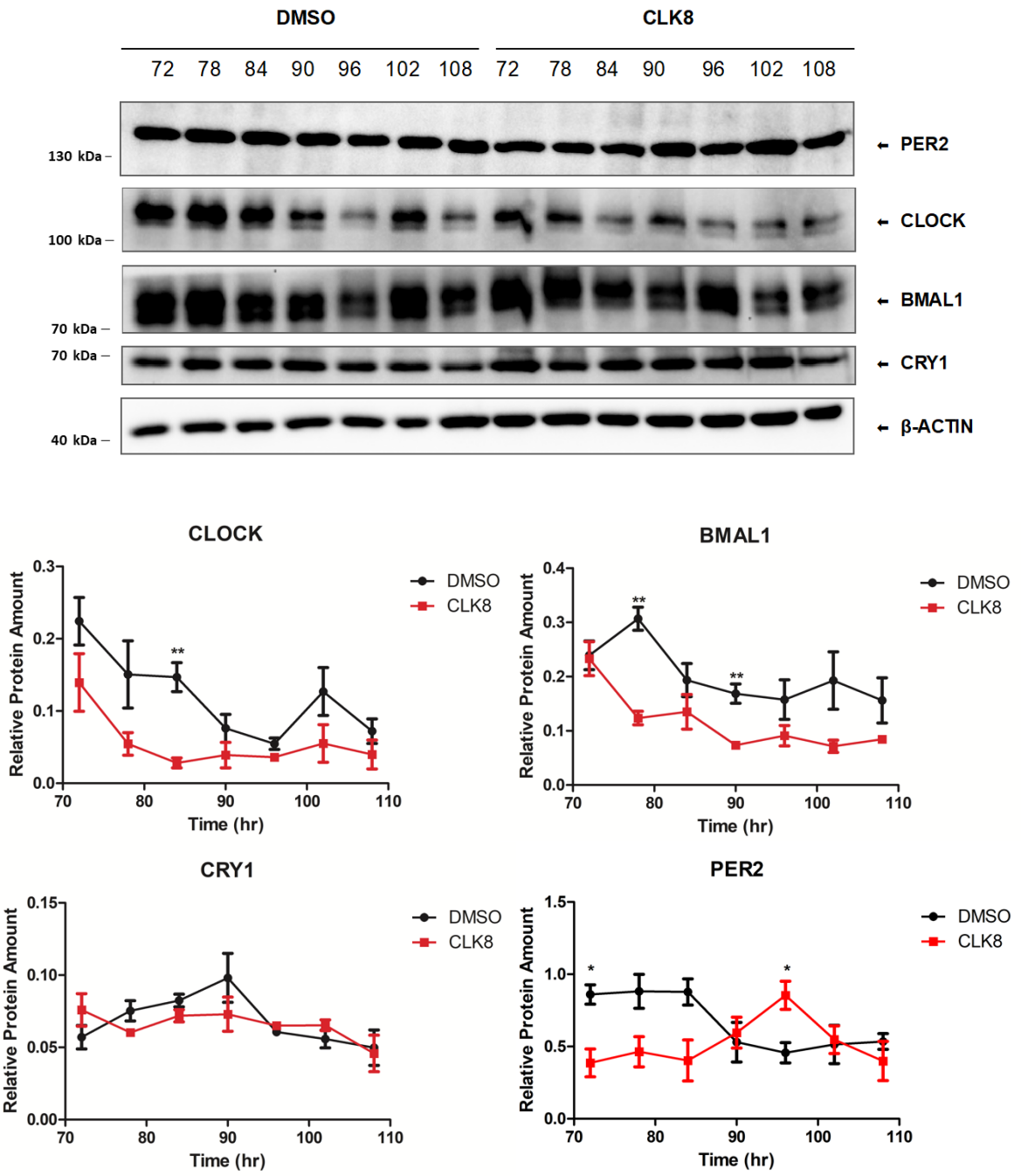

Figure 19. The effect of CLK8 on protein level of core clock components. Following dexamethasone treatment of confluent U2OS cells for synchronization, medium was replaced with fresh medium including CLK8 or DMSO and cells were harvested at indicated time points. Western blot analysis was performed to measure changes the amount of core clock proteins.(data are mean ± s.e.m.; n=3 independent experiments; western blot data is representative of 3 independent experiments); (One-way ANOVA with Tukey's test); [* p-value<0.05; ** p-value<0.01]

4.10. In vivo Effect of CLK8 on Mice Liver

While all in vitro studies shown that the CLK8 specifically binds to and affect CLOCK proteins in a way that the inflation of CLOCK with BMAL1, CLOCK abundance in nucleus and the transactivation activity of CLOCK were all reduced. In orated to assess effects of CLK8 in vivo, the CLK8 was administered into mice as 25mg/kg intraperitoneally (i.p.). 6 hours after the injection, those animals were sacrificed, and their livers were used in downstream analysis. Firstly, it was investigated whether the CLK8 alters the sub-cellular localization of core clock components. To do that, mice liver samples were fractionated into cytoplasm and nucleus and the samples were analyzed by western blot analysis where α-TUBULIN and HISTON H3, specifically localized in cytoplasm and nucleus, respectively were used to evaluate the purity of the fractionation. The results enlighten that the amount of CLOCK protein in nucleus reduced while the abundance of its partner BMAL1 in the positive arm of the TTFL as well as the amount of CRY1, the indicator of the negative arm of TTFL, were not changed. The outcome was parallel with in vitro studies. Further analysis was done for the effects of CLK8 on protein and transcription levels of clock components in mice livers. While there was reduction in CLOCK, BMAL1 and CRY1 did not change at protein level. Similar to in vitro, the expression level of *Clock* and *Bmal1* remain unchanged whereas *Cry1* expression was down regulated which was possible related to decrease in CLOCK protein amount, subsequently the transcription activity of CLOCK. All the results of in vivo analysis were consistent with the findings of in vitro assays. This suggested that CLK8 gives rise to similar circadian phenotype at molecular level both in vitro and in vivo.

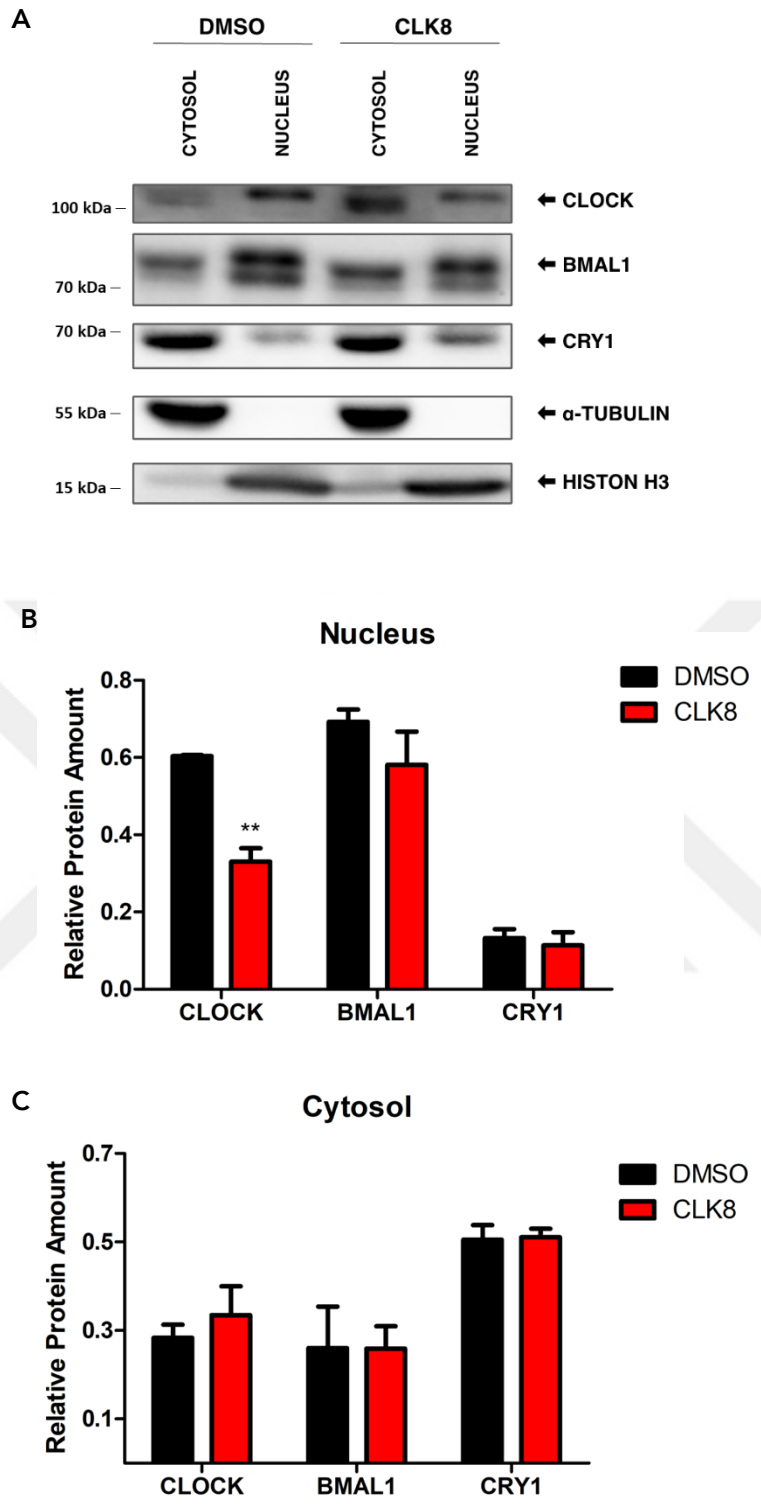

Figure 20. In vivo effect of CLK8 on sub-cellular localization of CLOCK. The livers of mice sacrificed 6 h after CLK8 treatment at 25 mg/kg were fractionated into cytoplasm and nucleus. (A) Western blot analysis, where CLOCK, BMAL1 and CRY1 were normalized by (B) HISTON H3 for nuclear fraction and by (C) α-TUBULIN for cytosolic fraction. (data are mean ± s.e.m.; n=5 for CLK8 and n=3 for DMSO groups, independent experiments; western blot data is representative of these independent experiments) (One-way ANOVA with Tukey's test [** p-value<0.01]

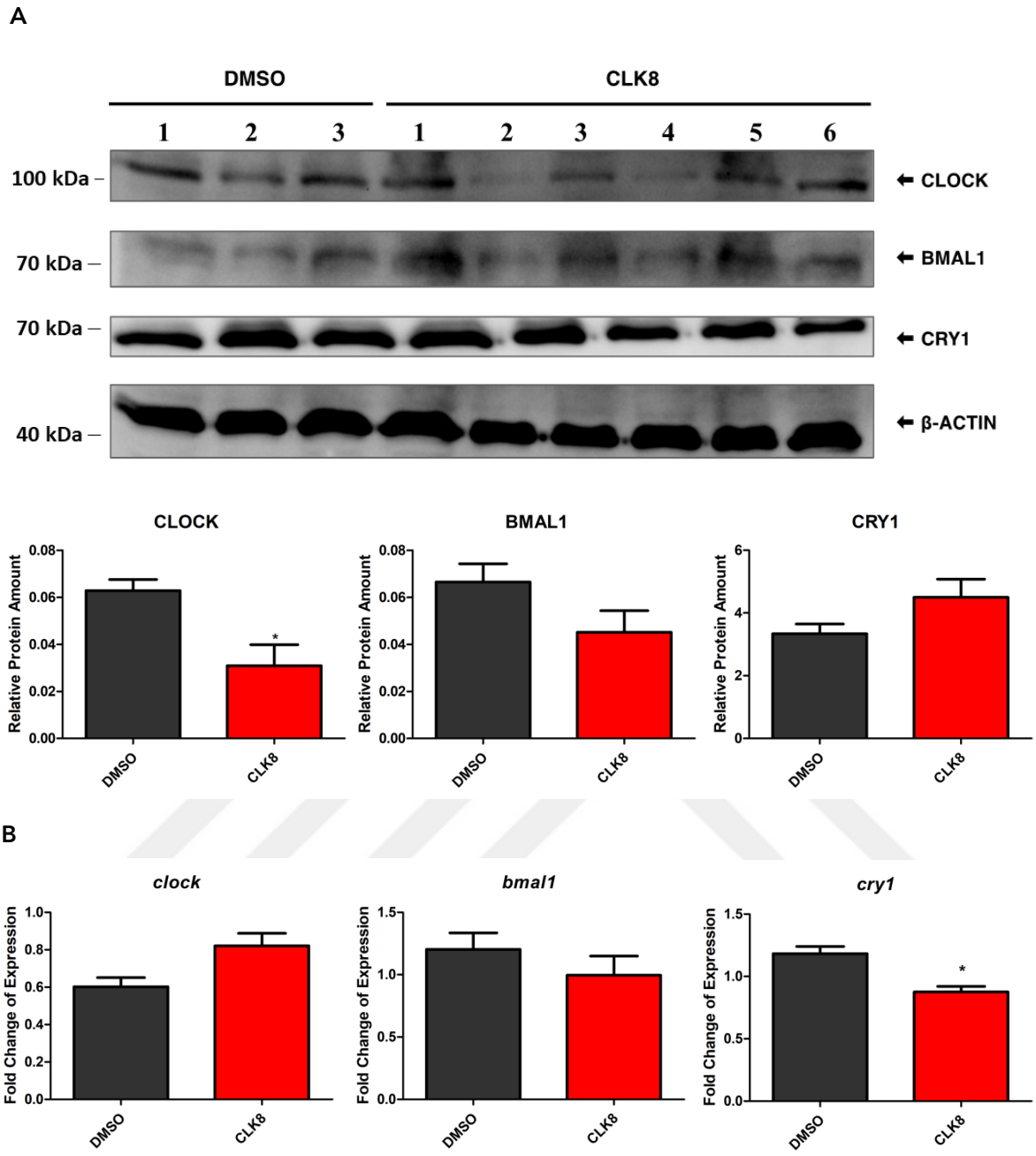

Figure 21. In vivo Effect of CLK8 on protein and transcription levels of clock components. The livers of mice sacrificed 6 h after CLK8 treatment at 25 mg/kg were used in western blot and RT-qPCR analysis. (A) The western blot results are quantified by normalizing according to β-ACTIN and shown the panel bewlo. (B) The results of RT-qPCR normalized to PRLP0 show the alteration in core clock gene expressions. (data are mean ± s.e.m.; n=3 independent experiments); [* p-value<0.05]

CHAPTER 5

DISCUSSION

Among the core clock components, only the repressor CRY1 has been targeted by small molecules including KL001 and KS15 [10,60]. The activators of TTFL are remained to be novel targets of small molecule modifiers of circadian clock. In this thesis, structure-based virtual screening was used to find chemical modulators. CLK8 was identified as a novel CLOCK-binding small molecule which enhances the circadian amplitude (Figure 10).

Computational studies revealed that CLK8 binds to Phe80 and Lys200 residues in the boundary of the cavity surrounded by the α2-helix of the bHLH domain and the Hβ strands within the PAS-A domain of CLOCK. F80A-K220A mutant CLOCK failed to bind CLK8 while wild type CLOCK strongly interacts with the molecule (Figure 12B, Figure 14B). Pull-down assays and LC-MS/MS results (Figure 12C, Table 2) also indicated that three out of four core clock components contain PAS domains but CLK8 specific binding to CLOCK and non-off its off targets play role in circadian oscillation. In fact, amplitude enhancement effect of CLK8 on circadian rhythm is also directly related to binding of CLK8 to CLOCK proteins as CLK8 could not provide a robust rhythm in Clock-deficient MDA MB 231 cells whereas it could do in Clock-wild type ones (Figure 11).

Even though multiple layers of regulations tightly control circadian rhythms and makes identification of exact working principal of CLK8 difficult, the studies in this thesis enlightened the action mechanism of this compound. It is shown that CLK8 partially prevents interaction of CLOCK with BMAL1 (Figure 15). Since the formation of CLOCK:BMAL1 complex is reduced by CLK8, nuclear translocation of CLOCK is decreased as well (Figure 17) . This result is in agreement with previous studies which shown that dimerization of CLOCK with BMAL1 leads its translocation into nucleus [40,41]. Time-dependent analysis of changes at protein and mRNA level in synchronized U2OS cells revealed that CLK8 alters the transcription of core clock components both in the positive and negative arm of the transcriptional-translational regulatory feedback loop (TTFL) while CLK8 only changes the amounts of proteins at the positive arm of the TTFL, but not that of the ones in the negative arm of the TTFL (Figure 18, Figure 19). Reduction in the expression level of clock output genes might be due to the decreasing effect of CLK8 on CLOCK and BMAL1 interaction which partially prevents CLOCK entry into nucleus. Meanwhile, the abundance of CRY1

and PER2 remains unaffected. This most probably strengthens the repression activity CRY/PER on E-Box driven transcription, in turn, mitigate transcription level of clock output genes.

Together with the reduction of CLOCK in nucleus, the outcomes suggest that CLK8 stabilizes the repression activity of PER2 and CRY1 proteins by reducing the CLOCK and BMAL1 amount which results in the enhancement of circadian amplitude. Such action mechanism of CLK8 shares similarities with previous genetic studies that alteration of stoichiometry between positive and negative arm of TTFL in favor of elevation of negative arm leads to enhancement of circadian clock [9,27] (Figure 22).

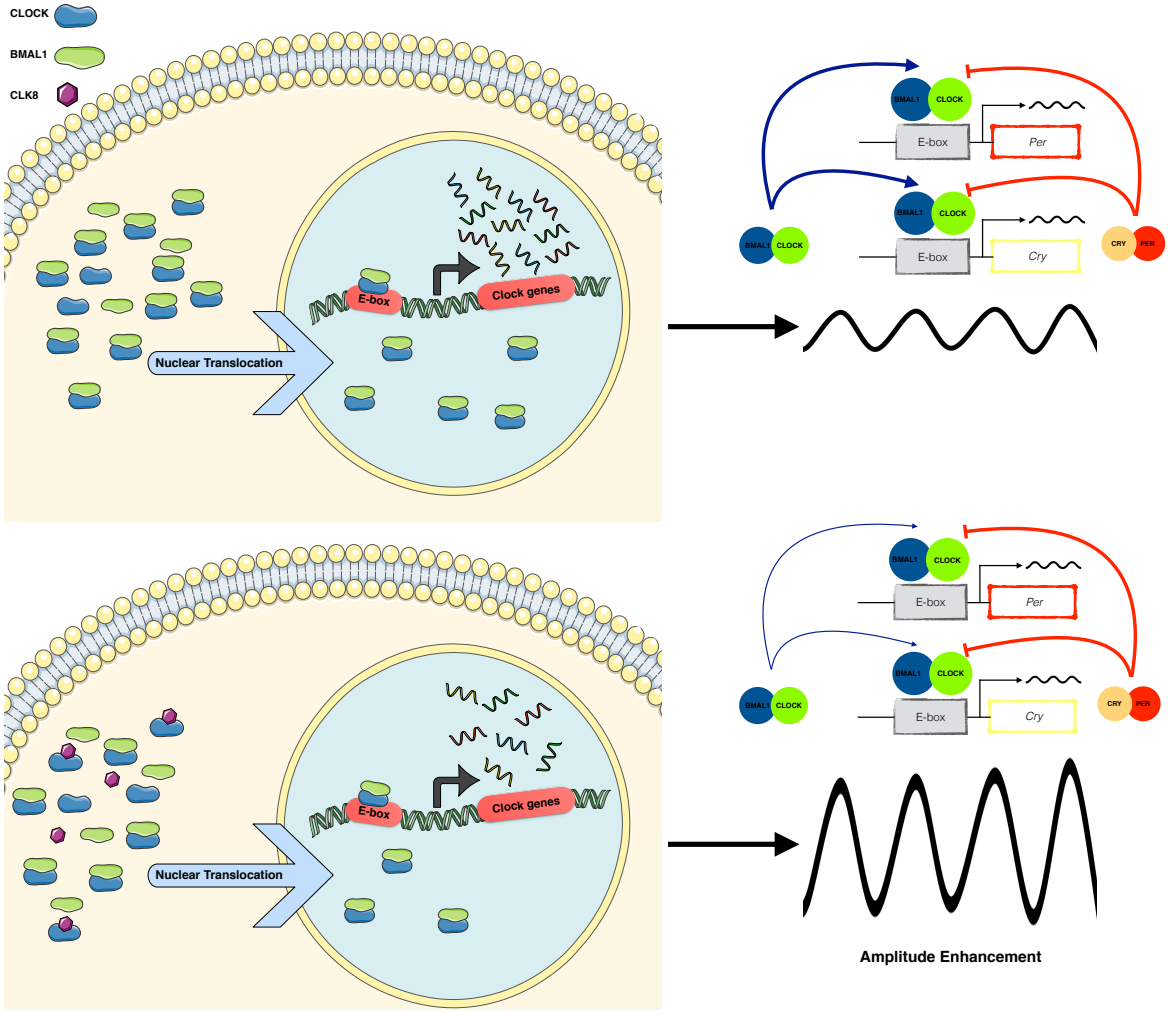

Figure 22. A schematic presentation of CLK8 affecting CLOCK and the circadian rhythm. CLOCK and BMAL1 are dynamically interacting. When CLK8 is present, it binds to CLOCK and reduces CLOCK and BMAL1 interaction. Upon binding of CLK8 to CLOCK, the translocation of the CLOCK into nucleus abolished. At the cellular level, CLK8 enhances the amplitude of the circadian rhythm. The role of CLOCK in regulating the circadian clock amplitude can be investigated using CLK8 to transiently modulate CLOCK. (This figure is created by using Servier Medical Art by Servier liciensed under a Creative Commons Attribution 3.0 Unported License)

Studies on Clock knockout mice and wild type or mutant Clock over-expressing cells have indicated the effects of CLOCK on the circadian phenotype including arrhythmicity and shortened or lengthened period [22,50]. However, the relationship between CLOCK and circadian amplitude has not been well-studied, yet. This brings a novelty to the results of this thesis as CLK8 specifically binds to CLOCK and modulate it transiently. In this way, CLK8 can be used to gain a better understanding of the mechanistic role of CLOCK in the circadian clock.

The second application area of CLK8 might be the development of therapeutic intervention to treat pathologies related to disrupted, especially declined, circadian rhythm. Chronic metabolic diseases as diabetes, mood disorders and accelerated aging are some of the health problems resulted from diminished circadian amplitude. For instance, exon 19 of CLOCK is found in the PAS-B domain of CLOCK and *Clock*Δ*19/*Δ*19* mutation in mice causes a range of metabolic disorder including obesity due to the attenuation of circadian gene expression and the rhythmicity of feeding [53]. Seasonal affective disorder (SAD) is a mood disorder happening when the circadian rhythm dampens in the scale of sleep-awake cycle, body temperature and hormone release. Such case causes dysregulated stress responses as a result, patients are suffered from mood unbalance [84]. Another example to the relationship between amplitude and health comes from αMUPA transgenic mice which have an enhanced circadian amplitude compared to wild-type mice [78]. These animals spontaneously eat less and display a higher amplitude of circadian rhythm both in the body temperature and expression of core clock genes compared to wild type controls [78]. The most interestingly, αMUPA mice have longer life-span than their counterparts [78]. These findings indicate the importance of a robust circadian amplitude for health. As CLK8 enhances the amplitude of the circadian rhythm, this small molecule modifier can be used to develop intervention to re-regulate dampened amplitude and to treat related pathologies. Actually, agreement in the results of in vitro and in vivo studies at molecular level implied that CLK8 has effect on mice. As future direction, pharmacodynamic and pharmacokinetic analysis of CLK8 should be investigated to elucidate the effects CLK8 on the physiological processes.

APPENDIX A: Primer Sequences

Table 3. Primers for the site-directed mutagenesis of CLOCK and for cloning of *Npas2:dluc* and *Clock:dluc* plasmid. The red color indicates either the mutated codons or the cut sites of restriction enzymes.

Gene	Forward Sequence	Reverse Sequence
F80A-hsClock	CAGAAAAGCATTGATGCTTTACGAAAACATAAAGAAATCACTGCACAG	CTGTGCAGTGATTTCTTTATGTTTTCGTAAAGCATCAATGCTTTTCTG
K220A-hsClock	GTAAAATTTATAGGAAATTTCGCATCTTTAAACAGTGTATCC	GGATACACTGTTTAAAGATGCGAAATTTCCTATAAATTTTAC
mClock	TTCTGCAGATATCCA ATGGTGTTTACCGTAAGCTGTAG	GGCGTCTTCCATGAGCGGCCGCCCCTGTGGCTGGACCTTGGAAGG
hsNpas2	GCTAGTTAAGCTTGGATGGATGAAGATGAGAAAGA	CATCTCGAGCGGCCGCCATCGGGGCGGCTGCTGGA
Pg5dLuc	TGGCGGCCGCTCGAGATGGAAGACGCCAAAAAC	GGGCCCTCTAGACTCCACGGCGATCTTTCC

Table 4. List of the primers used in RT-qPCR analysis

Gene	Forward Sequence	Reverse Sequence
hsPrlp0	TGTGGGAGCAGACAATGTGG	CATTCCCCCGGATATGAGGC
hsClock	GGCACCACCCATAATAGGGTA	TGTTGCCCCTTAGTCAGGAAC
hsBmal1	GCCCATTGAACATCACGAGTAC	CCTGAGCCTGGCCTGATAGTAG
hsCry1	ACAGGTGGCGATTTTTGCTTC	TCCAAAGGGCTCAGAATCATACT
hsPer2	AGTTGGCCTGCAAGAACCAG	ACTCGCATTTCCTCTTCAGGG
hsRev-erbα	TGGACTCCAACAACAACACAG	GTGGGAAGTAGGTGGGACAG
hsRor-α	ACTACACACCAGCATCAGGC	GCAGGTTTCCAGATGCGATT
hsDbp	GAGGAACTTAAGCCCCAGCC	CTCGTTGTTCTTGTACCGCC
mGapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
mClock	AGAGCGCGAAGGAAATCTGG	ACTGACCATCAGAACTCTTGCT
mBmal1	GACTACCAAGAAAGTATGGACACA	TTTGTCCCGACGCCTCTTTT
mCry1	CAACGTGGGCATCAACAGG	GATGTTCCATTCCTTGAAAAGCC

APPENDIX B: Maps of Expression Vectors

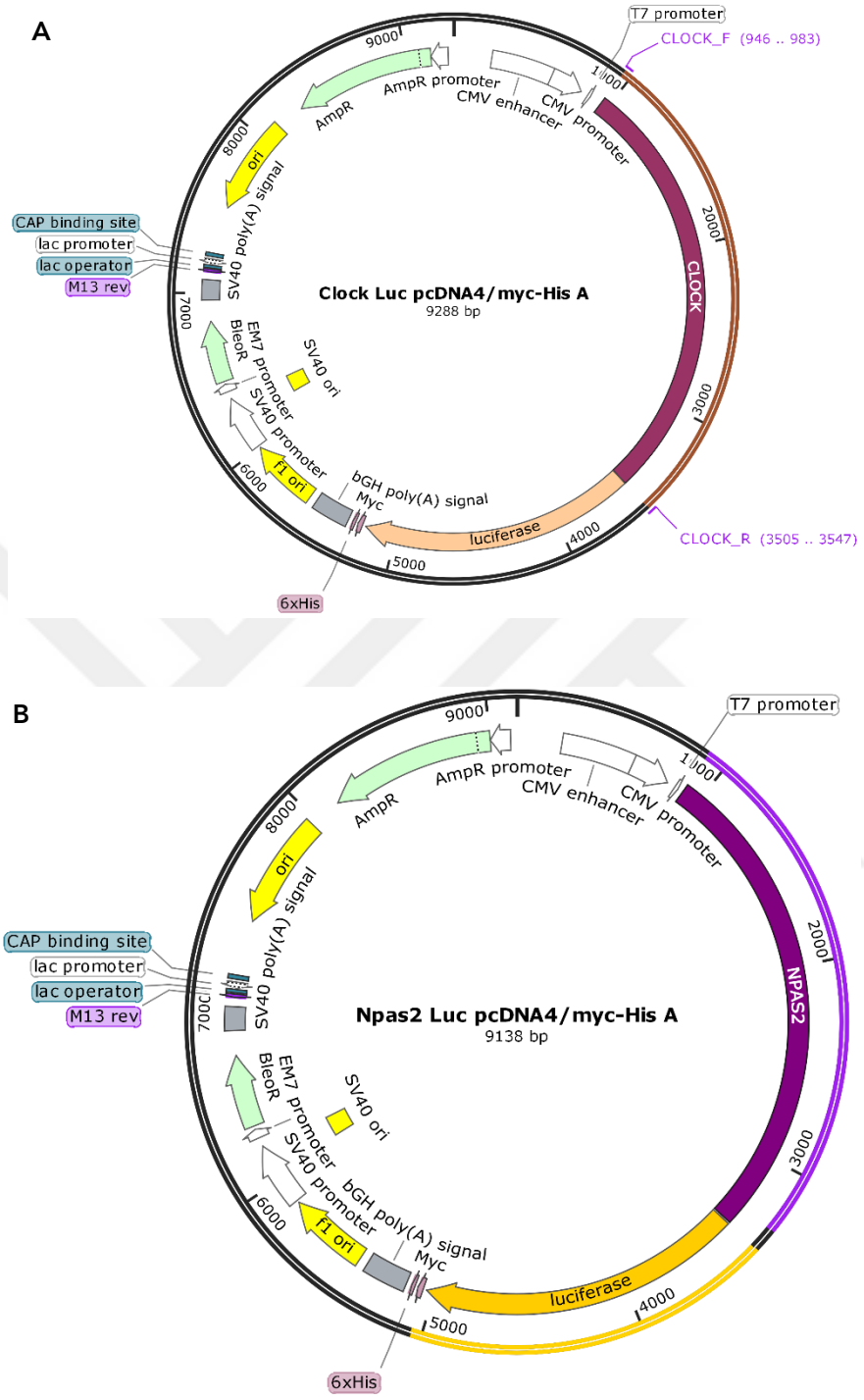

Figure 23. Maps of the expression vectors. (A) Clock:luc and (B) Npas2:luc genes were cloned into pcDNA4/myc-His A vector.

APPENDIX C: PROTEIN MARKER

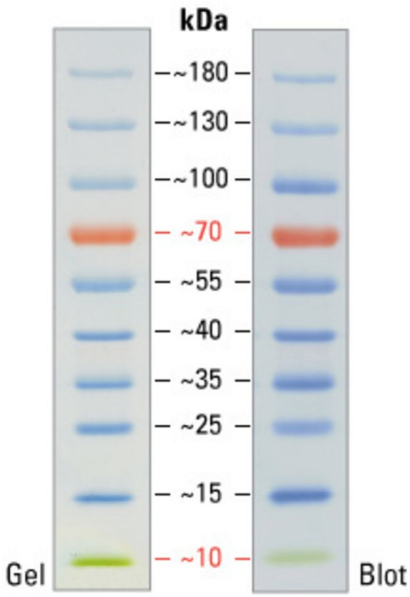

Figure 24. SDS-PAGE band profile of the PageRuler Prestained Protein Ladder within 4-20% Tris-glycine gel.

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